REPUBLIC OF NORTH MACEDONIA
„SS. CYRIL AND METHODIUS" UNIVERSITY IN SKOPJE
Faculty of Veterinary Medicine – Skopje

**Besart F. Jashari**

# CHARACTERISATION, GENETIC TYPING AND ANTIMICROBIAL RESISTANCE OF *LISTERIA MONOCYTOGENES* FROM FOOD CHAIN OF KOSOVO

**Doctoral Dissertation**

**Skopje, 2025**

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**Characterization, Genetic Typing and Antimicrobial Resistance of *Listeria monocytogenes* from Food Chain of Kosovo**

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BESART FAHRI JASHARI

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TITLE:

"CHARACTERISATION, GENETIC TYPING AND ANTIMICROBIAL RESISTANCE OF *LISTERIA MONOCYTOGENES* FROM FOOD CHAIN OF KOSOVO"

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# CHARACTERISATION, GENETIC TYPING AND ANTIMICROBIAL RESISTANCE OF *LISTERIA MONOCYTOGENES* FROM FOOD CHAIN OF KOSOVO

**-ABSTRACT-**

*Listeria monocytogenes* (*L. monocytogenes*) is one of the main pathogens, psychrotrophic, known for its ability to not only survive but also grow at different range of temperatures. This pathogen transmitted by the consumption of contaminated food and it causes a disease called listeriosis. This study provides a genetic characterization including phylogenetic lineages, molecular serotyping and clonal complexes of *L. monocytogenes* strains isolated from different food products within the food chain of the Republic of Kosovo. Given the limited data on antibiotic resistance among *L. monocytogenes* isolates in Kosovo, this study also aimed to assess the circulating CCs and sequence types (STs) and their antibiotic resistance profiles using a panel of 18 antibiotics.

These samples were categorized as follows: 648 from ready-to-eat (RTE) food products, 281 from food products typically consumed cooked (FPCC), 60 from raw materials (RM), and 6 from environmental sources. The overall of *L. monocytogenes* contamination across these samples was determined to be 11.76% (117 out of 995). Specifically, contamination rates were 6.33% (41 out of 648) in RTE products, 14.95% (42 out of 281) in FPCC, 55.00% (33 out of 60) in RM, and 16.66% (1 out of 6) in environmental samples. Sequence typing and antimicrobial resistance was performed on 114 isolates.

*L. monocytogenes* isolates were detected in the collected samples through the official EN ISO 11290-1:2017 method with two-phase enrichment. DNA was extracted from pure isolates using the specific kit for Gram-positive bacteria - Mericon DNA Bacteria Plus Kit (Qiagen). Strains were typed as follows: The concentration of DNA extracts was adjusted to between 0.1 and 1 ng/µL, then a real-time multiplex PCR analysis was performed using Taqman® PCR probes from TIB Molbio. Three different real-time PCR thermocyclers were used to perform the tests: A Mic-IV real-time PCR thermocycler from Bio Molecular Systems (Upper Coomera, Australia), a Rotor-Gene Q real-time PCR thermocycler from QIAGEN (Hilden, Germany), and a QuantStudio 5 real-time PCR thermocycler from Thermo Fisher Scientific (Waltham, USA). The following target genes were used for molecular serotyping lmo0737, lmo1118, ORF2819, ORF2110, prs, and plca. Clonal complexes (CCs) were identified by amplifying CC-specific genomic regions according to ANSES procedures Geno-Listeria. Seven housekeeping loci (*abc*Z, *bgl*A, *cat*, *dap*, *dat*, *ldh*, and *lhk*A) were selected for the characterization of *L. monocytogenes* isolates by MLST. Antibiotic susceptibility testing performed using the Sensititre GPN3F panel, revealed significant variability in resistance patterns.

All *L. monocytogenes* isolates underwent comprehensive molecular analyses, including serotyping and clonal complex (CC) identification using real-time PCR and multilocus sequence typing (MLST). The isolates were classified into four molecular serotypes: IIa (34.19%), IIb (3.48%), IIc (32.48%), and IVb (29.91%), representing two major lineages: Lineage I (33.33%) and Lineage II (66.66%). In total, 14 distinct CCs were identified among the 41 RTE isolates, with CC29 (7 isolates), CC2 (6 isolates), and CC6 (6 isolates) being the most prevalent. By contrast, CC9 was notably dominant in both FPCC (21 isolates) and RM (14 isolates). Importantly, 30 isolates were identified as belonging to CC1, CC2, CC4, or CC6, clonal complexes that are frequently linked to severe human infections.

Among the 114 isolates tested, 21 distinct sequence types (STs) were identified via MLST. CC9-ST9 was the most abundant in meat products, accounting for 38.75% of isolates, while CC29-ST29 was predominant in dairy products, representing 24.0% of isolates. Resistance was observed against several antibiotics, including levofloxacin (22.8%), gentamicin and rifampin (17.5%), quinupristin/dalfopristin (14.9%), erythromycin (11.4%), penicillin (7.89%), tetracycline (1.75%), and streptomycin (0.88%). Furthermore, 27 distinct multiple antibiotic resistance (MAR) phenotypes were identified among the isolates, with resistance ranging from 3 to 12 antibiotics. Alarmingly, the antimicrobial resistance index (ARI) for certain food categories, such as meat and meat products (MMP, ARI = 0.22) and fish meat products (FMP, ARI = 0.26), exceeded the permissible threshold of 0.2 defined by the Krumperman model. Notably, 34 isolates (29.8%) exhibited MAR indices above this critical threshold.

The discovery of multidrug-resistant *L. monocytogenes* strains, including those associated with high-risk sequence types, poses a significant threat to food safety and public health. These findings highlight the urgent need for enhanced surveillance systems to monitor the occurrence and spread of antimicrobial-resistant (AMR) *L. monocytogenes* within the food chain. Proactive measures, including stricter regulations and routine monitoring, are essential to mitigate the risks posed by AMR pathogens and to safeguard public health in Kosovo.

**Keywords:** *L. monocytogenes*, food chain, phylogenetic lineage, molecular serotype, clonal complex, sequence typing, antimicrobial resistance, intrinsic resistance, multidrug-resistant.

**КАРАКТЕРИЗАЦИЈА, ГЕНЕТСКО ТИПИРАЊЕ И АНТИМИКРОБНА ОТПОРНОСТ НА ЛИСТЕРИЈА МОНОЦИТОГЕНИТЕ ОД СИНЏИРОТ НА ХРАНА НА КОСОВО**

**- АПСТРАКТ -**

*Listeria monocytogenes* (*L. monocytogenes*) е еден од главните патогени, психотрофни, познат по својата способност не само да преживее, туку и да расте на различни температури. Овој патоген се пренесува со консумација на контаминирана храна и предизвикува болест наречена листериоза. Оваа студија обезбедува генетска карактеризација вклучувајќи филогенетски лоза, молекуларна серотипизација и клонални комплекси на соеви на *L. monocytogenes* изолирани од различни прехранбени производи во ланецот на исхрана на Република Косово. Со оглед на ограничените податоци за отпорност на антибиотици кај изолатите на *L. monocytogenes* во Косово, оваа студија исто така имаше за цел да ги процени циркулирачките CC и типови на секвенци (STs) и нивните профили на отпорност на антибиотици користејќи панел од 18 антибиотици.

Овие примероци беа категоризирани на следниов начин: 648 од готови за јадење (RTE) прехранбени производи, 281 од прехранбени производи кои обично се консумираат варени (FPCC), 60 од суровини (RM) и 6 од извори на животната средина. Целокупната на контаминација на *L. monocytogenes* низ овие примероци беше утврдена дека е 11,76% (117 од 995). Поточно, стапките на контаминација беа 6,33% (41 од 648) кај RTE производите, 14,95% (42 од 281) во FPCC, 55,00% (33 од 60) во RM и 16,66% (1 од 6) во еколошките примероци. Типизација на секвенци и антимикробна резистенција беше изведена на 114 изолати.

Изолатите на *L. monocytogenes* беа откриени во собраните примероци преку официјалната метода EN ISO 11290-1:2017 со двофазно збогатување. DNA беше извлечена од чисти изолати со помош на специфичниот комплет за грам-позитивни бактерии - Mericon DNA Bacteria Plus Kit (Qiagen). Видовите беа типизирани на следниов начин: Концентрацијата на DNA екстракти беше прилагодена помеѓу 0,1 и 1 ng/µL, а потоа беше извршена мултиплексна PCR анализа во реално време со користење на Taqman® PCR сонди од TIB Molbio. За извршување на тестовите се користеа три различни PCR термоциклери во реално време: Mic-IV PCR термоциклерот во реално време од Bio Molecular Systems (Upper Coomera, Австралија), Rotor-Gene Q PCR термоциклерот во реално време од QIAGEN (Хилден, Германија) и QuantStudio 5 во реално време FisciclerHer Sciencealt, USA TherWr. Следниве целни гени беа користени за молекуларна серотипизација lmo0737, lmo1118, ORF2819, ORF2110, prs и plca. Клоналните комплекси (CCs) беа идентификувани со засилување на CC-специфичните геномски региони според процедурите ANSES Гено-Листерија. Седум локуси за домаќинство (abcZ, bglA, cat, dap, dat, ldh и lhkA) беа избрани за карактеризација на изолатите на *L. monocytogenes* со MLST. Тестирањето за чувствителност на антибиотици, извршено со помош на панелот Sensititre GPN3F, откри значителна варијабилност во моделите на отпорност.

Сите изолати на *L. monocytogenes* беа подложени на сеопфатни молекуларни анализи, вклучително серотипизација и идентификација на клоналниот комплекс (CC) со користење на PCR во реално време и типирање на секвенца на повеќе локуси (MLST). Изолатите беа класифицирани во четири молекуларни серотипови: IIa (34,19%), IIb (3,48%), IIc (32,48%) и IVb (29,91%), кои претставуваат две главни линии: Lineage I (33,33%) и Lineage II (66,66%). Вкупно, 14 различни CC беа идентификувани меѓу 41 RTE изолати, при што CC29 (7 изолати), CC2 (6 изолати) и CC6 (6 изолати) беа најзастапени. Спротивно на тоа, CC9 беше особено доминантен и во FPCC (21 изолат) и во RM (14 изолати). Важно е дека се каже дека 30 изолати беа идентификувани дека припаѓаат на CC1, CC2, CC4 или CC6, клонални комплекси кои често се поврзани со тешки човечки инфекции.

Меѓу 114-те тестирани изолати, 21 различни типови на секвенци (STs) беа идентификувани преку MLST. CC9-ST9 беше најзастапен во месните производи, со 38,75% од изолатите, додека CC29-ST29 беше доминантен во млечните производи, што претставува 24,0% од изолатите. Беше забележана отпорност на неколку антибиотици, вклучително и левофлоксацин (22,8%), гентамицин и рифампин (17,5%), квинупристин/далфопристин (14,9%), еритромицин (11,4%), пеницилин (7,89%), тетрациклин (1,75%) и стрептомицин (0,88%) (0,88%). Понатаму, 27 различни фенотипови на повеќекратна отпорност на антибиотици (MAR) беа идентификувани меѓу изолатите, со отпорност од 3 до 12 антибиотици.

Алармантно, индексот на антимикробна отпорност (ARI) за одредени категории на храна, како што се месото и производите од месо (MMP, ARI = 0,22) и производите од месо од риба (FMP, ARI = 0,26), го надмина дозволениот праг од 0,2 дефиниран со моделот Круперман. Имено, 34 изолати (29,8%) покажаа MAR индекси над овој критичен праг.

Откривањето на соеви на *L. monocytogenes* отпорни на повеќе лекови, вклучително и оние поврзани со типови на низа со висок ризик, претставува значајна закана за безбедноста на храната и јавното здравје. Овие наоди ја нагласуваат итната потреба од засилени системи за надзор за следење на распространетоста и ширењето на антимикробно отпорни (AMR) *L. monocytogenes* во синџирот на исхрана. Проактивни мерки, вклучително и построги регулативи и рутински мониторинг, се од суштинско значење за да се ублажат ризиците од патогените AMP и да се заштити јавното здравје во Косово.

**Клучни зборови**: *L. monocytogenes*, синџир на исхрана, филогенетска лоза, молекуларен серотип, клонален комплекс, типизација на секвенци, антимикробна резистенција, внатрешна резистенција, мултирезистентна.

**Dedication to my loved ones**

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I declare that the doctoral thesis is an original thesis that I have prepared independently.

Signature of the doctoral student
(On the electronic version, signature and abbreviation)

(On the electronic version of the doctoral thesis)

I declare that the electronic version of the doctoral thesis is identical to the printed doctoral thesis.

Signature of the author,

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Abbreviations

| | |
|---|---|
| abcZ | abcZ (ABC transporter) |
| ALOA | Agar Listeria according to Ottaviani and Agosti |
| AMP | Ampicillin |
| AMR | Antimicrobial resistance |
| ANSES | French Agency for Food, Environmental and Occupational Health & Safety |
| ARI | Antimicrobial resistance index |
| ATCC | American Type Culture Collection |
| aw | Water activity |
| BA | Blood agar |
| bglA | bglA (beta glucosidase) |
| CAMP test | Christie–Atkins–Munch-Peterson test |
| cat | cat (catalase) |
| CC | Clonal Complex |
| CDS | Coding sequences |
| CEF | Ceftriaxone |
| cfu/g | Colony forming unit / gram |
| cgMLST | Core genome MLST |
| CIP | Ciprofloxacin |
| CLI | Clindamycin |
| CLSI | Clinical and Laboratory Standard Institute |
| DAP | Daptomycin |
| dapE | dapE (succinyl diaminopimelate desuccinylase) |
| Dat | dat (D-amino acid aminotransferase) |
| DFB | Demi Fraser Broth |
| DNA | Deoxyribonucleic acid |
| dsDNA | double stranded DNA |
| EC | European Commission |
| ECDC | European Centre for Disease Prevention and Control |
| EFSA | European Food Safety Authority |
| ERY | Erythromycin |

| | |
|---|---|
| EU | European Union |
| EUCAST | European Committee on Antimicrobial Susceptibility Testing |
| EURL | European Union Reference Laboratory |
| FBO | Food business operator |
| FP | Fish products |
| FPEC | Food products eating cocked |
| FSANZ | Food Standards Australia New Zealand |
| FSIS | Service of Food Safety and Inspection |
| G+C | Guanine + Cytosine |
| GAT | Gatifloxacin |
| GEN | Gentamicin |
| GIT | Gastrointestinal tract |
| GPN3F | Gram positive and negative sensititre |
| ISO | International Standard Organization |
| Ldh | ldh (L-lactate dehydrogenase) |
| LEVO | Levofloxacin |
| LFB | Listeria Fraser Broth |
| lhkA | lhkA (histidine kinase) |
| LM | *Listeria monocytogenes* |
| LOA | Listeria Oxford Agar |
| LZD | Linezolid |
| MARI | Multiple Antibiotic Resistance Index |
| Mb | Mega base |
| MDP | Milk and dairy products |
| MDR | Multi drug resistance |
| MHB | Mueller Hinton broth II |
| MIC | Minimum inhibitory concentration |
| mL | Milliliter |
| MLST | Multi-locus sequence typing |
| mm | Millimeter |
| MP | Meat products |
| MST | Minimum spanning tree |

| | |
|---|---|
| ng/µL | Nanogram/microliter |
| ºC | Degree Celsius |
| OXA+ | Oxacillin+2% NaCl |
| PEN | Penicillin |
| PFGE | Pulse Field Gel Electrophoresis |
| pH | Potential of hydrogen |
| RIF | Rifampin |
| RKS | Republic of Kosovo |
| RM | Raw material |
| RT PCR | Real Time Polymerase Chain Reaction |
| RTE | Ready to eat food |
| RTE | Ready to eat food |
| SNPs | Single nucleotide polymorphisms |
| ST | Sequence typing |
| STR | Streptomycin |
| SXT | Trimethoprim/ Sulfamethoxazole |
| SYN | Quinupristin/Dalfopristin |
| TET | Tetracycline |
| tRNA | Transfer ribonucleic acid |
| TSA | Tryptic Soy Agar |
| TSYEA | Tryptone Soy Yeast Extract Agar |
| USA | United State of America |
| USDA | US Department of Agriculture |
| VAN | Vancomycin |
| WGS | Whole Genome Sequencing |

# 1 INTRODUCTION

*Listeria monocytogenes* (*L. monocytogenes*) is a Gram-positive, non-spore-forming facultative anaerobic bacterium known for its resilience in harsh conditions. It can grow at refrigeration temperatures, survive freezing, tolerate high salt concentrations and nitrites, and is widespread in water, soil, and dust (Farber & Peterkin, 1991; Cole et al., 1990). *L. monocytogenes* is recognized as a serious foodborne pathogen responsible for listeriosis a severe disease with a high mortality rate, especially in vulnerable populations such as the elderly, immunocompromised individuals, pregnant women, and neonates (Buchanan et al., 2017).

In 2022, the European Union (EU) reported 2,738 confirmed listeriosis cases, 35 foodborne outbreaks, and a hospitalization mortality rate of 15.9% (EFSA & ECDC, 2023). Though incidence remains low, its severity is notable, with case fatality rates of 18.1% in Europe and 15.3% in the U.S. (Mohapatra et al., 2024). The disease is mainly associated with ready-to-eat (RTE) foods such as soft cheeses and RTE meat products (Fagerlund et al., 2020). A significant factor in outbreak persistence is the ability of *L. monocytogenes* to survive in food production environments (Carpentier & Cerf, 2011). The disease poses a substantial burden on public health and the economy (Olanya et al., 2019; Thomas et al., 2015), necessitating robust molecular surveillance to trace sources and control its spread.

France provides a successful example: robust monitoring and regulation led to a 68% reduction in sporadic listeriosis cases from 1987 to 1997, particularly through linking food and clinical isolates using molecular techniques (Goulet et al., 2001; Moura et al., 2017).

Kosovo, a small and developing country in the Balkans, has expanding animal production and food processing sectors. However, its surveillance systems, especially molecular-based monitoring of foodborne pathogens, remain underdeveloped. Studies in neighboring countries show occurrence of *L. monocytogenes* in beef RTE products between 2.7%–3.9% and 4.6% in hard cheeses (EFSA & ECDC, 2023). In Kosovo, *L. monocytogenes* has been identified in various sources, including clinical cases in sheep (Hamidi et al., 2020), raw milk (Mehmeti et al., 2017), cheese products (Studenica et al., 2022), and meat-based food items (Kukleci et al., 2019) However, these studies lacked molecular characterization and were limited in scale, reducing their comparability to international data.

Genetically, *L. monocytogenes* is divided into four lineages, with lineages I and II responsible for over 95% of human listeriosis cases (Orsi et al., 2011; Kathariou, 2002). Thirteen

serotypes and four molecular serogroups have been identified (Doumith et al., 2004, 2005), with serotypes IIa, IIc (lineage II), and IIb, IVb (lineage I) most frequently linked to invasive disease.

Traditional serotyping has largely been supplemented by multilocus sequence typing (MLST), which provides a standardized system for global comparison (Ragon et al., 2008; Salcedo et al., 2003). MLST categorizes isolates into sequence types (STs) based on alleles from seven housekeeping genes, and clonal complexes (CCs) based on shared alleles among STs. This method allows for tracking genetic relationships and identifying outbreak-related clones (Painset et al., 2019). Rapid identification has improved through multiplex PCR protocols for detecting up to 30 *L. monocytogenes* strains (Felix et al., 2023).

The identification of CCs and STs has proven valuable in tracing outbreaks and distinguishing related strains (Felix et al., 2022; Martin et al., 2006). MLST supports broader epidemiological assessments and enables more precise outbreak investigations (Chenal-Francisque et al., 2015; Moura et al., 2016).

In parallel, antimicrobial resistance (AMR) in *L. monocytogenes* is an emerging global concern. The widespread and sometimes unregulated use of antimicrobials in agriculture and food processing is accelerating resistance (Founou et al., 2016; Olaimat et al., 2018). First-line treatments for listeriosis include aminopenicillins (ampicillin, amoxicillin), benzylpenicillin (with gentamicin), trimethoprim (with or without sulfamethoxazole), erythromycin, and tetracyclines (Caruso et al., 2020; Scortti et al., 2006). In high-risk groups, alternative therapies such as vancomycin, carbapenems, or piperacillin-tazobactam may be used (Pagliano et al., 2017; Thønnings et al., 2016).

*L. monocytogenes* also displays intrinsic resistance to several antibiotics, including fosfomycin, fusidic acid, and second- and third-generation cephalosporins (Troxler et al., 2000; Scortti et al., 2006). Although overall resistance remains low, the first multidrug-resistant (MDR) strain was reported in 1988. Since then, MDR strains have been implicated in several outbreaks globally (Zhang et al., 2007).

To date, no studies in Kosovo have assessed the antimicrobial resistance profiles or molecular characterization of *L. monocytogenes* isolates in the food chain. The National Institute of Public Health of Kosovo reports only sporadic human listeriosis cases, and there is no public data on antibiotic use in food animals an important factor in resistance development. Meanwhile, neighboring countries have used phenotypic methods, such as disk diffusion and minimum

inhibitory concentration (MIC), to evaluate antibiotic susceptibility (Arslan & Özdemir, 2008; Caruso et al., 2020).

This study aimed to assess the diversity of *L. monocytogenes* strains isolated from the food chain in Kosovo using multiplex PCR. It further aimed to classify strains by lineage, molecular serotype, and CC, and to evaluate their resistance to 18 antibiotics commonly used in veterinary and human medicine. Ultimately, the study seeks to detect the presence of MDR strains and contribute to the establishment of molecular surveillance in the country.

# 2 LITERATURE REVIEW

## 2.1 *Listeria monocytogenes* - Historical overview

Unlike the aetiological agents of many diseases, listeriosis was not named after the scientists who first discovered it. *L. monocytogenes*, originally referred to as *Bacillus hepatis*, was first identified by Hülphers in 1910 when it was isolated from the necrotic liver of a rabbit in Sweden (Lamont et al., 2011; Matle et al., 2020; Relier, 1979). In 1926, Murray et al. isolated this bacterium linked to epizootics in rabbits and guinea pigs in Cambridge, England, during a laboratory animal outbreak, naming it *Bacterium monocytogenes* (Matle et al., 2020; Relier, 1979). The name went through several iterations, including *Erysipelothrix* and *Listerella*. The generic name "*Listeria*" was later proposed by Pirie in 1940 in honour of Lord Lister (Relier, 1979).

It 1921, the first human case of *Listeria* infection reported, involving a patient suffering from meningitis (Oevermann et al., 2010). By 1929, the first documented case of human listeriosis was reported in Denmark and was reported by Nyfeldt (Seeliger, 1988). In the early years following the discovery of *L. monocytogenes*, infections were more frequently observed among individuals working closely with farm animals and usually occurred sporadically, largely due to direct contact with infected livestock or contaminated environments, making it primarily an occupational hazard. Today, *L. monocytogenes* is recognized as a significant public health concern, chiefly known for causing listeriosis, a serious infection that primarily affects vulnerable populations, including pregnant women, newborns, the elderly, and individuals with weakened immune systems (YOPI). Listeriosis can manifest in various forms, ranging from mild flu-like symptoms to severe infections such as meningitis and septicemia (Kayode et al., 2020). Ingestion of contaminated food products such as unpasteurized dairy, ready-to-eat meats, and seafood, serves as the primary vector for transmitting *L. monocytogenes*. Public health measures have been established to monitor and mitigate the risk of infection, highlighting the importance of food safety protocols in the agricultural and manufacturing sectors.

Investigation of *L. monocytogenes* is continuously advancing as scientists strive to unravel its pathogenic mechanisms, ability to persist in diverse environments, and resistance to various stress conditions. Studies have also focused on exploring the genetic diversity of the species, which is critical for tracking outbreaks and developing targeted control measures. Ongoing research on *L. monocytogenes* not only enhances our understanding of foodborne

pathogens but also highlights the importance of robust surveillance systems to protect public health. In summary, *L. monocytogenes* is a pathogen with a complex history. First identified in the early 20th century, it is now recognized as a significant threat to human health, particularly, among vulnerable populations. Continuous research into biology and transmission control emphasizes the ongoing need to ensure food safety and public health protections.

## 2.2 Taxonomy, phylogeny and classification

Taxonomically, the family Listeriaceae includes two main genera: Listeria and Brochothrix, both of which are monophyletic. The genus *Listeria* currently comprises 28 species, which are divided into two recognized groups: *Listeria* sensu stricto and *Listeria* sensu lato (Figure 1) (Orsi et al., 2024; Orsi and Wiedmann, 2016). "*Listeria* sensu stricto" currently includes 10 species (*L. monocytogenes, L. seeligeri, L. welshimeri, L. innocua, L. ivanovii, L. marthii, L. farberi, L. immobilis, L. cossartiae and L. swaminathanii*) closely related to *L. monocytogenes.* "*Listeria* sensu lato" which currently includes 18 species (*L. grayi, L. fleischmannii, L. floridensis, L. aquatica, L. valentina, L. thailandensis, L. goaensis, L. ilorinensis, L. costaricensis, L. rustica, L. portnoyi, L. cornellensis, L. newyorkensis, L. rocourtiae, L. weihenstephanensis, L. grandensis, L. booriae and L. riparia*). These species have primarily been isolated from the intestinal tract of asymptomatic animals and animal-derived food products (Schardt et al., 2017). *L. monocytogenes* is one of several species in the genus *Listeria*, which belongs to the family Listeriaceae, order Bacillales, class Bacilli, and phylum Firmicutes within the kingdom Bacteria. *L. monocytogenes* is a rod-shaped Gram-positive bacterium that is motile at temperatures between 10°C and 25°C, facultatively anaerobic, non-spore-forming, and can be found in a variety of environments, including water, soil, effluent, and various foods, survive freezing conditions and high salt concentrations, and tolerate the presence of nitrites (Cole et al., 1990; Walker et al., 1990). Among the species in this genus, only *L. monocytogenes* and *L. ivanovii* are pathogenic, with *L. monocytogenes* being the one capable of causing disease in humans (Gómez-Camarasa, 2022; Kayode et al., 2020). *L. monocytogenes* is considered to be widespread in different environments, including water, dust, and soil (Farber and Peterkin, 1991).

*L. monocytogenes* is classified into four lineages (I-IV). Lineages I and II are the most prevalent, commonly isolated from food, natural environments, farms, and sporadic cases of

listeriosis in both animals and humans. These two lineages are also associated with a higher frequency in both animals and humans. These two lineages are associated with a higher frequency of human outbreaks accounting for over 95% of reported diseases (Chen et al., 2018a; Doumith et al., 2004).

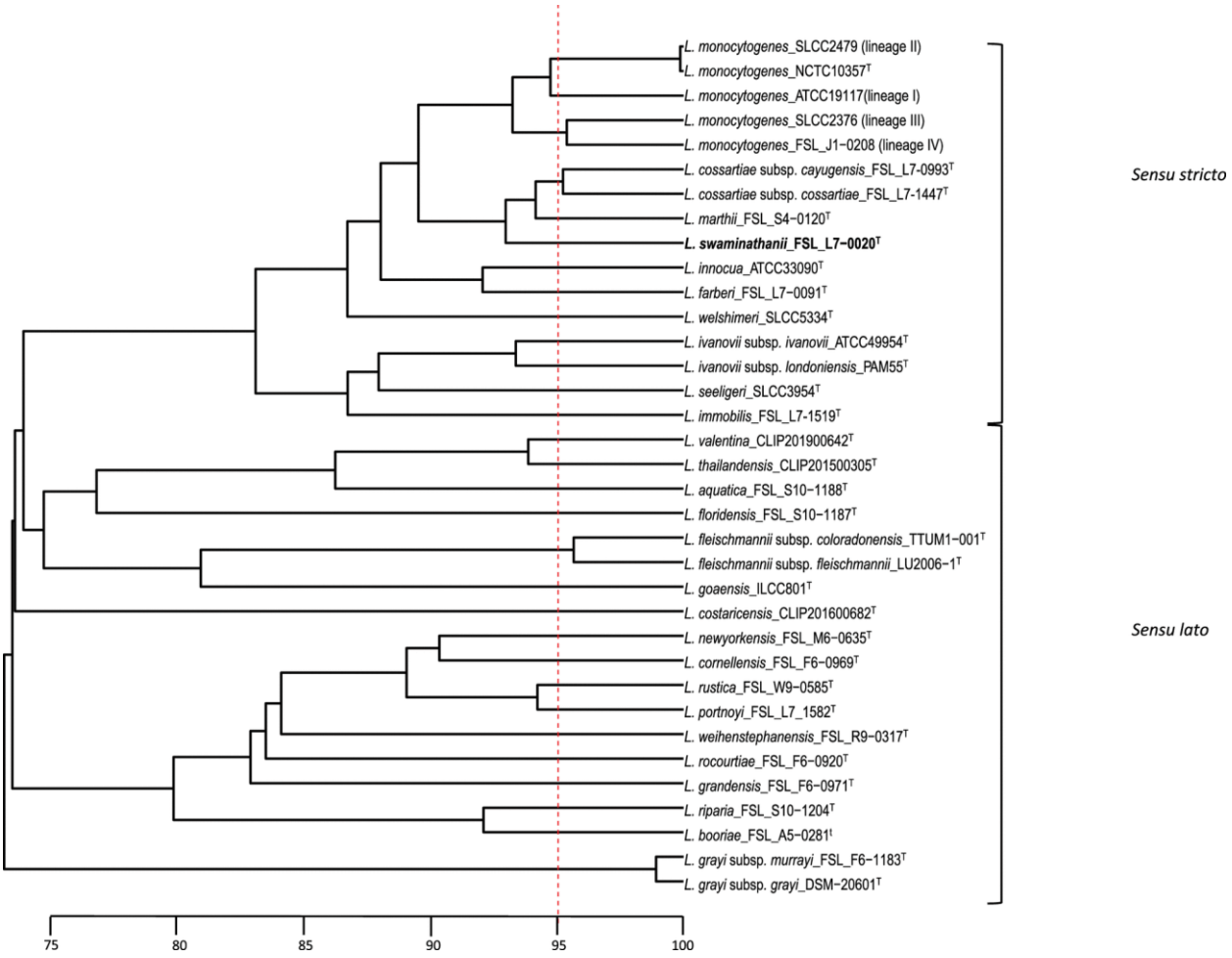



Figure 1. The 30 Listeria species and subspecies type strains are described (Carlin et al., 2021).

## *2.3* Characteristics of *Listeria monocytogenes*

*L. monocytogenes* are short rod-shaped coccobacillus that are gram-positive, non-spore-forming, and facultatively anaerobic. Some features and characteristics of *L. monocytogenes* characteristics were presented in Table 1. Their size varies, typically measuring 0.4 - 0.5 µm in diameter and 1-2 µm in length, with rounded edges and no capsule (Seeligeri&Jones, 1986). The activity of its flagella is temperature-dependent. For instance, it is motile below 30°C but non-flagellated and non-motile at 37°C (Hernandez-Milian and Payeras-Cifre, 2014). *Listeria* species

are psychrotrophic, able to grow in temperatures between -1.5°C and 45°C and they can also grow in pH ranges from 4 to 9.5 or in nutrient broth with up to 10% NaCl, even surviving at 20% (Osek et al., 2022). Furthermore, while it can survive but not grow in extreme conditions such as hydrostatic pressure, acidic pH as low as 2.5, and in the presence of bile (>0.3%) and bile acids (>5 mM), it is capable of surviving and growing in environments with relatively low water activity (aW < 0.90) (Lachtara et al., 2023).

Table 1. Some of the features and characteristics of *L. monocytogenes*.

| **Characteristics** | ***Listeria monocytogenes*** | **Citation** |
|---|---|---|
| Size | 0.4 - 0.5 diameter,<br>1 - 2 µm length | (Seeligeri&Jones, 1986) |
| Motile | < 30°C | (Hernandez-Milian and Payeras-Cifre, 2014) |
| Growth temperature | -1.5 to 45°C | (Lachtara et al., 2023) |
| pH | 4.0 to 9.5 | (Osek and Wieczorek, 2023) |
| Salt concentration | 10 - 20% | (Wieczorek et al., 2020) |
| Minimum water activity (aW) | < 0.90 | (Osek and Wieczorek, 2023) |

## 2.4 Molecular characterization

The estimated genome size of *L. monocytogenes* is 2.9 Mb, with a G+C content of 37.9%, including 2882 coding sequences (CDS), 67 tRNA genes, six 16S–5S-23S operons and 11 pseudogenes (Glaser et al., 2001; Wang et al., 2019). Table 2 presents the genomics data of *L. monocytogenes* adapted from National Center for Biotechnology Information, 2009, U.S. National Library of Medicine.

Table 2. *L. monocytogenes* genomic.

| *Listeria monocytogenes* | |
|---|---|
| Size of Chromosome (Mb) | ≈ 2.94 |
| GC content of protein-coding genes (%) | ≈ 38 |
| Total number of protein-coding genes | ≈ 2,846 |

Note. Adapted from National Center for Biotechnology Information, 2009, U.S. National Library of Medicine

The first complete genome sequence of *L. monocytogenes* EGDe (serovar 1/2a) and *L. innocua* CLIP 11262 (serovar 6a) was published in 2001. It compared the genome of a pathogenic strain of *L. monocytogenes* and that of a non-pathogenic strain of *L. innocua*. Figure 2 presents in detail the genome of this study, where *L. monocytogenes* had a lower G+C content and a genome consisting of 2 944 528 bp. In this study, a large number of putative protein-coding genes were also found, both in *L. innocua* (represented in green) and *L. monocytogenes* (represented in red), encoding surface and secreted proteins, transporters, and transcriptional regulators (Glaser et al., 2001).

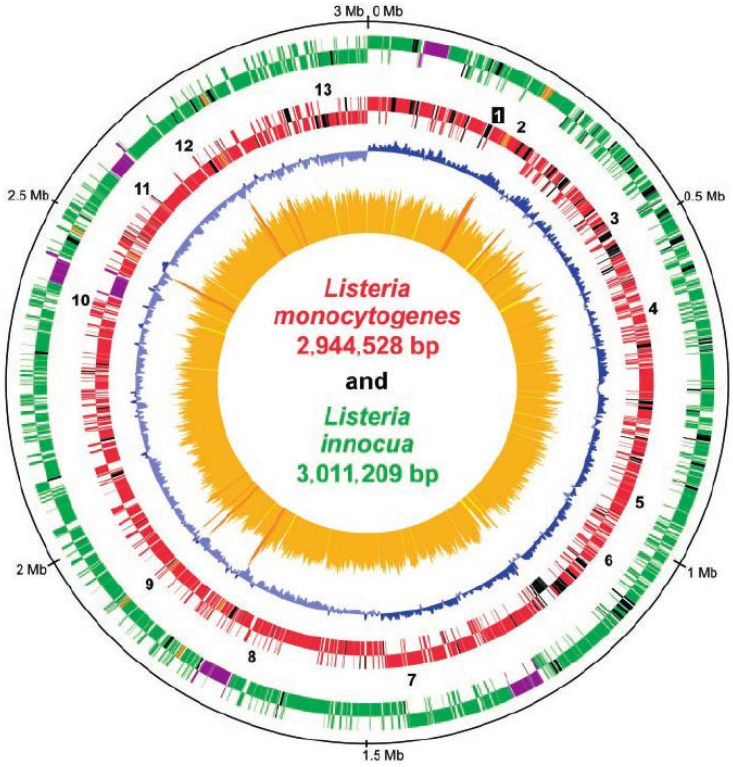



Figure 2. Genome maps of *L. monocytogenes* EGDe and *L. innocua* CLIP 11262 (Glaser et al., 2001).

The single chromosome of FSCNU0110 *L. monocytogenes* consisted of 2,982,685 bp with a G + C content of 37.99%, containing 2,913 genes, including 2,824 coding sequences (CDS), 67 tRNA genes, 18 rRNA genes, and 4 ncRNA genes (Figure 3). These data were supported by in silico analyses performed with PCR serotyping and MLST. Five antibiotic resistance genes were identified in this genome by Genome Comparator: fosX (fosfomycin), norB (quinolone), sul (sulfonamide), lin (lincomycin), and tetM (tetracycline). In addition, 64 virulence genes were found, including Listeria pathogenicity island 1 (LIPI-1) (prfA, plcA, hly, mpl, actA, plcB), 8 lls genes in LIPI-3, and 10 internal genes. No premature stop codons (PMSC) were detected in inlA (Jacquet et al., 2004).

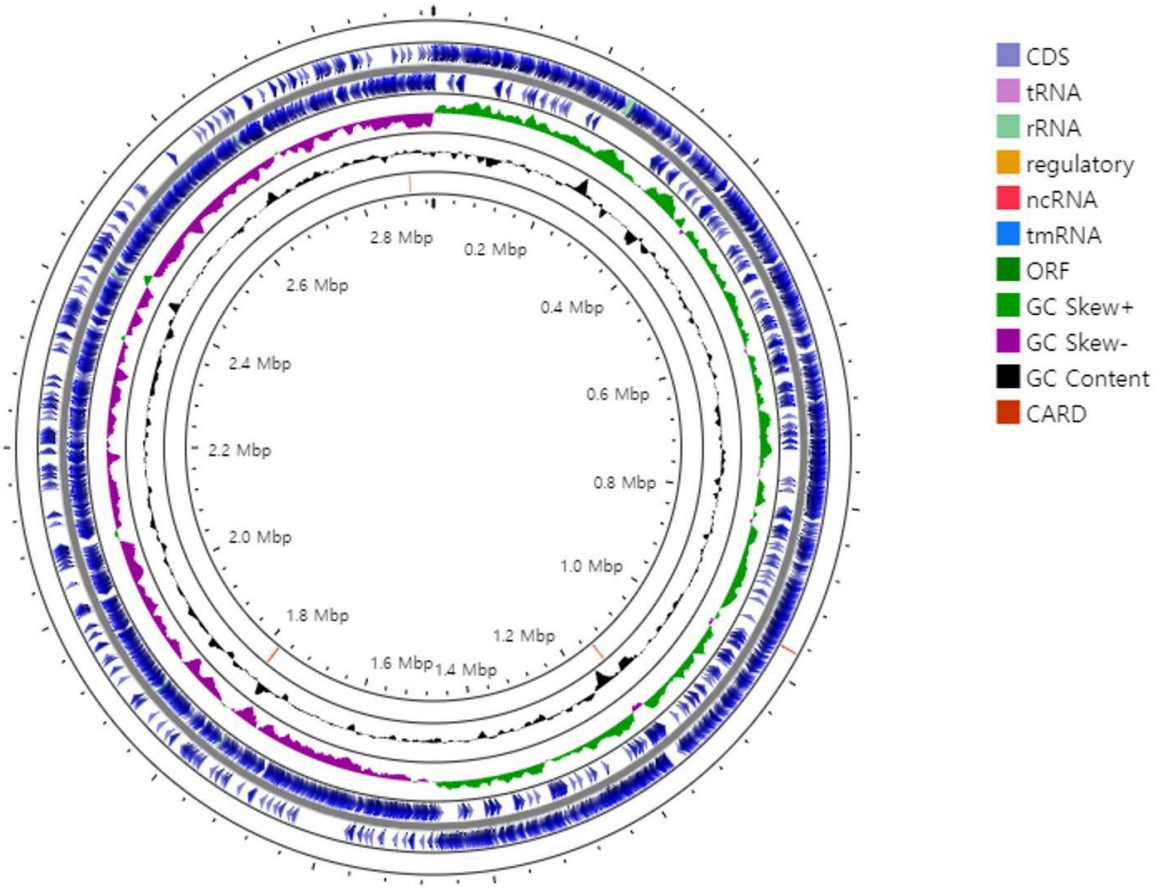



Figure 3. Circular genome map of the *L. monocytogenes* FSCNU0110 chromosome. The genome is 2,933,635, with an average GC content of 37.99%. Generated with Proksee (https://proksee.ca/) (Lee et al., 2023).

### *2.4.1 Molecular typing methods for Listeria monocytogenes*

The development of molecular techniques has helped to identify clonal complexes or epidemiological clones of *L. monocytogenes*, which are now used to distinguish genetically similar isolates that are implicated in single or massive outbreaks (Felix et al., 2022; Martin et al., 2006; Ragon et al. al., 2008). The investigation of *L. monocytogenes* has evolved significantly, transitioning from traditional methods like pulsed-field gel electrophoresis (PFGE) to more advanced genomic approaches. PFGE has long been considered the "gold standard" for typing *L. monocytogenes*, which due to its high discriminatory power in the pre-WGS era is fragment length restriction analysis (Dalmasso et al., 2014). This method was initially used by PulseNet to link disease cases by comparing their DNA fingerprints and thus to identify potential outbreaks. Among other disadvantages of PFGE is that it is time-consuming, difficult to standardize (Van Walle, 2018) and lacks the discriminatory power to define outbreaks.

Historically, pulsed-field gel electrophoresis (PFGE) served as the primary tool for investigating listeriosis outbreaks due to its superior discriminatory ability over serotyping. In PFGE, the bacterial genome is digested with two specific restriction enzymes, AscI and ApaI, and the resulting DNA fragments are separated by size in an alternating electric field, producing a unique band pattern - a strain-specific fingerprint used to determine genetic relatedness across multiple strains. PFGE offers advantages such as standardized laboratory protocols, consistent data analysis, result evaluation, and a shared database infrastructure for both food and clinical samples. However, PFGE also has limitations, including a high labour demand and limited resolution; genetic differences are only detected when they occur at restriction enzyme recognition sites, which can lead to inaccuracies in estimating genetic relationships among strains (Dangel et al., 2019; Lüth et al., 2018; Neoh et al., 2019).

Multiplex-PCR for classification of 5 serogroups - Consists of the amplification of 5 different genes (lmo0737, lmo1118, ORF2110, ORF2819 and prs). In order to facilitate and facilitate identification, this method was developed in order to easily distinguish serotypes and to more quickly classify *L. monocytogenes* into 5 serogroups (Borucki and Call, 2003; Doumith et al., 2004; Matle et al., 2020).

MLST (Multi-Locus Sequence Linkage) - this method, based on DNA sequencing, allows for the unambiguous identification of genetic changes by directly comparing allele sequences between samples, and sequencing information can be easily shared between laboratories. DNA

sequencing has provided a powerful solution for molecular typing using the 7-gene MLST (housekeeping genes) method for *L. monocytogenes* (Matle et al., 2020; Salcedo et al., 2003). Sequence types (ST) represent unique combinations of MLST alleles. Clonal complexes (CC) are groups of ST that differ by no more than one allele from another isolate belonging to the same CC (Henri et al., 2016; Ragon et al., 2008). This method enables a broader epidemiological investigation of *L. monocytogenes* and more precise tracing of the sources of specific strains during outbreaks (Chenal-Francisque V et al., 2015; Maiden, 2006; Moura et al., 2016; Ragon et al., 2008).

WGS (Whole Genome Sequencing) - Since 2005, NGS technology has improved the investigation of small-scale listeriosis outbreaks and is currently considered the new "gold standard" in the analysis of *L. monocytogenes* (Buermans and den Dunnen, 2014; Nadon et al., 2017). WGS provides information at the genomic level, allowing for highly discriminatory typing, including (cgMLST, wgMLST and SNP typing).

### *2.4.2 Serotyping*

*L. monocytogenes* strains group into 13 serotypes, strains of serotypes 1/2b, 4b, 3b, and 3c group into lineage I, while serotypes 1/2a, 1/2c, and 3a strains group into lineage II (Nadon et al., 2001). *L. monocytogenes* lineage III strains represent a third, distinct taxonomic group (Wiedmann, 2002), which predominantly includes serotype 4a and 4c strains (Nadon et al., 2001).

The serotyping of *L. monocytogenes* is based on variations in somatic (O) and flagellar (H) antigens, resulting in 13 distinct serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7). Serovars 1/2b, 3b, 4a, 4b, 4c, 4d, 5, 6a, 6b, and 7 contain H antigens A, B, and C. Additionally, serovars 1.2a, 1/2b, 12c, 3a, 3b, and 3c share O antigen II, while serovar 4ab, 4b, 4c, 4d, 4e, 5 6a and 6b all have O antigen (Chen et al., 2018b; Osek et al., 2022).

Serotyping has been crucial for evaluating the epidemiological strains of *L. monocytogenes* which has gained tremendous attention as a foodborne pathogen following a coleslaw-related outbreak in Canada. Currently, *L. monocytogenes* has 13 serotypes, with three 1/2a, 1/2b, and 4b accounting for over 90% of listeriosis cases. Among them, serotype 4b is responsible for slightly more than half of these cases (Borucki and Call, 2003). Table 3 present some of the possible sources of *L. monocytogenes* by serogroup (Kathariou, 2002).

Table 3. *L. monocytogenes* lineages, serotypes and potential sources.

| Lineage | Serotypes | Potential Source |
|---|---|---|
| I | 1/2b | Human Listeriosis cases |
| | 3b | |
| | 4b | |
| | 4d | |
| | 4e | |
| II | 1/2a | Food and environmental sources, animal listeriosis cases and sporadic human clinical cases |
| | 1/2c | |
| | 3a | |
| | 3c | |
| III | 4a | Animal sources |
| | 4c | |
| IIIc | 4c | Animal sources |
| IV | 7 and unusual 4a, 4b and 4c | Animal sources |

Adapted from (Kathariou, 2002).

Since serotype itself is not a virulence determinant and antisera used for serotyping are challenging to source and costly from a limited number of commercial suppliers, this method has become less favoured in outbreak investigations because of its low ability to differentiate strains. However, a few exceptions may apply:

1. The identification of a variant of serotype 4b, IVb (or 4bV), which appears to be expanding and has been associated with multiple recent outbreaks (Burall et al., 2017; Leclercq et al., 2011);

2. The emergence of a new serotype, 4h, within the hybrid sublineage of major lineage II (HSL-II), which displays hypervirulent characteristics and, in an orogastric mouse infection model, shows greater organ colonization capacity than well-known hypervirulent strains of *L. monocytogenes* (Yin et al., 2019); and

3. The use of molecular serotyping as a rapid approach to differentiate the four primary *L. monocytogenes* serovars commonly isolated from food and patients (1/2a, 1/2b, 1/2c, and 4b) into specific groups for investigative purposes or for managing critical control points in foodborne outbreaks (Doumith et al., 2004). Nevertheless, serotype information alone does not fully predict the health risk posed by strains of *L. monocytogenes*, limiting its value for epidemiological monitoring.

### *2.4.3 Phylogenetic lineages*

The clonal structure of *L. monocytogenes* is organized into four evolutionary lineages, with lineages I and II primarily linked to human disease (Nightingale et al., 2005; Orsi et al., 2011, Ward et al., 2008). The first clustering study using multilocus enzyme electrophoresis (MLEE) on *L. monocytogenes* isolates was conducted in 1989 (Piffaretti et al., 1989), classifying strains into specific phylogenetic divisions: clade I (Lineage I) comprised serovars 4b, 1/2b, and 4a, while clade II (Lineage II) included serovars 1/2a and 1/2c. Lineages III and IV were later identified as additional groups. Different genetic lineages show varied pathogenic potentials; historically, lineage I has been associated with severe disease and large outbreaks over 95% (Kathariou, 2002, Ward et al., 2008), whereas lineage II has been more often linked to isolated clinical cases. However, in some regions, lineage II is increasingly implicated in both clinical cases and outbreaks (Chen et al., 2018a; Orsi et al., 2011). Though lineages I and II contain most hypervirulent and hypovirulent strains of *L. monocytogenes*, respectively, lineage I also include some hypovirulent strains marked by *inlA* premature stop codons (PMSCs) (Doumith et al., 2004; Maury et al., 2016; Orsi et al., 2011). The *L. monocytogenes* species is divided into four molecular serotypes - IIa, IIc (Lineage II), IIb, and IVb (Lineage I) (Vitulo et al., 2013), as determined via the traditional, conventional serotyping methods.

### *2.4.4 Molecular serogroups*

Due to the time-intensive nature and subjective interpretation of traditional serotyping, efforts shifted toward faster, molecular-based methods. Until now, 13 serotypes and 4 molecular serogroups have been identified by serological and molecular methods (Doumith et al., 2005; Doumith et al., 2004), of which over 95% are the cause of invasive listeriosis in humans (Orsi et al., 2011; Vines and Swaminathan, 1998).

Initially, molecular serogroups were established using three primer sets along with a previously described primer set, effectively classifying 122 *L. monocytogenes* strains into groups [1/2a (3a), 1/2b, 1/2c (3c), 4b (d, e), and 4a/c] (Burucki and Call, 2003). Years later, *L. monocytogenes* was further divided into five molecular serogroups through multiplex PCR targeting specific genes - lmo1118, lmo737, ORF2110, and ORF2819 (Doumith et al., 2004), listed in the table 4. In 2005, in a study of 1204 strains collected from French food products, five molecular serotypes (IIa, IIb, IIc, IVa, IVb) were identified (Kérouanton et al., 2010).

Table 4. Classification of *Listeria monocytogenes* serogroups based on triplexes.

| Serogroup /serovars | Target genes | | | | | |
|---|---|---|---|---|---|---|
| | *ORF2819* | *ORF2110* | *Lmo1118* | *Lmo0737* | *plcA* | *prs* |
| IIa (1/2a, 3a) | - | - | - | + | + | + |
| IIb (1/2b, 3b) | + | - | - | - | + | + |
| IIc (1/2c, 3c) | - | - | + | + | + | + |
| IVb (4b, 4d, 4e) | + | + | - | - | + | + |
| IVb (atypical 4b) | + | + | - | + | + | + |
| Listeria spp. | - | - | - | - | - | + |

Adapted from (Doumith et al., 2004).

Additionally, triplex PCR with 5'-exonuclease in real-time proved to be an effective method for distinguishing these five serogroups and for differentiating *L. monocytogenes* from other *Listeria* species (Vitullo et al., 2013).

### *2.4.5 Clonal Complexes*

*L. monocytogenes* lineages can be further divided into various clonal complexes (CCs) based on multi-locus sequence typing (MLST). These CCs represent groups of genetically similar isolates believed to descend from a recent common ancestor, identified through genotypic methods such as MLST (Wiedmann, 2002). Recent epidemiological and microbiological research suggests that the gene content of a CC may be associated with its link to clinical disease. In 2016, Maury et al. reported that certain prevalent CCs were source-dependent: CC1, CC2, CC4, and CC6 were strongly linked to clinical cases, while CC121 and CC9 were commonly associated with food. Animal model studies in mice further categorized prevalent clones as infection-associated (CC1, CC2, CC4, and CC6), food-associated (CC9 and CC121), or intermediate (CC3, CC5, CC8, CC16, CC37, CC155) (Maury et al., 2016). These findings suggest that unique virulence or host specificity patterns may distinguish clonal groups, though reasons for differing infection potential remain unclear (Maury et al., 2016).

A clonal complex (CC) is defined by a 7-locus MLST framework, grouping strains whose sequence types (STs) differ by only one allele from at least one other ST in the group. This MLST-based grouping allows for detailed lineage sub-classification. In a global study examining 300 isolates from 42 countries across five continents, researchers identified 111 STs organized into 17 CCs, indicating the presence of globally distributed clones (Chenal-Francisque et al.,

2015). Broadly, three patterns emerge among major *L. monocytogenes* clones (Maury et al., 2019):

- Clones associated with hosts, often found in dairy products, show limited adaptation to food production environments and rarely carry genes for benzalkonium chloride tolerance (e.g., CC1 and CC4).
- Clones with low host adaptation persist effectively in food production environments, potentially due to reduced disinfectant sensitivity via benzalkonium chloride tolerance genes (e.g., CC9 and CC121). The co-selection of tolerance to disinfectants, such as quaternary ammonium compounds, alongside resistance to certain antibiotics (e.g., ciprofloxacin, gentamicin, amoxicillin) in *L. monocytogenes* could present emerging concerns for food safety and public health by heightening the risk of severe disease outcomes; and
- Intermediate clones, possibly transitioning from host-associated to saprophytic lifestyles, through either loss of virulence or acquisition of genes conferring disinfectant tolerance (e.g., CC2 and CC6).

### *2.4.6 Hypervirulent clonal complexes*

The primary hypervirulent clonal complexes, such as CC1, CC4, and CC6, are closely associated with human and animal clinical cases and are frequently linked to dairy products (Maury et al., 2019). Generally, these hypervirulent CCs (including CC1) demonstrate superior gut lumen and tissue colonization compared to their hypovirulent strains. Notably, CC1 shows adaptation for survival, persistence, and faecal shedding within the host. The enhanced growth of hypervirulent clones at 37°C in salty conditions may partially explain their superior gut colonization ability (Cardenas-Alvarez et al., 2019). Classifying clones as hypervirulent or hypovirulent can help food business operators (FBOs) and risk managers respond quickly and effectively during a food recall or listeriosis outbreak (Maury et al., 2019). Recently, novel hypervirulent sublineages linked to listeriosis in goats have been identified, carrying both *L. monocytogenes* LIPI-1 and a truncated LIPI-2 locus encoding sphingomyelinase (SmcL), along with additional chromosomal segments from *L. ivanovii*. These isolates possess a unique wall teichoic acid structure essential for resistance to antimicrobial peptides, bacterial invasion, and virulence (Quereda et al., 2021; Yin et al., 2019).

### *2.4.7 Multilocus Sequence typing*

Various sequence types (STs) have been implicated in foodborne listeriosis with some demonstrating a higher prevalence. For example, ST6 has been linked to several outbreaks worldwide (Table 9). Additionally, hypervirulent sequence types appear to be region-specific, such as ST87, which has been reported in China and Spain. Additionally, hypervirulent sequence types appear to be region-specific, such as ST87, which has been reported in China (Li et al., 2020) and Spain (Pérez-Trallero et al., 2014).

### *2.4.8 Whole genome sequencing*

Whole genome sequencing (WGS) has been the standard method for national surveillance of human listeriosis and monitoring of *L. monocytogenes* in food products. Retrospective sequencing of patient *L. monocytogenes* isolates from 2016 was conducted at the Center for Infectious Disease Control of the National Institute for Public Health and Environment (RIVM) (Friesema et al., 2023). While isolates from 2010 to 2015 were sequenced during the ELiTE study (European *Listeria* Typing Exercise Extension to Whole Genome Sequencing) led by the European Center for Disease Prevention and Control (Friesema et al., 2023; Walle et al., 2018). From 2016 to 2019, sequencing of both human and food isolates was carried out by a commercial sequencing service using Illumina HiSeq (2×100 bp) and NovaSeq (2×150 bp) platforms. From 2020 onwards, sequencing was performed at RIVM with Illumina NextSeq (2×150 bp). All sequences underwent quality control and were de novo assembled using an in-house pipeline. Sequences with a phred score above 30, draft genome lengths between 2,700,00 and 3,230,000 bp, N50 greater than 10,000 bp, GC content ranging from 37.6% to 38.2%, and average read coverage of at least 10× were included in further analysis. Multilocus sequence typing (MLST) of seven loci (Ragon et al., 2008) and core genome MLST cgMLST as described by Ruppitsch et al. (Ruppitsch et al., 2015) as well as sequence type (ST), and clonal complex (CC) as described by Ragon et al. (Ragon et al., 2008) were determined using Ridom SeqSphere+ (version 5.0.0, Ridom GmbH, Münster, Germany). Assembled genomes were considered high quality if they identified 98.1–100% of loci (<33 loci missing) and were included in subsequent analyses. Pairwise distances were calculated as Hamming distances across all cgMLST loci, with clusters defined using single-linkage hierarchical clustering and a maximum threshold of seven allelic differences across 1701 loci (Ruppitsch et al., 2015).

These clusters, although independently calculated, correspond to cgMLST types reported in the literature and were denoted using a combination of CC and cluster labels (e.g., CC1_613). Clusters were considered persistent if sequences from the same cluster were detected over a period exceeding one year, regardless of continuity. Genetic differentiation of *L. monocytogenes* between and within food categories was analyzed using allele frequencies from the 1701 cgMLST loci, employing the R package hierfstat (v 0.5–10) (Goudet, J. and Jombart, 2020). For source attribution, human *L. monocytogenes* sequences without an identified food source cluster were linked to specific food categories based on the minimum pairwise Hamming distances. The comparison between cgMLST and pulsed-field gel electrophoresis (PFGE) typing was assessed using the Fowlkes-Mallows (FM) index (Fowlkes and Mallows, 1983), implemented in the R package profdpm v 3.3 (Shotwell, 2013). Concordance was evaluated for pulsotypes defined by ApaI (95% similarity), AscI (85% similarity), the combination of both enzymes (ApaI/AscI), and varying allele difference thresholds for cgMLST (1 to 50 alleles). Cluster correspondence was visualized through summary statistics and persistence data using the R package alluvial v 0.1–2 (Bojanowski and Edwards, 2016).

## 2.5 Regulation - Food Microbiology Criteria

The criteria for determining whether *L. monocytogenes* levels in a food product are acceptable for sale and consumption are outlined in the EU Regulation on Microbiological Criteria for Foodstuffs (No. 2073/2005). The Republic of Kosovo (RKS) established a legal framework in 2012, aligning with EU Regulation No. 2073/2005 through Regulation No. 27/2012, which was later updated with Regulation No. 09/2018. While some countries, such as the United States, enforce a zero-tolerance policy for the presence of *L. monocytogenes*, others, including the European Union, Canada, Australia, and New Zealand, permit minimal amounts of the pathogen in food products that do not support its growth. Notably, these more lenient standards do not necessarily correlate with higher listeriosis incidence rates (Dufour, 2011; FSIS, 2014; Guston, 2012; Kayode and Okoh, 2022a; Zhang et al., 2020).

Chapter 1 of the food safety criteria regarding *Listeria monocytogenes* focuses on products intended for direct human consumption without the need for cooking or other processing

to reduce harmful microorganisms to acceptable levels. These products are categorized as follows:

- **Category 1.1**: Ready-to-eat (RTE) foods for children and special medical purposes.

- **Category 1.2**: Foods that support the growth of *L. monocytogenes* intended for children and special medical purposes.

- **Category 1.3**: Foods that inhibit the growth of *L. monocytogenes* intended for children and special medical purposes.

The criteria for these categories apply to all products throughout their shelf life. For products in Category 1.2 (those that support the growth of *L. monocytogenes*), the criteria must be met before the product leaves the immediate control of the food business operator (FBO). It is the responsibility of the FBO to conduct inspections and ensure compliance with regulatory standards. Under Regulation 2073/2005, *L. monocytogenes* must not be present in foods intended for infants or for special medical purposes. For other RTE foods, different criteria are applied based on the food's composition (e.g., water activity (aw), pH, and NaCl concentration). Stricter standards are imposed on RTE foods that can support the growth of *L. monocytogenes*. Testing methods, batch sample requirements, and legal limits for *L. monocytogenes* in various RTE food categories, as outlined in Regulation (EC) No. 2073/2005, are summarized in Table 5.

Table 5. Microbiological criteria for *L. monocytogenes* in RTE foods according to Regulation (EC) No. 2073/2005.

| Food Category | Microorganisms their toxins, metabolites | Sampling plan | | Limits | | Analytical reference method | Stage where the criteria apply |
|---|---|---|---|---|---|---|---|
| | | N | c | M | M | | |
| 1.1. Ready-to-eat foods intended for infants and ready-to-eat foods for special medical purposes (1) | *Listeria monocytogenes* | 10 | 0 | Absence in 25 g | | EN/ISO 11290-1 | Products placed on the market during their shelf-life |
| 1.2. Ready-to-eat foods able to support the growth of L.monocytogenes, other than those intended for infants and special medical purposes | | 5 | 0 | 100 cfu/g (2) | | EN/ISO 11290-2 (3) | Products placed on the market during their shelf-life |
| | | 5 | 0 | Absence in 25 g (4) | | EN/ISO 11290-1 | Before the food has left the immediate control of the food business operator, who has produced it |
| 1.3. Ready-to-eat foods unable to support the growth of L.monocytogenes, other than those intended for infants and special medical purposes (1) (5) | | 5 | 0 | 100 cfu/g (2) | | EN/ISO 11290-2 (3) | Products placed on the market during their shelf-life |

(1) **Exemptions from Regular Testing**: Routine testing for *Listeria monocytogenes* is generally not required for certain ready-to-eat (RTE) foods under normal circumstances. These include:

– Foods that undergo heat treatment or other processes that effectively eliminate *L. monocytogenes*, provided recontamination is impossible (e.g., products heat-treated in their final packaging).

– Fresh, uncut and unprocessed fruits and vegetables, excluding sprout seeds – bread, biscuits and similar products,

– Bottled or packaged waters, soft drinks, beer, cider, wine, spirits and similar products,

– Sugar, honey and confectionery, including cocoa and chocolate products, - live bivalve molluscs.

(2) **Limit of 100 CFU/g at End of Shelf-Life**: This criterion applies if the producer can demonstrate to the satisfaction of the relevant authority that the product will not exceed *L. monocytogenes* levels of 100 CFU/g by the end of its shelf life.

(3) **Testing Procedure**: For testing, 1 ml of inoculum is plated either on a 140 mm Petri dish or a 90 mm Petri dish.

(4) **Control Before Distribution**: If the food business operator cannot demonstrate compliance with the 100 CFU/g limit throughout the product's shelf life, this criterion must be applied before the product leaves their immediate control.

(5) **Automatic Inclusion in Low-Risk Category**: Products with the following characteristics are automatically considered low-risk:

–pH ≤ 4.4 or water activity (aw) ≤ 0.92.

–pH ≤ 5.0 and aw ≤ 0.94.

–Shelf life of less than five days.

–Other products may also be classified as low risk if supported by scientific evidence.

## 2.6 Listeriosis

This bacterium is recognized as a major foodborne pathogen and causes listeriosis, an illness that often occurs in the form of sporadic infections and is associated with high mortality rates in susceptible individuals (Buchanan et al., 2017). *L. monocytogenes* and *L. ivanovii* are recognized pathogenic species within the *Listeria* genus. While *L. monocytogenes* predominantly causes listeriosis in humans and animals, *L. ivanovii* mainly affects ruminants, with rare sporadic cases in humans (Chen et al., 2017; Kayode et al., 2020). Other *Listeria* species, such as *L. innocua* and *L. grayi*, have been occasionally implicated in human infections, typically under conditions of predisposing health factors (Cummins et al., 1994; Guillet et al., 2010; Spickler, 2019). Historically, listeriosis was rare, and its epidemiology was poorly understood. However, from the late 1970s to the early 1980s, increasing cases and epidemic outbreaks in North America and Europe highlighted *L. monocytogenes* as a significant pathogen for foodborne diseases (Farber and Peterkin, 1991; Goulet et al., 1998; Vaillant et al., 2005). Despite its low annual incidence compared to other foodborne pathogens, invasive listeriosis poses a severe threat, particularly when ingestion involves RTE foods containing more than 2,000 CFU/g of the bacterium. Notably, about one-third of cases are linked to pathogen growth during the consumer phase (Kayode and Okoh, 2022a). In the European Union (EU) in 2022, *L. monocytogenes* was considered the cause of the most serious human zoonosis that mainly comes from contaminated food, with 2738 confirmed cases of the disease and 35 outbreaks of listeriosis of foodborne origin. During this period in the EU, among hospitalized patients, a mortality rate of (15.9%) was reported (EFSA&ECDC, 2023).

The majority of listeriosis cases occur in immunocompromised individuals, pregnant women, newborns, and the elderly, often referred to as ‘at-risk population’ (Buchanan et al., 2017; Hamon et al., 2006; Mylonakis et al., 2002; Wambogo et al., 2020). Manifestation of listeriosis illness can occur in various forms, including mild gastroenteritis to more serious cases of meningitis, encephalitis, and septicemia (de Noordhout et al., 2014). Infected pregnant women can also experience miscarriages, stillbirths, preterm birth, neonatal sepsis, and meningitis (Hassoun et al., 2014; Silk et al., 2013). Unlike other foodborne pathogens, it is uncommon for healthy individuals to experience severe listeriosis symptoms after consumption of contaminated foods (de Noordhout et al., 2014; Mook et al., 2011). However, febrile gastroenteritis can occur in healthy individuals that have ingested foods contaminated with a high number of *L.*

*monocytogenes* cells (Dalton et al., 1997; Maurella et al., 2018; McIntyre et al., 2015). Symptoms associated with febrile gastroenteritis can include stomach cramps, sleepiness, headache, joint and muscle pain, fever, nausea, diarrhea, and vomiting (Aureli et al., 2000; Carrique-Mas et al., 2003). Although the number of *L. monocytogenes* infections is relatively low (1,600 illnesses/year in the U.S.) compared to other foodborne pathogens (39-fold lower than pathogenic E. coli and 625-fold lower than Salmonella infections; (Scallan et al., 2011), the high morbidity and mortality (19-23%) rates associated with vulnerable populations demands an equitable amount of attention.

The virulence of *L. monocytogenes* in ingested food, along with the health status of the consumer, plays a crucial role in determining the outcome of infection (Gambarin et al., 2012). Both invasive and non-invasive forms of listeriosis can occur across the general population. The diagnosis of these forms differs: the invasive form is confirmed by isolating *L. monocytogenes* from sterile sites such as blood or cerebrospinal fluid, while the non-invasive gastrointestinal form is identified through the presence of *L. monocytogenes* in faeces (Gambarin et al., 2012, Kayode and Okoh, 2022a; Schoder et al., 2022). Approximately 5% of the population are asymptomatic faecal carriers of this bacterium. Although the gastrointestinal tract is the primary entry point for foodborne *L. monocytogenes*, the disease manifests predominantly as meningitis or septicemia (Vázquez-Boland et al., 2001). Healthy individuals may experience mild, flu-like symptoms or gastroenteritis, often without recognizing the infection. While the infectious dose is not definitively known, it is believed to be significantly lower in individuals with weakened immunity, with estimates suggesting as few as 100 bacterial cells can trigger illness (Vázquez-Boland et al., 2001). As an invasive intracellular pathogen, *L. monocytogenes* utilizes various adhesion and invasion mechanisms to colonize the gastrointestinal tract and penetrate the intestinal barrier (Osek and Wieczorek, 2022). It invades through junctions between mucus-secreting goblet cells and enterocytes, via epithelial M cells in Peyer's patches, or at the tips of intestinal villi, where apoptotic epithelial cells extrude into the lumen (Radoshevich et al., 2015; Radoshevich and Cossart, 2018). Once ingested, the bacterium crosses the intestinal wall into the circulatory system and is transported via the portal vein to the liver and spleen, where it replicates. It then disseminated throughout the body via the bloodstream, infecting the brain and placenta. In pregnant women, this can lead to fetal infection (Vázquez-Boland et al., 2001). In hosts with impaired cell-mediated immunity, *L. monocytogenes* can cause bacteremia, cross the blood-brain barrier to induce meningitis or traverse the placenta during pregnancy, leading to invasive

listeriosis (Disson and Lecuit, 2012; Osek and Wieczorek, 2022; Sibanda et al., 2022). The pathophysiology of this infection highlights its complex invasion strategies and the severe systemic consequences of listeriosis is illustrated in Figure 4.

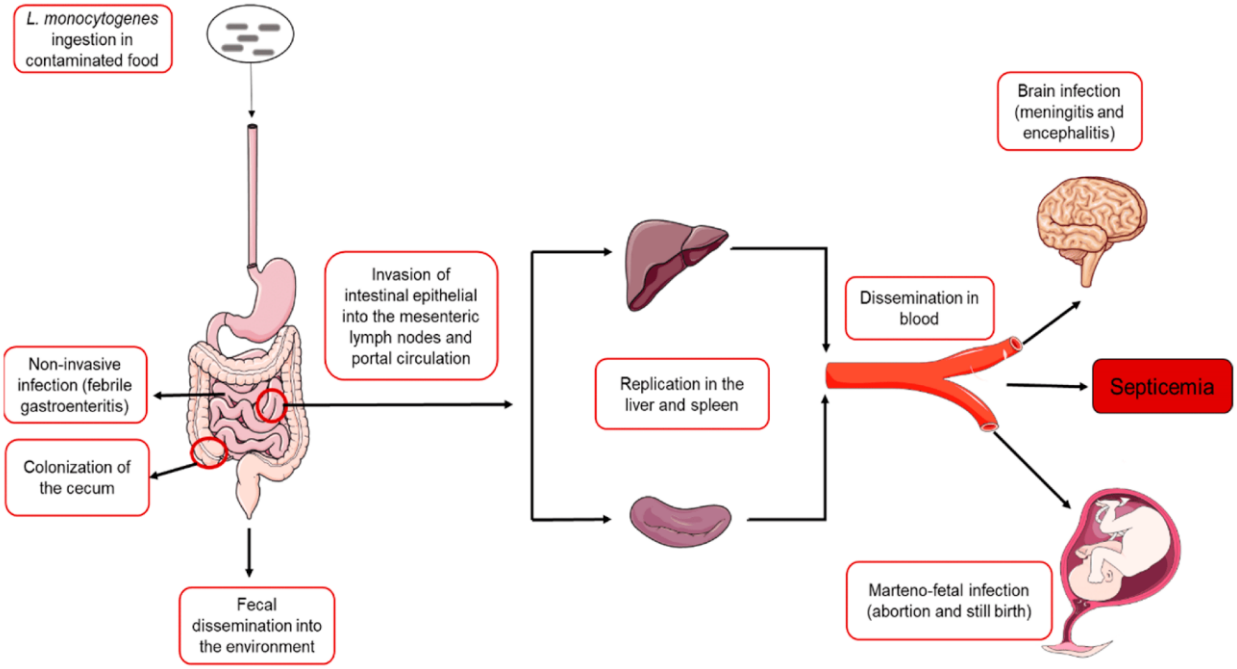



Figure 4. Illustrating the human infection cycle of *L. monocytogenes.* This figure was taken from Servier Medical art (https://smart.servier.com (accessed March 1, 2022), (Sibanda et al., 2022).

The pathogenic mechanism of *L. monocytogenes* involves four major steps: adhesion and invasion of host cells, vacuole escape, intracellular replication, and cell-to-cell spread (Kim and Bhunia, 2008; Petrisic et al., 2021; Osek and Wieczorek, 2022), summarized in Figure 5. The primary virulence factor facilitating intracellular infection is listeriolysin O (LLO), a pore-forming toxin belonging to the cholesterol-dependent cytolysin family (Heuck et al., 2010). Other critical virulence factors include ActA, which promotes actin polymerization, and phospholipases, which aid in the pathogen's survival and dissemination within the host (Vázquez-Boland et al., 2001).

Virulence regulation is primarily controlled by six genes including prfA, PI-PLC, LLO, Mpl, ActA and PC-PLC which are part of the PrfA-dependent virulence gene cluster. Additional virulence-related genes, such as those encoding internalins are located outside this cluster (Vázquez-Boland et al., 2001).

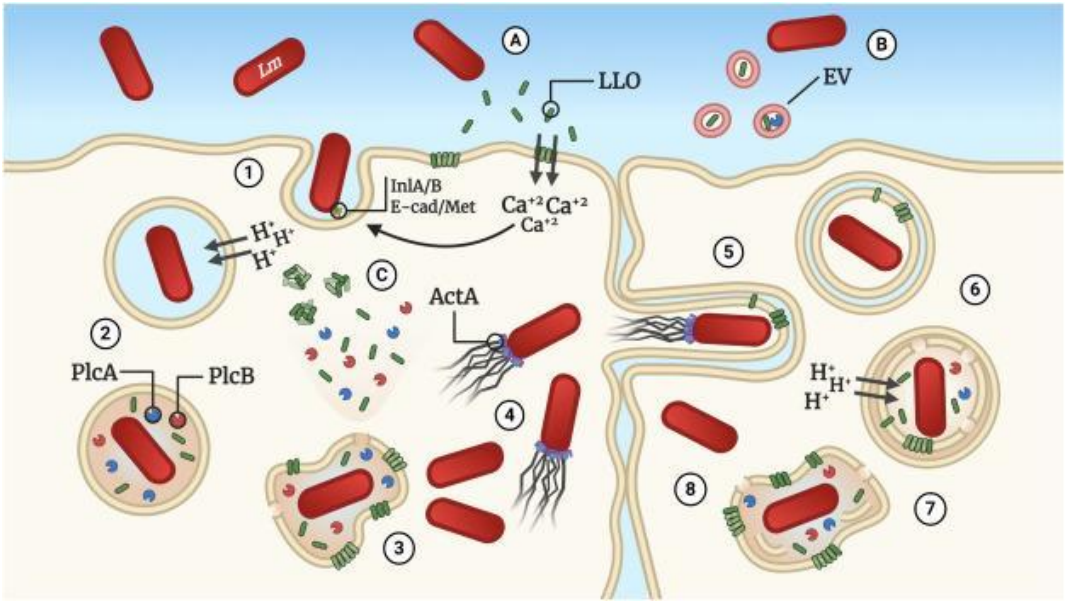



Figure 5. The intracellular life cycle of *L. monocytogenes* and the use of its various virulence factors (Petrisic et al., 2021).

### *2.6.1 Epidemiology and global incidence of Listeriosis*

The increase in listeriosis cases across developed regions has been widely reported in the literature, reflecting a concerning trend over the past decade. According to the latest ECDC report (2022), there has been a continuous rise in the notification rate of cases in EU/EEA countries, reaching 0.62 cases per 100,000 populations in 2022 one of the highest incidences recorded since the start of EU-level surveillance in 2007. This increase is particularly notable in countries such as Germany, France, Spain, and Italy, which reported a high number of cases and fatalities compared to previous years (ECDC, 2022).

Earlier studies from the CDC also report a stable but lower incidence in the United States, at approximately 0.3 cases per 100,000 populations per year (CDC, 2013; Silk et al., 2012). Despite the lower incidence in the U.S., listeriosis remains a severe infection with high hospitalization and mortality rates, particularly among vulnerable population groups. The literature also highlights that ready-to-eat foods and meat and dairy products are among the most common sources of contamination (Kayode and Okoh, 2022b, 2022c), drawing attention to food safety across all stages of the production chain.

Furthermore, the rise in reported cases may be partly attributed to improved diagnostic and monitoring systems, as well as to enhanced national and international reporting protocols.

However, the literature suggests that despite these improvements, controls and interventions in the food production and processing sectors remain inconsistent or insufficient in certain countries. ECDC reports indicate significant differences among member states in terms of detection capacity, implementation of *L. monocytogenes* control policies, and the resources dedicated to infection prevention (ECDC, 2022).

In this context, a range of studies recommend integrated approaches involving reference laboratories, efficient systems for tracing contamination sources, and harmonization of food safety standards (EFSA, 2022; WHO/FAO, 2021). Systematic studies on the incidence, distribution, and trends of listeriosis at the global level remain essential to better understand the spread of the disease and to enable evidence-based public health interventions.

Its epidemiology includes the study of its incidence in the United States is approximately 0.3 cases per 100,000 people, with around 1,600 cases reported each year, according to the (Rogalla D, 2024). Geographic differences in incidence often correlate with regional dietary habits, particularly the consumption of unpasteurized dairy products and undercooked meats. Contaminated food is the primary source of *L. monocytogenes*, which is capable of growing at refrigeration temperatures, making it persistent in the food supply chain (Jackson et al., 2018; Li et al., 2024). Foods such as unpasteurized milk, and soft cheeses. Raw meats and processed items like deli meats and hot dogs are common vectors of contamination. Transmission occurs mainly through ingestion, though environmental exposure to contaminated soil or water and food-processing environments can also contribute. However, direct person-to-person transmission is rare (Gilmour et al., 2010; Liu et al., 2020). Certain populations are disproportionately affected. Pregnant women face a 20-fold increased risk, with potential outcomes including miscarriage, stillbirth, or preterm delivery (Kayode et al., 2020). The elderly and immunocompromised individuals are also at heightened risk, often experiencing severe complications such as meningitis and sepsis (Jacquet et al., 2000). Listeriosis exhibits seasonal and regional variability, with higher case rates during summer and early fall, attributed to increased consumption of fresh produce and high-risk foods (War et al., 2022). Countries with rigorous food safety regulations tend to report lower incidence rates compared to those with less stringent measures (Boatemaa et al., 2019). Outbreaks, such as the 2011 U.S. outbreak linked to contaminated cantaloupes, highlight the challenges of controlling Listeria in the food supply and emphasize the need for robust food safety practices (Belias et al., 2024).

## 2.7 Foodborne listeriosis: Global and regional perspectives

### *2.7.1 Epidemiology and impact of listeriosis*

In the context of listeriosis outbreaks, numerous studies have identified a strong association with the consumption of ready-to-eat (RTE) foods, particularly those that undergo minimal processing. Soft cheeses and, most notably, RTE meat products have been frequently implicated as common sources of contamination (Fagerlund et al., 2020).

While the incidence of listeriosis is low, it is a severe disease with a reported case fatality rate of 18.1% in Europe in 2022 (EFSA&ECDC, 2023) and a similar mortality rate has been reported in the United States over the past decade (15.3%) (Mohapatra et al., 2024). One major reason for listeriosis outbreaks reported in recent investigations (Gilmour et al., 2010) is the persistence of *L. monocytogenes* in food processing environments (Carpentier and Cerf, 2011). Listeriosis is a burden to society, public health and the economy (Olanya et al., 2019; Thomas et al., 2015) and requires constant molecular surveillance to link human cases to the food vehicles and their sources of contamination.

*L. monocytogenes* as one of the most concerning foodborne pathogens due to its severe clinical outcomes and significant economic burden. Although less common than other foodborne agents, listeriosis results in a disproportionately high number of hospitalizations and deaths. According to the Centers for Disease Control and Prevention (CDC, 2014), listeriosis contributes to a notable share of the estimated 3,000 annual deaths caused by foodborne illnesses in the United States. From an economic perspective, *L. monocytogenes* imposes one of the highest costs among foodborne pathogens. In a comprehensive analysis by the Economic Research Service of the United States Department of Agriculture, estimated that the annual economic burden of listeriosis alone amounts to approximately $3.19 billion. This figure includes direct medical costs, productivity losses, and the economic value of premature deaths (Hoffmann et al., 2015). The high cost reflects not only the severity of the disease but also the challenges in controlling *L. monocytogenes*, particularly in ready-to-eat food products where post-processing contamination can occur.

Listeriosis is one of the most severe foodborne diseases, characterized by low incidence (about three cases per million people annually) but a high hospitalization rate (94%) and significant mortality (18%), according to reports from the European Food Safety Authority (EFSA) and the European Center for Disease Prevention and Control (ECDC, 2022). Outbreaks

of listeriosis are increasingly detected, particularly in high-income countries where robust surveillance systems and diagnostic capabilities exist (Ricci et al., 2018). To enhance the detection, investigation, and control of such outbreaks, whole genome sequencing (WGS) is now widely adopted by public health and food regulatory authorities. WGS provides precise identification of outbreak-related cases and accurate attribution of the outbreak source, even differentiating strains with fewer than 50-100 single nucleotide polymorphisms (SNPs) (Brown et al., 2019).

Polyclonal outbreaks, where multiple pathogenic strains are linked to a single food source, highlight the complexity of managing foodborne illnesses. For example, in 2015, an outbreak in the United States linked to contaminated ice cream revealed two different *L. monocytogenes* clones. The largest recorded listeriosis outbreak occurred in South Africa from 2017 to 2018, with 1,060 reported cases over 1.5 years (Conrad et al., 2023).

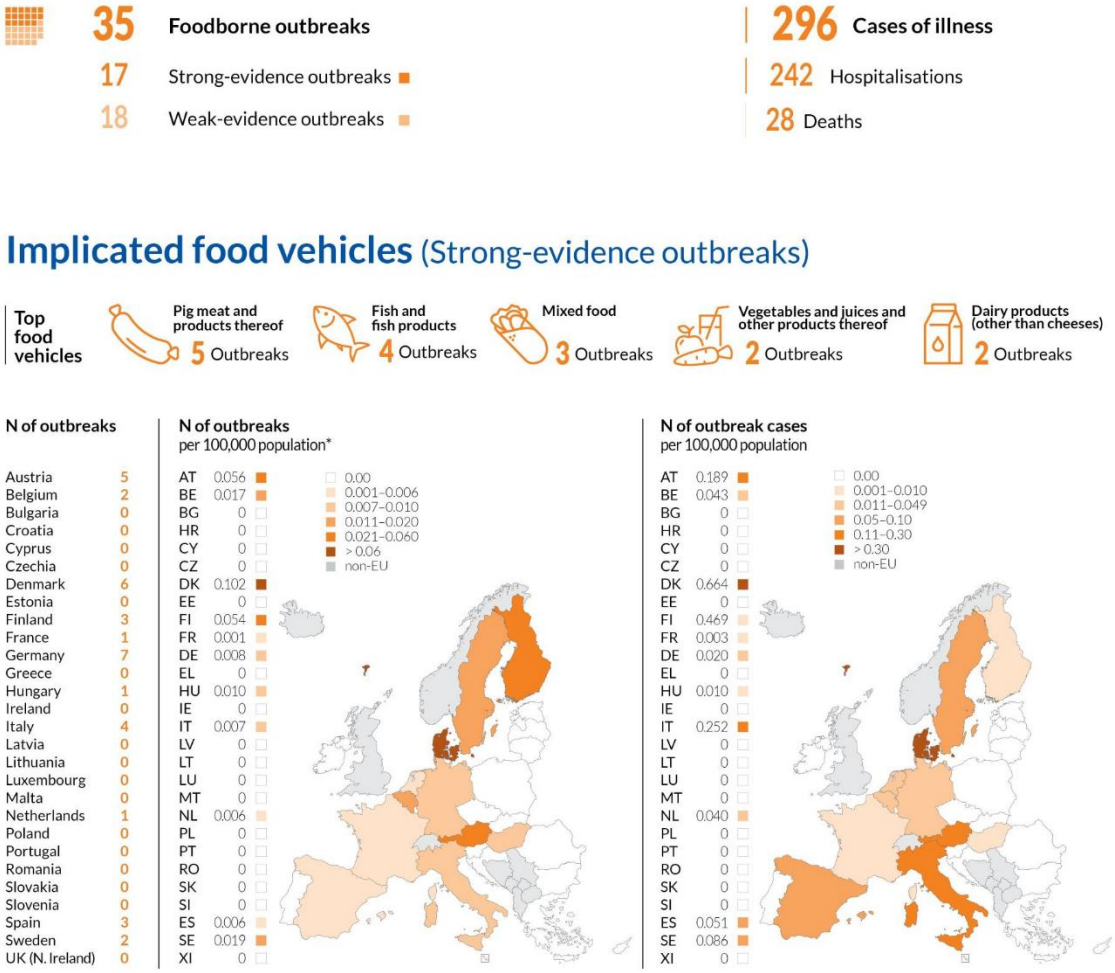



Figure 6. Data from the European Union One Health 2022 Zoonoses Report – Foodborne outbreaks and related cases.

Investigations identified a single sequence type (ST6) in 93% of cases, with 216 deaths among 806 patients, resulting in a case-fatality rate of 27% (Olanya et al., 2019). Foodborne outbreaks and associated cases are reported in the 2022 "European Union Zoonoses Report - One Health", Figure 6.

*L. monocytogenes* causes two distinct types of illnesses: Invasive listeriosis, commonly referred to as listeriosis, and non-invasive listeriosis, known as febrile gastroenteritis (Kayode et al., 2020). The incubation period for invasive listeriosis ranges from 1 to 67 days but varies significantly depending on clinical presentation. Pregnancy-related cases last between 17-67 days, the central nervous system (CNS) infection is between 1-14 days while bacteremia infection is between 1-12 days (Goulet et al., 2013; Hernandez-Milian and Payeras-Cifre, 2014). In pregnant women, complications may include miscarriage, perinatal infections, or neonatal illnesses like sepsis and meningitis (Kayode et al., 2020). The infectious dose required for invasive listeriosis ranges between 105 and 109 colony-forming units (CFU) (Farber et al., 1996). The incubation period for non-invasive listeriosis ranges from 6 to 240 hours, averaging 24 hours (Goulet et al., 2013). Symptoms typically include diarrhoea, fever, muscle pain, headache, abdominal cramps, and vomiting. These symptoms generally subside within a week (Ooi and Lorber, 2005). The infectious dose for non-invasive listeriosis is approximately 106 CFU (Ooi and Lorber, 2005; Ricci et al., 2018). Both forms underline the importance of proper food safety practices to prevent contamination and outbreaks.

### *2.7.2 Food vehicles and contamination sources*

In recent years, ready-to-eat (RTE) foods, dairy products (especially cheese), smoked fish, cooked and marinated items, meat products, and vegetables have been identified as major sources of listeriosis outbreaks (Martinez-Rios and Dalgaard, 2018; Meloni et al., 2009). Within the EU, *L. monocytogenes* was detected in 2.1% of tested samples (60,952 units), with contamination rates varying between members states. Most data originated from Poland (69.1%) and several other countries including Bulgaria, Italy, and Spain (EFSA & ECDC, 2023). Historically, soft cheese consumption was linked to nearly half of sporadic listeriosis cases in France, but prevention measures reduced these by 68% between 1987 and 1997 (Goulet et al., 2001). This progress was supported by molecular surveillance linking food and human cases (Moura et al., 2017) and the enforcement of EU microbiological criteria (EC, 2005).

Among analyzed samples, 59.8% were related to four animal species: pigs, cattle, broilers, and turkeys, with pig products, especially RTE meats, being the most frequently tested (52.7%) (EFSA & ECDC, 2023). In 2022, the EU reported 2,738 listeriosis cases, including 12 travel-associated infections and 948 cross-country transmissions, resulting in 1,330 hospitalizations and 286 deaths (ECDC, 2022).

Notable listeriosis outbreaks worldwide have been linked to various food products, such as pasteurized milk cheese in Germany 189 cases, 27 deaths, (Koch et al., 2010), RTE deli meats in Canada 57 cases, 22 deaths (Gilmour et al., 2010), cantaloupes in the USA 147 cases, 33 deaths (Laksanalamai et al., 2012), processed meat in South Africa 1,060 cases, 216 deaths, (Thomas et al., 2020), and packaged salads and ice cream in the USA (ECDC, 2013–2022). Table 6 summarizes these outbreaks by source, cases, deaths, country, and year.

Table 6. Listeriosis outbreaks associated with *Listeria monocytogenes*.

| Source | Number of cases | Number of deaths | Country | Year | Reference |
|---|---|---|---|---|---|
| Cheese (pasteurized milk) | 189 | 27 | Germany | 2006-2007 | Koch et al., 2010 |
| Scalded sausages | 16 | 5 | Germany | 2006-2007 | Winter et al., 2009 |
| Pasteurized milk | 5 | 3 | USA (Massachusetts) | 2007-2008 | CDC, 2008 |
| Brie and camembert cheese | 165 | 14 | Chile | 2008 | Montero et al., 2015 |
| Cheese (pasteurized milk) | 38 | 2 | Canada | 2008 | Gaulin et al., 2012 |
| Jellied pork | 12 | 0 | Austria | 2008 | Pichler et al., 200 |
| RTE deli meats | 57 | 22 | Canada | 2008 | Gilmour et al., 2010 |
| Beef meat | 8 | 2 | Denmark | 2009 | Smith et al., 2011 |
| Quargel cheese | 34 | 8 | Austria, Germany, Czech Republic | 2009-2010 | Fretz et al., 2010 |
| Cantaloupes | 147 | 33 | USA (28 states) | 2011 | Laksanalamai et al., 2012 |
| Cheese (ricotta) | 22 | 4 | USA (14 states) | 2012 | Heiman et al., 2016 |
| Smoked fish | 20 | 7 | Denmark | 2013-2015 | Gillesberg et al., 2016 |
| Prepackaged caramel apples | 35 | 7 | USA (12 states) | 2014 | Salazar et al., 2016 |
| Ice cream | 10 | 3 | USA (4 states) | 2015 | Pouillot et al., 2016 |
| Soft cheeses | 30 | 3 | USA (10 states) | 2015 | ECDC, 2015 |
| Packaged salads | 19 | 1 | USA (9 states) | 2016 | ECDC, 2013 |

| | | | | | |
|---|---|---|---|---|---|
| Salmon | 4 | 1 | Denmark | 2017 | FQN, 2017 |
| Processed meat | 1060 | 216 | South Africa | 2017-2018 | Thomas et al., 2020 |
| Rockmelons | 20 | 7 | Australia | 2018 | WHO, 2018 |
| Ready-to-eat meat | 21 | 3 | Netherlands, Belgium | 2018-2019 | ECDC&EFSA, 2019 |
| Bloody sausages | 112 | 2 | Germany | 2018-2019 | Halbedel et al., 2020 |
| Chilled roasted pork meat | 222 | 3 | Spain | 2019 | Gomez-Laguna et al., 2020 |
| Enoki mushrooms | 36 | 4 | USA (17 states) | 2020 | ECDC, 2020 |
| Packaged salads | 18 | 3 | USA (13 states) | 2021 | ECDC, 2021 |
| Ice cream | 23 | 1 | USA (10 states) | 2022 | ECDC, 2022a |
| Ready-to-eat fish products | 17 | 2 | Austria, Belgium, Italy, Germany, and the Netherlands | 2022-2023 | EFSA, 2023 |
| Queso Fresco and Cotija Cheese | 26 | 2 | USA (11 states) | 2024 | ECDC, 2024 |

### *2.7.3 Regional focus: The Republic of Kosovo*

The Republic of Kosovo (RKS) is a small-sized, newly-established country in the Balkans, with small-scale farming and food processing facilities and a need for economic growth to satisfy the needs of its population, especially in the sector of animal production. As animal production grows, this growth will need to be supported by a robust surveillance system for foodborne pathogens, including molecular surveillance to mirror requirements in the EU countries. Prior to this study, there was a paucity of information regarding molecular typing data on bacterial isolates circulating in Kosovo, including those originating from and from clinical cases of listeriosis. Limited data have been published on the prevalence of *L. monocytogenes* in North Macedonia (Jankuloski et al., 2010) and in Serbia (Jovanović et al., 2022). For countries surrounding RKS, the prevalence of *L. monocytogenes* in meat RTE products of beef origin was between 2.7-3.9%, and in hard cheeses was 4.6% (EFSA&ECDC, 2023). Listeriosis cases from animals and levels of contamination in foods have already been reported in Kosovo from ovine clinical cases (Hamidi et al., 2020), on-farm milk products (Mehmeti et al., 2017), retail cheeses (Studenica et al., 2022), and the meat sector (Kukleci et al., 2019). However, these studies did not provide molecular characterization of the strains and were conducted in a limited number of retail sites, processing plants, or farms.

In recent years, foods in general, especially RTE, are the main causes involved in listeriosis outbreaks. Ready-to-eat foods, dairy products, especially cheeses, smoked fish, cooked

marinated products, meat products, and vegetables were found to be contaminated with *L. monocytogenes* (Martinez-Rios & Dalgaard, 2018; Meloni et al., 2009). In the EU, the overall occurrence of *L. monocytogenes*, all matrices included, was 2.1% (N = 60,952 tested units for detection), varying from 0% to 13.8% according to the MS. 69.1% of reported data were provided by Poland. Excluding Poland, five MSs contributed 76% of the remaining reported data (Bulgaria, Czech, Italy, Romania and Spain). In all, 59.8% of tested units were assigned to the four main animal species (pigs, bovine animals, broilers, and turkeys). The remaining 40.2% of tested sampling units were reported from other or unspecified animals. RTE meat and meat products from pigs were the most frequently tested (52.7%). RTE meat and meat products from bovine animals, broilers and turkeys represented 4.4%, 1.8% and 0.21% of all tested units, respectively (EFSA&ECDC, 2023). In EU countries during 2022, about 2738 cases of listeriosis were registered, of which 12 cases were from non-EU countries and 948 cases from transmission through people from different countries. From this number, about 1330 cases were hospitalized, of which 286 ended in death (ECDC, 2022).

## 2.8 Resistance of *L. monocytogenes* to different antimicrobials

Antimicrobial agents have been used in a wide range of settings to eliminate or inhibit bacterial growth. Most of these compounds target unique bacterial cell features including cell wall synthesis, the bacterial membrane, particular stages of protein synthesis, DNA and RNA synthesis, and folic acid metabolism, and depending on the nature of the drug, these can result in bacterial cell death or the inhibition of growth (Wright, 2010).

In recent times, the use of compounds with bacteriostatic and bactericidal effects against *L. monocytogenes* is growing unchecked. The tendency of this phenomenon is increasing by using antimicrobials of synthetic, semi-synthetic and natural nature and their unrestricted application is causing the phenomenon of antimicrobial resistance (Founou et al., 2016; Olaimat et al., 2018; Srinivasan et al., 2005). In the fight against *L. monocytogenes,* the antibiotics that are applied and which are considered the "gold standard of treatment" are from the group of aminopenicillins (ampicillin or amoxicillin), benzylpenicillin (penicillin G and gentamicin) used in combination with aminoglycosides, trimethoprim alone (or combined with sulfamethoxazole), erythromycin and tetracyclines (Caruso et al., 2020; Granier et al., 2011; Scortti et al., 2006). Vancomycin, trimethoprim-sulfamethoxazole, erythromycin, carbapenems or piperacillin-

tazobactam respectively are given as alternative therapy in the immunocompromised population or in pregnant women (Pagliano et al., 2017; Thønnings et al., 2016).

Antimicrobial resistance (AMR) in *L. monocytogenes*, the bacterium responsible for listeriosis, is an emerging concern in both clinical treatment and food safety management. The increasing occurrence of AMR in *L. monocytogenes* complicates treatment options, posing significant challenges to public health (Rippa et al., 2024).

Traditionally, *L. monocytogenes* has been susceptible to antibiotics such as ampicillin and penicillin. However, recent studies indicate a growing resistance to these antibiotics. For instance, a study found that 89.5% of *L. monocytogenes* isolates exhibited resistance to ampicillin, and 47% showed resistance to penicillin (Hanes and Huang, 2022). Additionally, all 17 *L. monocytogenes* strains in another study were resistant to nalidixic acid, ampicillin, penicillin G, linezolid, and clindamycin (Sanlibaba et al., 2018).

The mechanisms behind this resistance include the production of β-lactamases, which deactivate β-lactam antibiotics like penicillins, and alterations in penicillin-binding proteins (PBPs) that reduce antibiotic binding efficacy (Luque-Sastre, L., 2018). These adaptations not only hinder clinical treatment but also complicate food safety management, as *L. monocytogenes* is a common contaminant in various food products (Luque-Sastre, L., 2018).

Bacteria possess two types of resistance: intrinsic, or naturally occurring, resistance and acquired resistance via mutations in chromosomal genes and by horizontal gene transfer (Blair et al., 1999).

Intrinsic resistance usually arises as a result of inherent structural or functional characteristics of the microorganism. Also, the phenomenon of natural resistance or intrinsic resistance of *L. monocytogenes* to a wide spectrum of antibiotics such as fosfomycin, fusidic acid, cephalosporins, then to the second and third generation (cefetam, cefotaxime, ceftriaxone, cefuroxime) etc. is known (Scortti et al., 2006; Troxler et al., 2000).

An example of intrinsic resistance arises due to the lack of affinity of the antimicrobial compound for its bacterial target in *L. monocytogenes*, and this is found in two cases of β-lactam-based compounds, monobactams and broad-spectrum cephalosporins. In these cases, intrinsic resistance was caused by the low affinity of these drugs for PBP3, the enzyme that catalyzes the final step during cell wall synthesis (Charpentier et al., 1999; Godreuil et al., 2003). Although a few intrinsic resistance mechanisms in *Listeria* species have been described, most cases of resistance to antimicrobial compounds in this bacterium were due to acquired mechanisms, such

as mobile genetic elements including self-transferable plasmids and conjugative transposons (Charpentier et al., 1999; Godreuil et al., 2003).

### *2.8.1 Antibiotics*

Antibiotics typically have a single mechanism of action, such as inhibiting cell wall synthesis (beta-lactam antibiotics; e.g., penicillin), inhibiting DNA replication (quinolones; e.g., ciprofloxacin), or interfering with protein synthesis (aminoglycosides and tetracyclines; e.g., streptomycin and tetracycline, respectively) (Aase et al., 2000; Kapoor et al., 2017; Schmidt, 1997). Descriptions of these mechanisms of action are provided in Table 7. The classification of antimicrobial resistance within a bacterium is defined and the methodology to test the efficacy of antibiotics has been standardized by the Clinical and Laboratory Standards Institute (CLSI). Antibiotic resistance of bacterial isolates is classified by their ability to grow in the presence of antibiotic concentrations that would be achieved by clinical dosage schedules (CLSI, 2015).

The widespread use of penicillin and the subsequent reduction in effectiveness that followed, signaled a significant trend in the development of antibiotic resistance raisig growing concerns among public health experts worldwide (CDC, 2019; World Health Organization, 2018). This concern-extends to newly developed antibiotics, as bacteria may overcome the mechanisms of action through stress and similar adaptive responses. The CDC released the Antibiotic Resistance Threats in the United States report in 2013 and again in 2019, detailing the mounting antibiotic resistance threats within the U.S. (CDC, 2013, 2019). The latest CDC report communicates the comprehensive challenges of antibiotic resistance to the healthcare system and the community, including some aspects of resistance within the food industry, specifically in animals for food use. Until now, the resistance of *L. monocytogenes* to a wide group of antibiotics is not evident, however, the appearance of the first type resistant to several antibiotics (MDR) dates back to 1988. Until now, the sensitivity to a wide group of antibiotics is not known, however, the appearance of the first multidrug-resistant (MDR) strain of *L. monocytogenes* dates back to 1988. Since then, MDR of *L. monocytogenes* resistant to more antibiotics has been identified in cases of listeriosis outbreaks (Zhang et al., 2007).

Table 7. Antibiotics in the literature employed to treat listeriosis, their cellular targets, and resistance mechanisms.

| Class | Antibiotics | Targets | Resistance mechanisms | References |
|---|---|---|---|---|
| **Aminoglycosides** | Amikacin<br>Gentamicin<br>Kanamycin<br>Streptomycin | Positively charged molecules that attach to the negatively charged outer membrane causing the formation of large pores and allowing for antibiotics to enter the cell. Once inside the cell they target 16S r-RNA of the 30S subunit near the A site through hydrogen bonding causing misreading and premature termination of translation. | There are three main mechanisms of resistance reported: 1) alteration of the ribosomal binding sites; 2) a decreased uptake and accumulation in the cell; and 3) production of enzymes that inactivate the compound. | Kapoor et al. (2017); Pagliano et al. (2017); Temple and Nahata (2000) |
| **B-lactams** | Penicillin G<br>Amoxicillin<br>Ampicillin1 | Interact with the penicillin binding proteins (PBP) which play a necessary role in cross-linking the cell wall resulting in inhibition of cell wall synthesis. Resistance can be mediated through alterations in the PBP. | Resistance can be mediated through alterations in the PBP. | Kapoor et al. (2017); Pagliano et al. (2017); Temple and Nahata (2000) |
| **Quinolones** | Ciprofloxacin | Binds to DNA gyrase or topoisomerase IV enzymes, which both work to assist in DNA replication, resulting in inhibition of DNA synthesis. | Resistance development is mediated through alterations in the target enzyme (either DNA gyrase or topoisomerase IV) and multidrug resistance membrane-associate efflux pumps which prevent the compound | Blondeau (2004); Kapoor et al. (2017) |

| | | | | |
|---|---|---|---|---|
| | | | from reaching its target. | |
| **Glycopeptides** | Vancomycin | Binds to D-alanyl Dalanine portion of the peptide side chain, disrupting crosslinking of the cell wall structure and leading to inhibition of cell wall synthesis | The alteration of Dalanyl-alanine to Dalanyl-lactate, preventing the binding of the glycopeptide can result in resistance development. | Kapoor et al. (2017); Pagliano et al. (2017) |
| **Macrolides** | Erythromycin | Effect early stages of protein synthesis targeting the 23S rRNA of the 50S resulting in a premature detachment of incomplete peptide chain. | Resistance is mediated through alterations in the 50S subunit | Kapoor et al. (2017); Temple and Nahata (2000) |
| **Sulfonamide** | Trimethoprim/ suflamethoxazole | Inhibits steps in folic acid metabolism. Inhibits dihydropteroate synthase and dihydrofolate reductase | Resistance can occur due to mutations in folP gene encoding dihydropteroate synthase | Kim et al. (2019); Pagliano et al. (2017); Temple and Nahata (2000); Thønnings et al. (2016) |

While the report focuses on antibiotic resistance in pathogens directly associated with animal production, namely *Salmonella* spp. and pathogenic *E. coli*, other foodborne pathogens that are widespread in agricultural settings (e.g., *L. monocytogenes*) and that have the potential to develop antibiotic resistance in the future, are not discussed.

For the first time in 1950, some clinical microbiology laboratories in the United States began using the disk diffusion method to determine the "susceptibility of bacteria to antimicrobials". The interpretation of susceptibility and resistance was very simple; it was based solely on the presence or absence of an inhibition zone surrounding the disk, and two or three different concentrations of the same antimicrobial were routinely tested against the pathogen (Bauer et al., 1959). A standardized procedure for the disk diffusion susceptibility test was later developed, called the Kirby-Bauer disk diffusion test (Bauer et al., 1966).

One of the methods that has facilitated the determination of the lowest concentration of an antimicrobial agent that inhibits the visible growth of a microorganism in vitro is the Minimum Inhibitory Concentration (MIC) method.

The microtiter plates, first introduced in the 1970s, can be utilized to conduct MIC experiments in low volumes (Ashour et al., 1987). Their primary benefits include low sample volumes, high experimental throughput, and simplicity of usage.

In different countries of the region and beyond, studies have been conducted regarding the sensitivity to antibiotics of *L. monocytogenes* by means of phenotypic methods, disc diffusion and micro inhibitor concentration (MIC) (Arslan and Özdemir, 2008; Caruso et al., 2020; Gómez et al., 2014; Kevenk and Gulel, 2016).

# 3 RESEARCH OBJECTIVES

These objectives focus on various aspects such as phylogenetic origin, molecular serotype, clonal complexes, sequence typing, antimicrobial resistance patterns and multidrug resistance of *L. monocytogenes* in the food chain and food categories:

1. To identify and categorize the different phylogenetic lineages of *L. monocytogenes* present in food chain samples and food categories, utilizing genetic and genomic analyses.
2. To analyze the molecular serotypes of *L. monocytogenes* isolated from various food sources and assess their distribution across different food categories.
3. To investigate and classify the Clonal Complexes (CC) within *L. monocytogenes* populations to elucidate their genetic diversity and relatedness and investigate their occurrence and variation among food chain samples and food categories.
4. To utilize sequence typing methods such as to acquire a detailed genomic profile of the *L. monocytogenes* strains found in the food chain and food categories.
5. To apply Multilocus Sequence Typing (MLST) methods for classifying and comparing the sequence types (ST) of *L. monocytogenes* strains based on genetic sequences and to assess their genetic diversity.
6. To evaluate the antimicrobial resistance of *L. monocytogenes* isolates, to identify resistance patterns, assess implications for food safety and public health and determine the extent of multidrug resistance.
7. To investigate the occurrence and patterns of multidrug resistance in *L. monocytogenes* strains from different food categories and their implications for food safety.
8. To analyse the phylogenetic, molecular serotype, clonal, and resistance profiles of *L. monocytogenes* in relation to specific food sources and categories to better understand the transmission dynamics and potential risk factors.

# 4 MATERIALS AND METHODS

## 4.1 Materials

A total of 995 samples were tested, with five tests per sample, making a total of 4975 tests. Samples analyzed represented RTE foods, food products consumed cooked, raw materials, and environmental samples. The sampled food products were categorized into two groups based on their ability to support the growth of *Listeria monocytogenes*, in accordance with Regulation (EC) No. 2073/2005: one group included foods that support the growth of *L. monocytogenes*, while the other comprised foods that do not.

The samples were analyzed for the presence of *L. monocytogenes* from January 2016 to March 2022. The tested samples of animal origin were collected in the RKS at all stages of food chain processing. Out of these samples, 648 samples were RTE foods (meat products n=286, milk products n=361, fish products n=1), 281 food products consumed cooked (meat products n=249, milk products n=32), 60 were raw materials (meat products n=39, milk products n=8, fish n=8, combined food products n=5). Six were from various environments (food contact sample n=5, sample from personnel n=1), summarized in the Table 8. Most were typical local products reflecting the taste of Kosovar gastronomic traditions and artisanal food culture. The samples were collected under sterile conditions in accordance with the food codex. Each one was marked and registered for identification purposes prior to storage under optimal storage conditions (+5°C). Based on the Regulation on Microbiological Criteria 2073:2005, a representative sample consisted of five units of 25 g for each sample tested. *L. monocytogenes* isolates were detected in the collected samples via the official method with two-phase enrichment (EN ISO 11290-1).

Table 8. Samples tested during the period 2016-2022.

| Years | Ready-to-eat | Food products eating cooked | Raw material | Environmental sample | Total samples by year |
|---|---|---|---|---|---|
| **2016** | 68 | 25 | 10 | - | 103 |
| **2017** | 34 | 12 | 1 | - | 47 |
| **2018** | 134 | 54 | 8 | - | 196 |
| **2019** | 74 | 45 | 31 | - | 150 |
| **2020** | 53 | 29 | 1 | - | 83 |
| **2021** | 138 | 59 | 5 | 6 | 208 |
| **2022** | 147 | 57 | 4 | - | 208 |
| **Total:** | 648 | 281 | 60 | 6 | 995 |

Figure 7 shows some of the food product samples that were collected and used for microbiological testing.

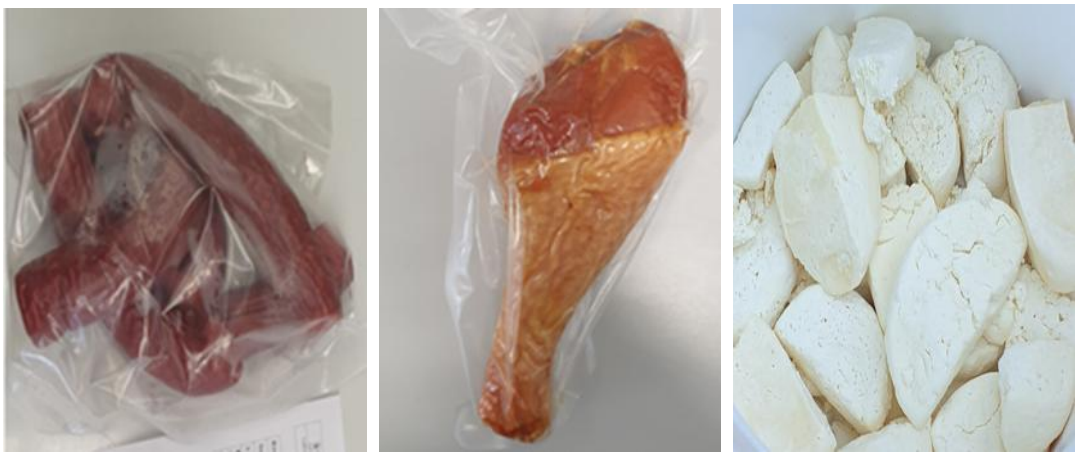


Figure 7. Some of samples of food products for microbiological testing.

A total of 149 inspection points such as dairies, meat processing plants, farms, fisheries, food warehouses, cold rooms, retail, restaurants and slaughterhouses were included in this study. Among them were business operators, of which 10 had implemented and applied HACCP - Hazard Analysis and Critical Control Points, while another 139 inspection points including small farms did not implement it.

Multilocus sequence typing and antimicrobial susceptibility testing were conducted on a total of 114 strains of *L. monocytogenes* isolated from food products in the food production chain in Kosovo, collected between 2016 and 2022. The samples include products of animal origin such as meat and meat products, milk and milk products, combined animal-based food products, fish, and environmental samples from food processing facilities. Food products of animal origin were collected from food business operators from meat and meat products ($n$ = 80) with the subcategories of beef meat products ($n$ = 69); pork meat products ($n$ = 9); chicken meat products ($n$ = 1); sheep meat products ($n$ = 1); milk and dairy products ($n$ = 25) with the following subcategories of dairy products ($n$ = 21): fresh milk ($n$ = 4) and fish meat products ($n$ = 4); combined food products ($n$ = 4) with the following subcategories: burgers with cheese ($n$ = 2); chicken fillets with cheese ($n$ = 2); and an environmental sample of a food-contact surface ($n$ = 1). Expressed as a percentage, the representation of samples from food categories in this study was 70.2% isolates from meat and meat products; 21.9% milk and milk products; 3.51% fish

products; 3.51% combined food products; and 0.88% were from food contact surface. Samples were collected under aseptic conditions from business operators in Kosovo, including factories, slaughterhouses, retail shops, cold rooms, and restaurants, and were transported in an insulated cooling box to the microbiology laboratory for processing. The study was conducted at the University of Veterinary Medicine Vienna in Austria, the Institute of Food Safety, Food Technology and Veterinary Public Health, and the Food Microbiology Unit. All samples were processed within 6 h after collection.

## 4.2 Methods

For the primary isolation of the strains tested in this work, the following nutrient medium and microbiological reagent were used, which were prepared in the microbiological kitchen at the Food Microbiology Laboratory near the Food and Veterinary Agency in Pristina, based on the manufacturer's instructions and EN ISO 11133-1:2014.

### *4.2.1 Isolation and Identification of L. monocytogenes*

The testing procedures for the isolation and identification of the strains started immediately after the acceptance of the sample where the sample was first verified as to whether it is suitable for testing by measuring weight and temperature.

*L. monocytogenes* isolates were detected in the collected samples via the official EN ISO 11290-1:2017 method with two-phase enrichment Half-Fraser Broth (LFB) – Liofilchem, Italy and Fraser Broth (LFB) – Liofilchem, Italy (International Association for Standardization, 2017) (Figure 8). In accordance with Mandate M381 by the European Commission for CEN, inter-laboratory comparisons validated the reference technique for the detection and reporting of *L. monocytogenes* in food (standard EN ISO 11290-1:2017). The specificity and the sensitivity of the detection method in these food matrices varies from 97.6% to 100% and from 91.1% to 100%, respectively (Gnanou Besse et al., 2019).

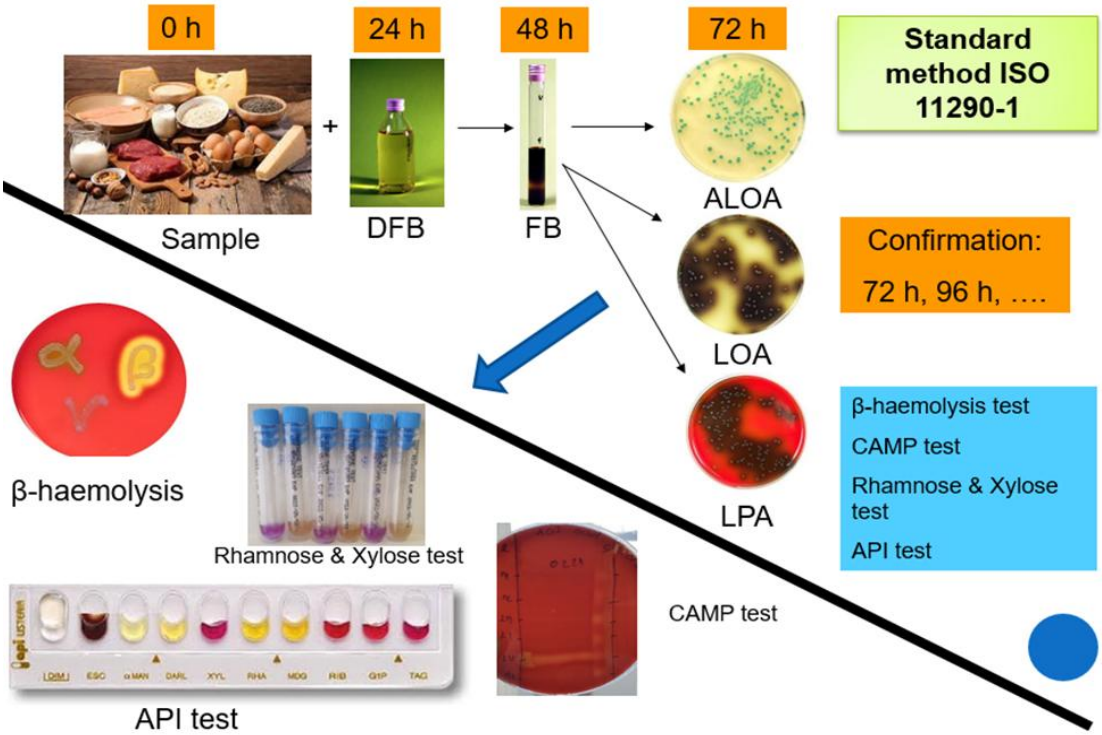



Figure 8. Testing of the samples was performed by ISO 11290 part one, the detection method.

From each sample, 25 g was extracted and inoculated into 225 mL of Demi Fraser Broth (DFB, Liofilchem® S.r.l., Roseto degli Abruzzi, TE, Italy) for initial selective enrichment (Figure 9).

Figure 9. Demi Fraser Broth after incubation.

After incubation at 30°C ± 1°C for 24 h ± 2 h, 0.1 ml of the liquid culture was inoculated into 10 ml of full-strength Fraser broth (FB, Liofilchem® S.r.l., Roseto degli Abruzzi, TE, Italy) for the second enrichment and cultured at 37°C ±1ºC for 24 h ± 2 h (Figure 10).

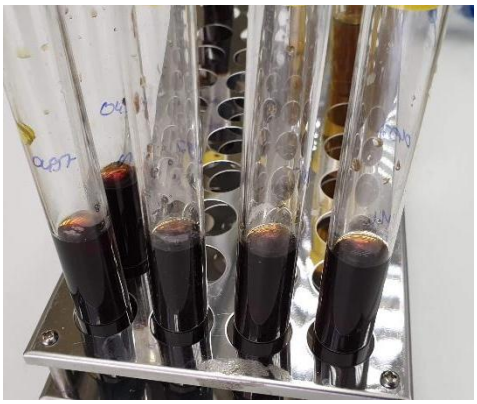


Figure 10. Listeria Fraser broth.

Listeria Ottaviani and Agosti (ALOA, Liofilchem® S.r.l., Roseto degli Abruzzi, TE, Italy) agar, and (LOA, Liofilchem® S.r.l., Roseto degli Abruzzi, TE, Italy) Oxford Listeria Selective Agar were employed for selective and differential plating, with Petri dishes incubated at 37°C ±1°C for 24 h to 48 h (Figure 11). Five typical and five atypical colonies were subcultured onto tryptic soy agar supplemented with 0.6% yeast extract (TSA-YE, Liofilchem® S.r.l., Roseto degli Abruzzi, TE, Italy) as a non-selective medium and incubated at 37°C for 24 h.

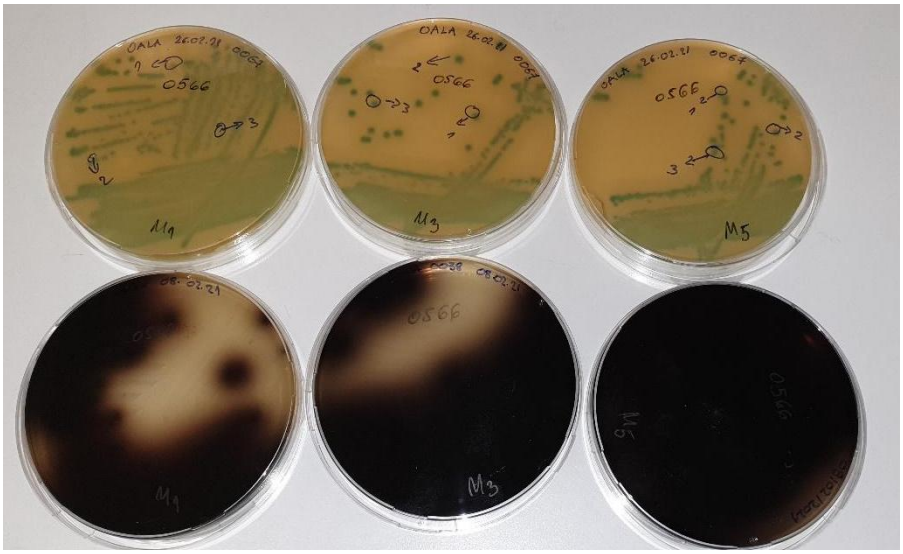


Figure 11. *L. monocytogenes* on ALOA and LOA.

The isolates were confirmed as *L. monocytogenes* via Gram staining, catalase reaction, oxidase testing, carbohydrate utilization, hemolysis testing, CAMP testing and motility at 20°C to 25°C. *L. monocytogenes* was confirmed and phenotypically identified to the species level using standard API Listeria biochemical tests (Bio Mérieux, Marcy l'Etoile, France) (Bille et al., 1992), following the manufacturer's instructions. *L. monocytogenes* ATCC 13932 and ATCC 35152 were utilized as control strains.

### *4.2.2 Biochemical tests*

Hemolysis test (obligatory) - Sowing is done on blood agar, first the soil is well dried, then the sowing is done, incubation at 37°C for 24h ±2h.

Typical colonies of *L. monocytogenes* form bright narrow and clear hemolytic zone (β-hemolysis), and the reaction is positive L-Rhamonse test (obligatory) - Planting is done in liquid soil, incubation at 37°C for 24h-48h. Positive reaction turns yellow (acid formation) and the reaction is positive.

D-Xylose test (mandatory) - Planting is done in liquid soil, incubation at 37°C for 24h-48h. Negative reaction - purple color and the reaction is Negative (Figure 12).

Figure 12. Rhamnose and Xylose test.

### *4.2.3 API test*

The API Listeria system (BioMerieux, La Balme-les-Grottes, France) is a standardized system for identification of Listeria that uses miniaturized tests, as well as a database. The API Listeria strip consists of 10 microtubes containing dehydrated substrate that enable the performance of enzymatic tests or sugar fermentations. During incubation, metabolism produces color changes that can either be spontaneously detected or need to be revealed by the addition of reagents. The reactions were read according to the reading table and the identification was obtained by consulting the profile list using the identification software. A 24-hour culture of bacteria on TSA slant were suspended in 2 mL of sterile distilled water (BioMerieux) to reach the same opacity as the MacFarland 0.5 Standard No. 1 on the scale. The suspension was simply homogenized using a vortex mixture (Vortex Genie 2, Fisher Scientific, USA). Three milliliters (3mL) of sterile distilled water was poured into the tray to create a moist atmosphere, and then the reaction strip was removed from its packaging and placed in the tray. The bacterial suspension was then distributed into ten (10) microtubes (100 µL for the DIM test and 50 µL for other cupules, ESC to TAG). Afterwards, the strip box was closed and incubated at 37ºC for 18 to 24 hours. After incubation, one drop of ZYM B (supplied by the manufacturer) was added to the DIM microtube and allowed to react for 3 minutes, and then all of the reactions were noted (Figure 13 a, b).

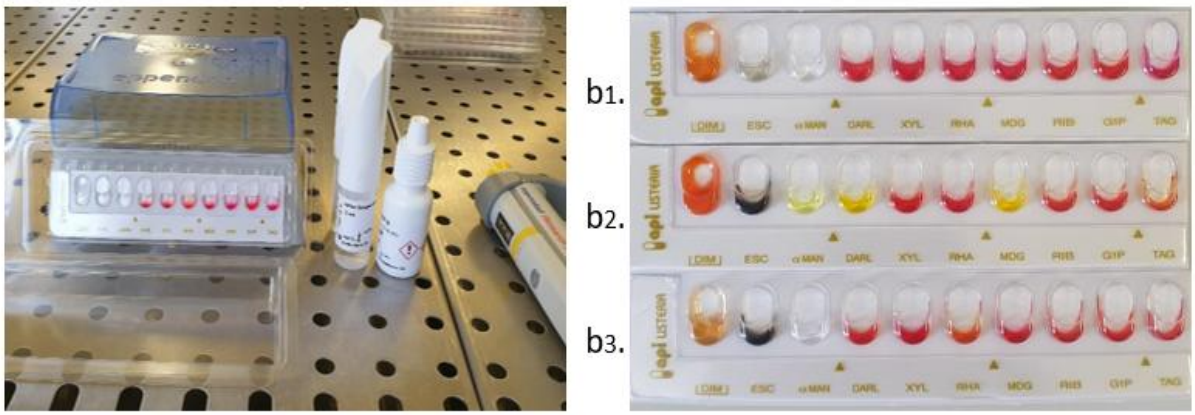



*Figure 13. a.* Reagents of API test, b. Interpretation of API results, b1 and b3 negative, b2 positive.

### *4.2.4 Christie, Atkins, Munch-Petersen (CAMP) test*

The CAMP test is used to differentiate between the haemolytic species in the genus Listeria namely; *L. monocytogenes, Listeria ivanovii and L. seeligeri.* It is done by streaking β hemolysin-producing *Staphylococcus aureus* and *Rhodococcus equi* parallel to each other on 5% sheep blood agar followed by streaking a colony of the suspect Listeria cultures at right angles in between the parallel streaks. A positive reaction creates a spade-shaped formation in the area of hemolysis of *S. aureus*. The plate is then incubated for 18-24 hours at 37°C. Hemolysis by *L. ivanovii* is enhanced within the vicinity of *Rhodococcus equi,* while that of *L. monocytogenes* and *L. seeligeri* is enhanced in the vicinity of *S. aureus* (Gasanov et al., 2005) (Figure 14 a, b).

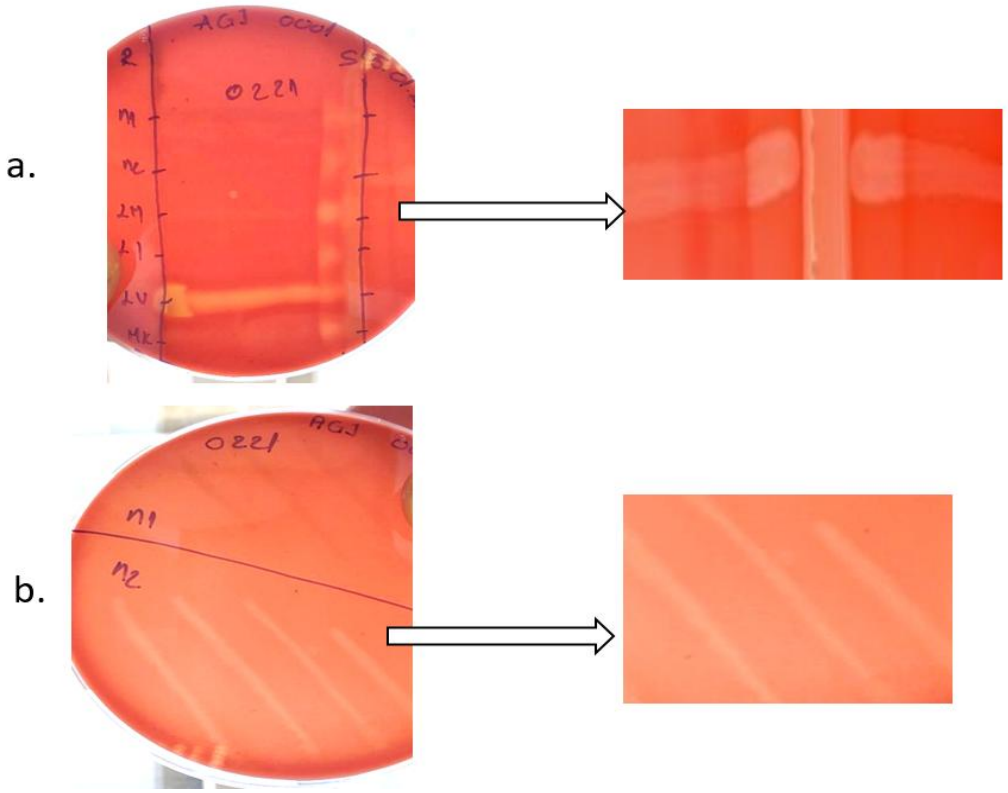



Figure 14. a. CAMP test and b. β-Hemolysis.

Figure 15 shows a typical colonies of *L. monocytogenes* on the selective chromogenic ALOA medium. Colonies with a typical morphological appearance are surrounded by a distinct halo-like area of dark precipitation.

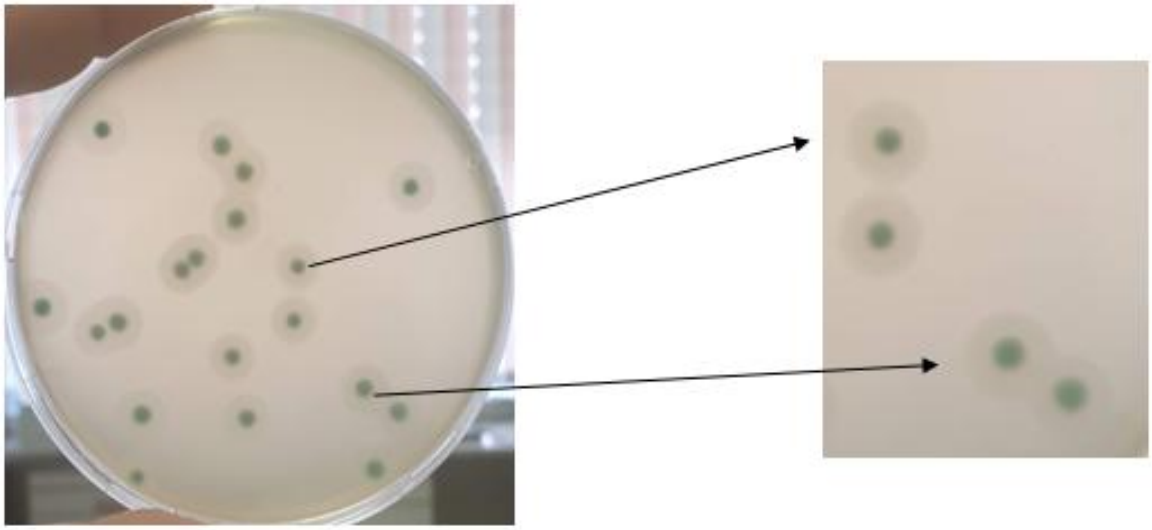


Figure 15. Colony of *L. monocytogenes* in ALOA surrounded by a distinct area of dark halo-like precipitation.

Figure 16 shows typical colonies of *L. monocytogenes* on RAPID'L medium. Colonies with a blue color (pale blue, gray-blue to dark blue) without a yellow halo.

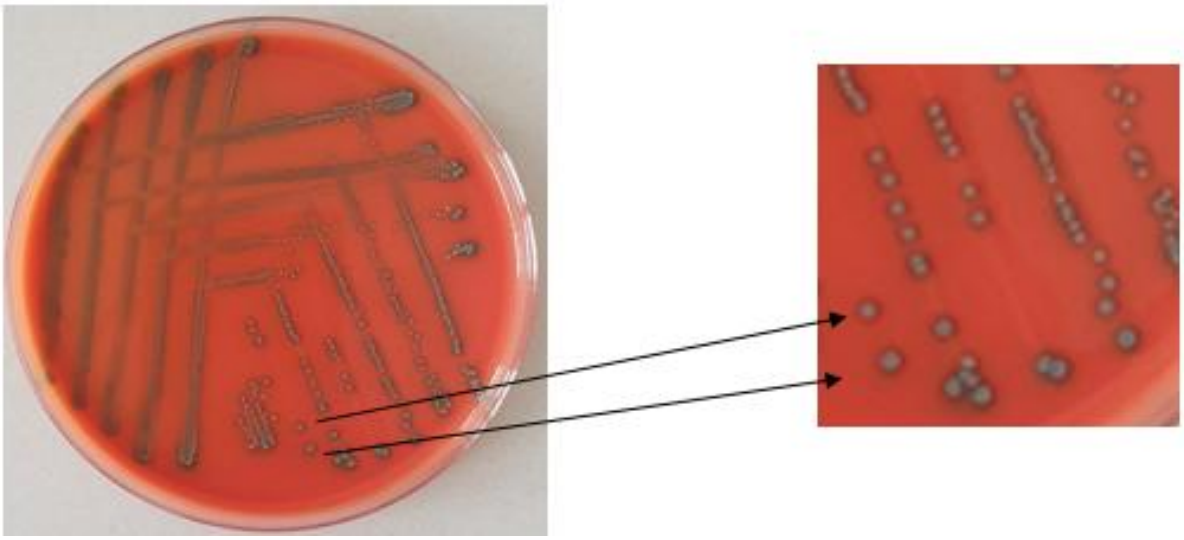


Figure 16. *Listeria monocytogenes* in RAPID'L. Mono agar blue pale blue, grey-blue to dark blue) colonies without a yellow halo.

Isolation of pure colonies of *L. monocytogenes* on OALA and RAPID'L.mono Agar, storage in cryo tubes at -80°C (Figure 17).

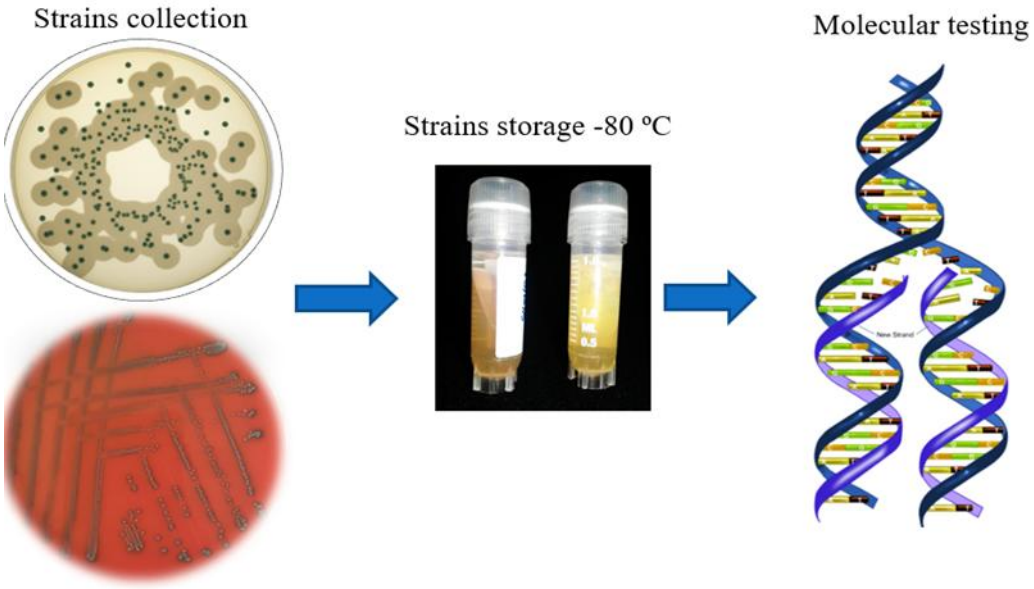



Figure 17. Storage of isolates for molecular testing.

### *4.2.5 Serological serotyping*

Serotyping was performed by a slide agglutination assay using commercially prepared antisera (*Listeria* antisera Seiken kit; Denka Seiken Co., Tokyo, Japan) according to the manufacturer's instructions (Figure 18).


Figure 18. Antisera for serotyping of *L. monocytogenes*.

### *4.2.6 DNA extraction*

Pure colonies were streaked on RAPID'L. Mono agar (Bio-Rad, Hercules, CA, USA) for 24 h at 37°C, then one colony was re-seeded on Tryptic Soya Agar (TSA, Merck, Darmstadt, Germany) and incubated for 24 h at 37°C. For the extraction of DNA from TSA plates, 3–4 colonies were taken and suspended in 2 mL tubes containing 100 µL of 0.01 M Tris HCI pH 7 (Thermo Scientific, Rockville, MD, USA). Four hundred (400) microliters of Chelex 100 Resin solution were then added (Bio-Rad, Hercules, CA, USA). The contents were vortexed and then boiled at a temperature of 100°C for 10 min. After boiling, the tubes were centrifuged for 5 min at 15,000 rcf. Using a pipette, 100 ul of supernatant was taken and transferred to a fresh 1.5 mL microcentrifuge tube and stored at −20°C until required for further analysis (Figure 19 a, b, c).


Figure 19. a. Water bath; b. Eppendorf 5415R refrigerated centrifuge; c. Thermomixer.

### *4.2.7 DNA concentration measuring*

The DNA concentration was measured using the Qubit™ dsDNA HS with a Qubit® 2.0 fluorometer (Thermo Fisher Scientific, Waltham, USA) (Figure 20). A DNA extract was retained when its concentration was above 0.1 ng/µL.

The DNA concentration measurement procedure begins by diluting Qubit® dsDNA HS reagent 1:200 in Qubit® dsDNA HS buffer known as working solution. The final volume in each tube should be 200 µL. Each standard tube requires 190 µL of Qubit® working solution and each sample tube requires anywhere from 180-199 µL. The working solution was prepared in a clean tube and an aliquot was taken for as many samples as were tested.

Prepare enough Qubit® working solution to accommodate all standards and samples. For example, for 118 samples and 2 standards, ~200 µL per tube and 2 mL working solution (120 µL Qubit® reagent plus 19800 µL Qubit® buffer) were taken. Then it is mixed by rotating it for 2-3 seconds without creating bubbles. Then all tubes were incubated at room temperature for 2 minutes. The reading of the results was done where first the calibration of the device was done with the prepared standards and then each tube is read by pressing on the Qubit® 2.0 screen, press DNA, then select dsDNA High Sensitivity as the type of analysis and then press the button " Run".

Calculation of the concentration of your sample = QF value × 200/x, where QF value = the value given by the Qubit® 2.0 fluorometer, x = the number of microliters of sample added to the assay tube. This equation generates a result with the same units as the value given by Qubit® 2.0 Fluorometer. For example, if the Qubit® 2.0 fluorometer gives a concentration in ng/mL, the result of the equation is in ng/mL.


Figure 20. Qubit.

### *4.2.8 DNA extraction materials*

-Gram-positive bacteria - Mericon DNA Bacteria plus Kit (Qiagen, Hilden, Germany).

-Brain heart infusion broth (BHI) (Liofilchem® S.r.l, Roseto degli Abruzzi TE, Italy

-Primers and probes set for identification of lineages, molecular serogroups, and clonal complexes of *L. monocytogenes* are listed on (Table 9) (Vitullo et al., 2013).

Table 9. Primers and probes for the detection of GenoListeria targeted genes.

| Multiplex | Primers and probes | Probe sequence (5'-3') | Forward primer sequence (5'-3') | Reverse primer sequence (5'-3') | Reference |
|---|---|---|---|---|---|
| 1 | prs | FAM-CATGACAACCACGGATACTTTCTTCAATGTTAATTTG-BBQ | CAGGRTTACTCGTTGATTGAATAAC | GCTGAAGAGATTGCGAAAGAAG | Vitullo et al. 2013 |
| 1 | plcA | HEX-TCAAGATGACTACAATGGTCCGAGTGTGAAAA-BBQ | CGGCGCACCTAACCAAGTAA | CAGTCTGGACAATCTCTTTGAATTTT | |
| 1 | Lmo0737 | Cy5-CCAACACTTTCTCATCAATACCATCTTCCC-BBQ | GCATCTTGTTTAGCAAGTGGATC | GAGCACGGAAGTTGCTAGGT | |
| 2 | Lmo1118 | FAM-CCTTTATCTTCTCCTGAGTGTATACGCCTC-BBQ | CTTAGTATTCCAGGATTTAAGACC | CCAAAGAACCAAATTGATCGAATC | |
| 2 | ORF2110 | HEX-TCTCCGTCATTTGTTACCGTTTCCCCAAC-BBQ | CACTAATCTCATCGACTATAAACTC | TGCACAAGCAGCAGAGGAAG | |
| 2 | ORF2819 | Cy5-CTCGTAAGATCGATATACGTCATGGCAGTTTCC-BBQ | GGAAGATTTCCACGCAATACTC | GGAAGATTTCCACGCAATACTC | |
| IVb-1 | 149_CC1_1911 | Cy5-TTCCAGCACTCAATGCAATCGC-BBQ | GTTCGATAGTGTCATAGGA | GCTCTCTATTCAATATTGGTAA | - |
| IVb-1 | 149_CC2_8862 | FAM-TCATCTTGTCCGATAGGTTCTGATTCT-BBQ | GCGTTTATTGGAAGGAAA | TGGGAAAGATTTCTTCTCA | - |
| IIb-1 | 4711_CC3_6907 | Cy5-AGTCGCTTTGACGAATATCAAACTCAC-BBQ | ACCCAAATAGATCAAAGC | CGGATTCTCTCTATTCTTG | - |
| IVb-2 | 4711_CC4_3572 | FAM-TGCCTCCTACCAACTGTACTGAAG-BBQ | CATCGTAGCCTTTTCATC | GGAACTAACCGAGGATTA | - |
| IIb-1 | 4711_CC5_9574 | HEX-AGACACATTAATTTCCGCTTGGCAA-BBQ | CCTTGCTAGCTTCTGTAG | GAAGGTACTTTTACAGACAAA | - |
| IVb-1 | 149_CC6_4418 | HEX-AACGGATTCTATTAAACACGCAAGCAA-BBQ | GGCAGTGTTTGATACATG | CTGGTAGAATAGATTACTTTAGAC | - |
| IIa-3 | 258_CC7_6968 | Cy5-AACTGCAACTCCAGAGTCAACATAAT-BBQ | GGTGAAATATGAGTAAATGGA | GAACCTATATTTTGAGGCATTA | - |
| IIa-1 | 258_CC8_952 | Cy5-AGTCACAGAAACTTCTAAGCCGG-BBQ | GGTACGGGTAGTTTTGTTA | GCCTTTTCAATGAAGTGAA | - |
| IIa-7 | 111 CC451_9204 | FAM-TTAAGACTCGCGCATGTTGCTGTGCAC-BBQ | GATGGGAGTTAATGATTTTATGGATA | ACACCTATTCTTTCTTGATTATACAG | - |
| IIa-3 | 258_SNP_LMO002932_ST14 | FAM-TCAGGACAAATCAGTGCATTTGGCC-BBQ | GATGCAACTGCTATTAGG | GTTCGTACATTCGCTTAG | - |

| | | | | | |
|---|---|---|---|---|---|
| IIa-4 | 258_SNP_LMO00317_ST91 | HEX-TTTTCCACTTTTAAGTTGCTCATTACC-BBQ | AGTGGTAAGGTTACATTA | CGAGCGAAATTAAATTAAATG | - |
| IIa-4 | 258_CC18_7946 | Cy5-TCTAACTCCTCTGTGTAAGAAGCAATT-BBQ | GTCACATTGGTTATATTTCAAG | CAGGTTTATTTCACTAGTTTG | - |
| *IIa-7* | *111-Lmo01705_ST398_1* | *HEX-CGTTAGTTTATCAATACGTCGCAACTTTAGC-BBQ* | *CTTGCTTCCGCTGATATCAATG* | *GTCCCAAACACGGTCAGAAA* | - |
| IIa-5 | 21322_CC20_1143 | Cy5-TCTAGCCTGTTCAATTTCTTGATTGG-BBQ | TGTCCTAATAGTGTAAGCA | CGTGAAAATGAACAACTAAA | - |
| IIa-5 | 21322_CC21_5690 | FAM-TCAACTTTGCTTGTTTTAAACCAAGA-BBQ | GCAACTAAAATAACTATCTCAA | GGATGAAAATTACTGATGAAG | - |
| IIa-6 | 3511_CC26_4338 | Cy5-AGACATAATGAATCATGGACGCTTCTT-BBQ | ACACGACGTATGACTTTA | CAGCATCTTCAAACAGAG | - |
| IIa-6 | 3511_CC29_2081 | FAM-TTGACGCTGAACTTGCTAATGC-BBQ | AACGGCTATTAAACGGAG | GGCAAAGTTACTACAGTTG | - |
| IIa-2 | 1610_CC31_8696 | Cy5-ATGAAACGAGCTAAATCTCCTCAATT-BBQ | GAGTGTATGGCATATGAAAG | GATCGTTGATAGAGAATTAAAATC | - |
| IIa-2 | 1610_CC37_4509 | FAM-TCAGGCAGCACTTCATAATCCA-BBQ | CCAGAGAATGGCTAGATA | GAACCAATAGAAGAATTGATAC | - |
| IVb-2 | 1_CC54_731 | HEX-AGCCTCCCGTACCGTAAACCGGT-BBQ | AGGACATATTAGATGTTCGTTCTG | GCTTCACCAACACTTAGCATA | - |
| IIb-2 | 359_CC59_5912 | Cy5-AAAGAATCTCCGACGAAACGCT-BBQ | CAGCAAAAGACAGCAGATA | AGCCAGAATAAATAAATTTACTTAC | - |
| IIb-2 | 359_CC77_3146 | FAM-ACAGAACCAATTCCTCCAACCAA-BBQ | CACGAATCAAACTGTGAA | CTTCGCAGGCATTTTATC | - |
| IIb-1 | 258_CC87_8655 | FAM-ATCCTTTGAGTGATAAACATCGCCTAC-BBQ | GTGACACCATGTAAATCTC | GCAGAAAACTTGGAATGA | - |
| IIa-5 | 21322_SNP_LMO00485_CC101 | HEX-CACTCTTAATGTTATGTGCTAAGCCG-BBQ | ATGGCACTTGAATTATTCA | GCCATACGTTCGATTTTAC | - |
| IIa-1 | 258_CC121_3159 | FAM-TTAGATTGCTACTACCGCCAATT-BBQ | ATGGCTACTGAATATATCCC | TCGGAATTTATCATTATATGTTCTA | - |
| IIa-2 | 1610_CC155_5334 | HEX-ATATTCAGAATCCATCCCTATTTGCG-BBQ | GTCAGAGTCGAATTCATTA | TCTGGAATTTTCAAAAGTATTG | - |
| IIa-6 | 3511_CC193_5118 | HEX-TGATGAGGAACCATATCATTTCCAATG-BBQ | CTGTCATGTGTTATCCTTG | TGGGAATAACGAGTCAATA | - |
| IIa-7 | 111_CC199_711 | Cy5-AGACTCTCCACTTCCAGCAAACGCTTCTGT-BBQ | CGGAGCATTCACTATATCATTTACA | GTCAGTTGGATGTTAGACCAAA | - |

| | | | | | |
|---|---|---|---|---|---|
| IIa-3 | 258_CC204_2959 | HEX-TGTGGACAACTTTCTCTAATTTCATCT-BBQ | CCTCTTGGTACTTCTAAATTATC | CAGAGCCGAAGATTATCC - | - |
| IIb-2 | 359_CC224_7283 | HEX-TCTTGTCCAAATTGTTTCACTATTATCGTAAGTA-BBQ | GAACGTATCTCTCTAGTAGC | GAAGGATTTATTAGAAATGAAAGTA - | - |

The *L. monocytogenes* MLST scheme uses internal fragments of the following seven housekeeping genes: *abcZ* (ABC transporter), *bglA* (beta glucosidase), *cat* (catalase), *dapE* (succinyl diaminopimelate desuccinylase), *dat* (D-amino acid aminotransferase), *ldh* (L-lactate dehydrogenase), *lhkA* (histidine kinase).

Table 10. The list of primers and probes used for MLST of *L. monocytogenes*.

| Target | Primer | Sequence (5'-3') | Ref. | Amplicon size (bp) |
|---|---|---|---|---|
| *abcZ* | abcZoF | GTTTTCCCAGTCACGACGTTGTATCGCTGCTGCCACTTTTATCCA | Salcedo et al., 2003 | 537 |
| | abcZoR | TTGTGAGCGGATAACAATTTCTCAAGGTCGCCGTTTAGAG | | |
| *bglA* | bglAoF | GTTTTCCCAGTCACGACGTTGTAGCCGACTTTTTATGGGGTGGAG | Salcedo et al., 2003 | 399 |
| | bglAoR | TTGTGAGCGGATAACAATTTCCGATTAAATACGGTGCGGACATA | | |
| *Cat* | catoF | GTTTTCCCAGTCACGACGTTGTAATTGGCGCATTTTGATAGAGA | Salcedo et al., 2003 | 486 |
| | catoR | TTGTGAGCGGATAACAATTTCAGATTGACGATTCCTGCTTTTG | | |
| *dapE* | dapEoF | GTTTTCCCAGTCACGACGTTGTACGACTAATGGGCATGAAGAACAAG | Salcedo et al., 2003 | 462 |
| | dapEoR | TTGTGAGCGGATAACAATTTCATCGAACTATGGGCATTTTTACC | | |
| *Dat* | datoF | GTTTTCCCAGTCACGACGTTGTAGAAAGAGAAGATGCCACAGTTGA | Salcedo et al., 2003 | 471 |
| | datoR | TTGTGAGCGGATAACAATTTCTGCGTCCATAATACACCATCTTT | | |
| *Ldh* | ldhoF | GTTTTCCCAGTCACGACGTTGTAGTATGATTGACATAGATAAAGA | Ragon et al., 2008 | 453 |
| | ldhoR | TTGTGAGCGGATAACAATTTCTATAAATGTCGTTCATACCAT | | |
| *lhkA* | lhkAoF | GTTTTCCCAGTCACGACGTTGTAAGAATGCCAACGACGAAACC | Salcedo et al., 2003 | 480 |
| | lhkAoR | TTGTGAGCGGATAACAATTTCTGGGAAACATCAGCAATAAAC | | |

Table 10 shows the *L. monocytogenes* MLST PCR panel for target genes (abcZ, bglA, cat, dapE, dat, ldh, lhkA) designed by Ragon et al., 2008.

The preparation of the reaction mixture (mastermix) for performing PCR reactions used in the molecular typing MLST (Multi-Locus Sequence Typing) of *L. monocytogenes* is provided in Table 11, which includes the mastermix preparations for six genes (*abcZ*, *bglA*, *dapE*, *dat*, *lhkA*), while Table 12 presents the preparations for two other genes (*cat* and *ldh*), based on the methodology described by Ragon et al. (2008). Table 13 describes the PCR cycling conditions used for these experiments.

Table 11. Mastermix preparation for *L. monocytogenes* MLST – PCR (*abcZ, bglA, dapE, dat, lhkA*), (Ragon et al., 2008).

| Mastermix | Final concentration | | Stock concentration | | 1x | Unit |
|---|---|---|---|---|---|---|
| DEPC water | | | | | 30.8 | µl |
| 5xPCR buffer | 1x | | | | 10 | µl |
| $MgCl_2$ | 2.5 | mM | 50 | mM | 0 | µl |
| Primer F | 200 | nM | 5000 | nM | 2 | µl |
| Primer R | 200 | nM | 5000 | nM | 2 | µl |
| dNTP's | 200 | µM | 5000 | µM | 0 | µl |
| Biozym HS Taq | 1 | U | 5 | U/ µl | 0.2 | µl |
| Mastermix | | | | | 45 | µl |
| Template | | | | | 5 | µl |
| Reaction volume | | | | | 50 | µl |

Table 12. Mastermix preparation for *L. monocytogenes* MLST – PCR (*cat, ldh*) (Ragon et al., 2008).

| Mastermix | Final concentration | | Stock concentration | | 1x | Unit |
|---|---|---|---|---|---|---|
| DEPC water | | | | | 15.2 | µl |
| 10xPCR buffer | 1x | | | | 5 | µl |
| $MgCl_2$ | 2.5 | mM | 50 | mM | 2.5 | µl |
| Primer F | 1000 | nM | 5000 | nM | 10 | µl |
| Primer R | 1000 | nM | 5000 | nM | 10 | µl |
| dNTP's | 200 | µM | 5000 | µM | 2 | µl |
| Platinum Taq | 1.5 | U | 5 | U/ µl | 0.3 | µl |
| | | | | | | |
| Mastermix | | | | | 45 | µl |
| Template | | | | | 5 | µl |
| Reaction volume | | | | | 50 | µl |

Table 13. PCR conditions

| Stages | Temperature | Time | Cycles |
|---|---|---|---|
| Initial denaturation | 94 ºC | 4 min | |
| Denaturation | 94 ºC | 30 sec. | 35 |
| Annealing | 52 ºC, (bglA: 45ºC) | 30 sec. | |
| Elongation | 72 ºC | 2 min | |
| Final elongation | 72 ºC | 10 min | |
| | 4 ºC | Hold | |

### *4.2.9 Identification of L. monocytogenes isolates with PFGE*

The PFGE typing was performed using a CHEF-DRII apparatus (Bio-Rad Laboratories, Des Plaines, USA). Sample plugs were prepared by combining 400 µL of bacterial cell suspension with 20.00 µL of lysozyme solution (20.00 mg $mL^{-1}$, Sigma, St. Louis, USA) and incubating the mixture at 56.0ºC for 20 minutes. The plugs were digested with 160–200 U of ApaI (New England Biolabs Inc., Ipswich, USA) at 30°C for 5 hours and 25 U of AscI (Fermentas, USA) at 37°C for 3 hours.

Electrophoresis was conducted by loading the plugs onto a 1.00% agarose gel in 0.5X Tris-borate EDTA (TBE) buffer under the following conditions: voltage of 6.00 V, initial switch time of 4 seconds, final switch time of 40 seconds, and a runtime of 22 hours. The gels were stained with 25.0 mL of ethidium bromide solution (10.0 mg $mL^{-1}$) in 400 mL of 0.5X TBE for 30 minutes. After staining, the gels were destained in deionized water (two washes of 20–30 minutes each with 400 mL) and subsequently visualized and photographed using the Gel Documentation System (Alpha Imager; ProteinSimple, California, USA).

PFGE patterns generated by this method were analyzed using Phoretix 1D Pro Gel Analysis Software (TotalLab, Newcastle, UK). The restriction patterns were normalized against the Lambda Ladder PFG Marker No. 340 S (New England Biolabs). Clustering analysis was performed using the unweighted pair group method with the Dice correlation coefficient. The results were validated through visual comparison of the PFGE profiles.

### *4.2.10 Detection of lineages, molecular serogroups and clonal complexes*

The concentration of DNA extracts was adjusted to between 0.1 and 1 ng/µL, then a real-time multiplex PCR analysis was performed using Taqman® PCR probes from TIB Molbio, Berlin, Germany (https://www.tib-molbiol.de) according to the methodology described by Félix et al. (2023). Three different real-time PCR thermocyclers were used to perform the tests: A Mic-IV real-time PCR thermocycler from Bio Molecular Systems (Upper Coomera, Australia), a Rotor-Gene Q real-time PCR thermocycler from QIAGEN (Hilden, Germany), and a QuantStudio 5 real-time PCR thermocycler from Thermo Fisher Scientific (Waltham, USA) (Figure 21 a, b, c).

a.  b.  c. 


Figure 21. a. MIC 4 real-time PCR thermocycler Bio Molecular System (Upper Coomera, Australia); b. Rotorgen Q real-time PCR thermocycler QIAGEN (Hilden, Germany); c. QuantStudio 5 real-time PCR thermocycler Thermo Fisher Scientific, (Waltham, USA).

### *4.2.11 Real time PCR analysis in the form of triplex/duplex PCRs*

The following target genes were used for molecular serotyping (Vitullo et al., 2013): lmo0737, lmo1118, ORF2819, ORF2110, prs, and plca.

This method requires to use conventional real time PCR instruments with three detection channels: dye FAM 520 nm, dye HEX 554 nm (compatible with VIC 549 nm), dye Cy5 669 nm. In this analysis configuration the PCRs are performed as triplex FAM-HEX-Cy5 or duplex. The triplex mixes all have the same composition, as well as the duplex mixes. The reaction volume could be performed in 15 µL and 20 µL format.

The PCR was performed in two rounds:

1) For all samples triplex PCR 1 (prs, plcA Lmo0737) and PCR 2 (ORF2819 ORF2110, Lmo1118).

2) The other triplex or duplex PCR may be performed according to the molecular serotype deduced. Testing can be performed for each triplex or duplex separately or they can be performed at the same time.

### *4.2.12 Preparation of the PCR mix*

The preparation of the Master Mix was performed in the PCR mixing chamber, which was adapted to the PCR mix used (Table 14).

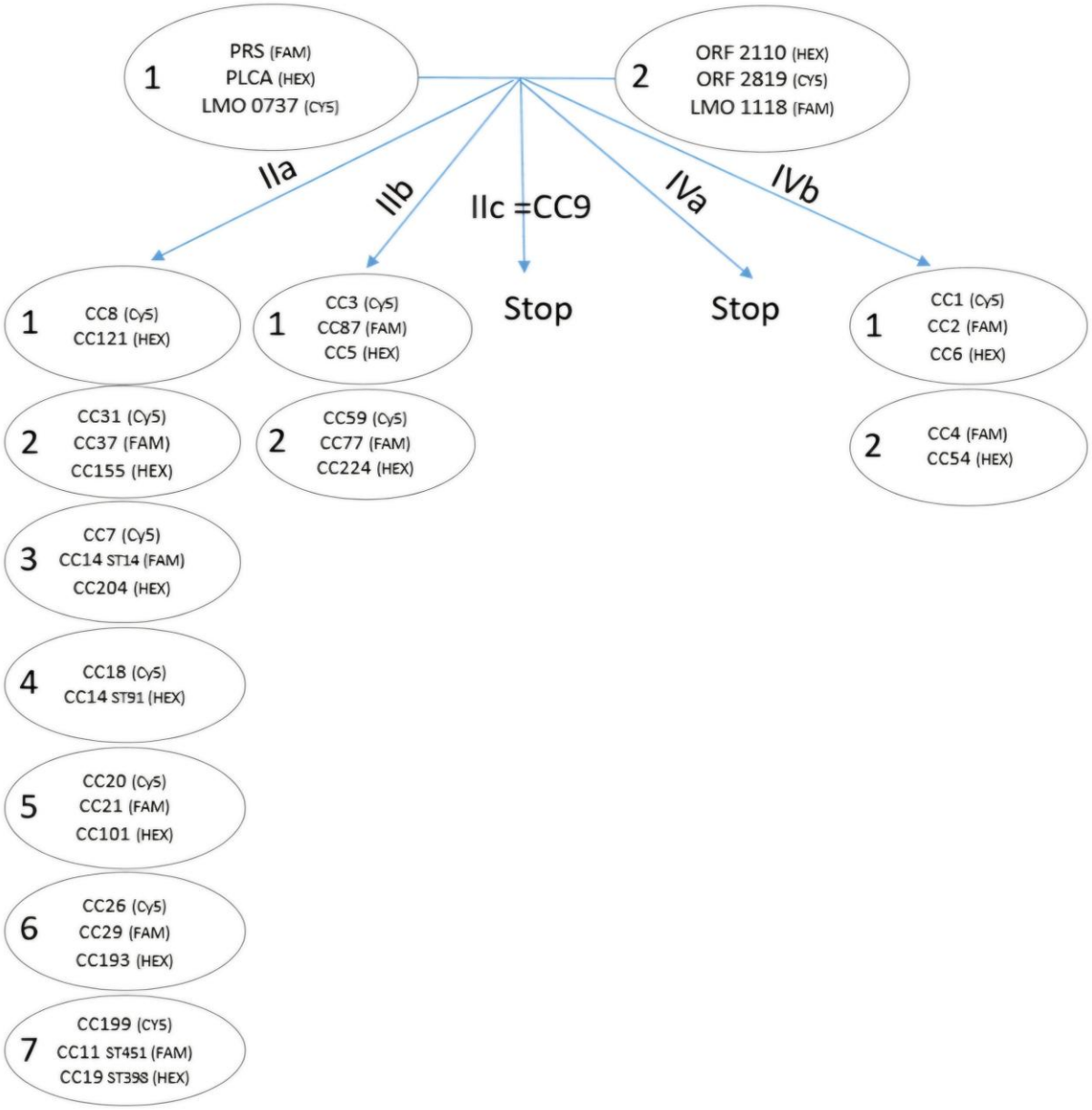
Table 14. Preparation of the master mix for the identification of triplexes and duplexes.

| **TRIPLEX** Reagent | 20 µL µl/reaction | 15 µL µl/reaction |
|---|---|---|
| $H_2O$ | 5.3 | 3.975 |
| 2x master mix | 10 | 7.5 |
| 20 µM Forward Primer 1 | 0.3 | 0.225 |
| 20 µM Reverse Primer 1 | 0.3 | 0.225 |
| 20 µM Probe 1 | 0.3 | 0.225 |
| 20 µM Forward Primer 2 | 0.3 | 0.225 |
| 20 µM Reverse Primer 2 | 0.3 | 0.225 |
| 20 µM Probe 2 | 0.3 | 0.225 |
| 20 µM Forward Primer 3 | 0.3 | 0.225 |
| 20 µM Reverse Primer 3 | 0.3 | 0.225 |
| 20 µM Probe 3 | 0.3 | 0.225 |
| Total: | 18 | 13.5 |

| **DUPLEX** Reagent | 20 µL µl/reaction | 15 µL µl/reaction |
|---|---|---|
| $H_2O$ | 6.2 | 4.65 |
| 2x master mix | 10 | 7.5 |
| 20 µM Forward Primer 1 | 0.3 | 0.225 |
| 20 µM Reverse Primer 1 | 0.3 | 0.225 |
| 20 µM Probe 1 | 0.3 | 0.255 |
| 20 µM Forward Primer 2 | 0.3 | 0.225 |
| 20 µM Reverse Primer 2 | 0.3 | 0.225 |
| 20 µM Probe 2 | 0.3 | 0.225 |
| Total: | 18 | 13.5 |

The concentration of DNA extracts was adjusted to between 0.1 and 1 ng/µL; then, real-time multiplex PCR was performed using Taqman® PCR probes from TIB Molbio, (Berlin, Germany), according to the methodology described by Félix et al. 2023 (Felix et al., 2023). Three different real-time PCR thermocyclers were used to perform the tests: a Mic-4 real-time PCR thermocycler from Bio Molecular Systems (Upper Coomera, Australia), a Rotor-Gene Q real-time PCR thermocycler from QIAGEN (Hilden, Germany), and a QuantStudio 5 real-time PCR thermocycler from Thermo Fisher Scientific (Waltham, MA, USA). The following target genes were used for molecular serotyping (Vitullo et al., 2013): *lmo0737*, *lmo1118*, *ORF2819*, *ORF2110*, *prs*, and *plca*. Clonal complexes (CCs) were identified by amplifying CC-specific genomic regions (Felix et al., 2023): CC1, CC2, CC3, CC4, CC5, CC6, CC7, CC8, CC9, CC11-ST451, CC14-ST14-206-399, CC18, CC19-ST398-802-1308, CC20, CC21, CC26, CC29, CC31, CC37, CC54, CC59, CC77, CC87, CC101, CC121, CC155, CC193, CC199, CC204, and CC224 (Table 15).

Table 15. GenoListeria multiplex scheme (Felix et al., 2023).



### *4.2.13 Multilocus sequence typing of L. monocytogenes*

Seven housekeeping loci ABC transporter (abcZ), D-amino acid aminotransferase (dat), L-lactate dehydrogenase (ldh), catalase (cat), succinyl diaminopimelate dessucinylase (dapE), beta-glucosidase (bglA), and histidine kinase (lhkA) (*abc*Z, *bgl*A, *cat*, *dap*, *dat*, *ldh*, and *lhk*A) were selected for the characterization of *L. monocytogenes* isolates by MLST. The primers that were used in this study to determine the MLST scheme is based on the panel designed by (Ragon et al., 2008; Salcedo et al., 2003).

The following temperature conditions were used: 1 cycle 94 °C, 4 min; 35 cycles (94°C, 30 s; 52°C, 30 s; 72°C, 2 min); 1 cycle 72°C, 10 min for the PCR amplification. An annealing temperature of 52°C was used for all genes except for *bgl*A (45°C) (https://bigsdb.pasteur.fr/listeria, accessed on 25 March 2024) (Figure 22 and Table 16).

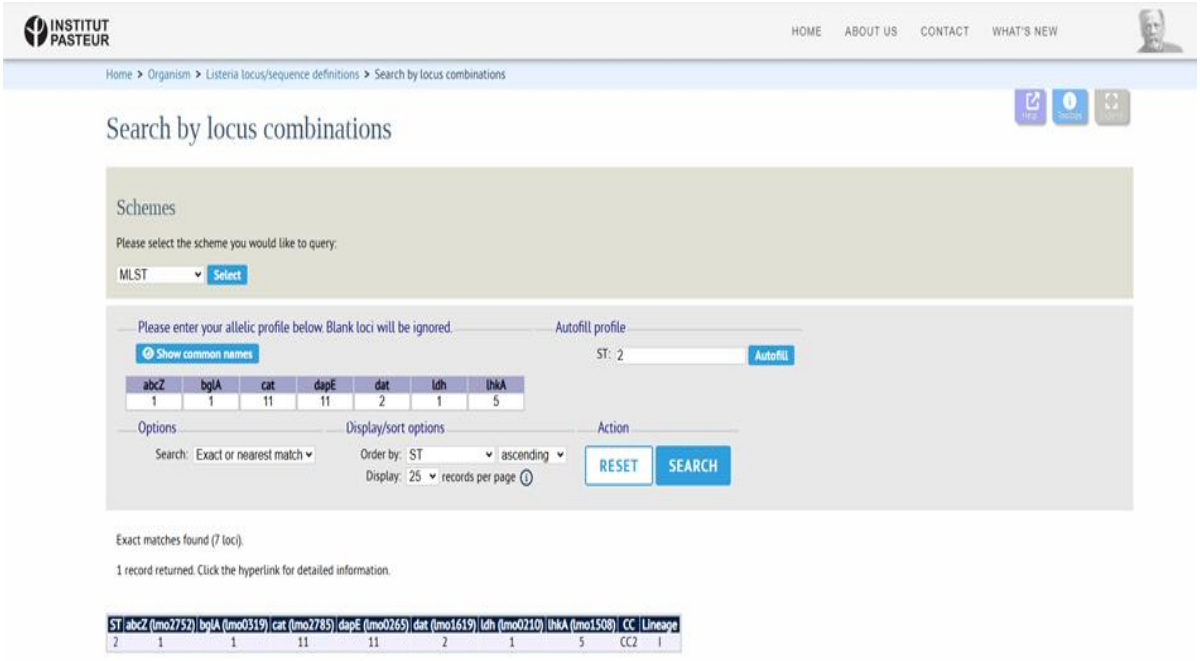



Figure 22. https://bigsdb.pasteur.fr/listeria, accessed on 25 March 2024.

Table 16. Allelic profile.

| ST | abcZ (lmo2752) | bglA (lmo0319) | cat (lmo2785) | dapE (lmo0265) | dat (lmo1619) | ldh (lmo0210) | lhkA (lmo1508) | CC | Lineage |
|---|---|---|---|---|---|---|---|---|---|
| ST3 | 4 | 4 | 4 | 3 | 2 | 1 | 5 | CC3 | I |
| ST4 | 1 | 2 | 12 | 3 | 2 | 5 | 3 | CC4 | I |
| ST6 | 3 | 9 | 9 | 3 | 3 | 1 | 5 | CC6 | I |
| ST7 | 5 | 8 | 5 | 7 | 6 | 2 | 1 | CC7 | II |
| ST8 | 5 | 6 | 2 | 9 | 5 | 3 | 1 | CC8 | II |
| ST9 | 6 | 5 | 6 | 4 | 1 | 4 | 1 | CC9 | II |
| ST12 | 5 | 8 | 5 | 7 | 6 | 22 | 1 | CC7 | II |
| ST14 | 8 | 6 | 13 | 6 | 5 | 2 | 1 | CC14 | II |
| ST26 | 5 | 10 | 8 | 21 | 6 | 2 | 1 | CC26 | II |
| ST29 | 15 | 10 | 18 | 18 | 1 | 3 | 1 | CC29 | II |
| ST32 | 1 | 18 | 27 | 2 | 4 | 1 | 5 | CC32 | I |
| ST37 | 5 | 7 | 3 | 5 | 1 | 8 | 6 | CC37 | II |
| ST87 | 12 | 1 | 4 | 14 | 3 | 39 | 4 | CC87 | I |
| ST121 | 7 | 6 | 8 | 8 | 6 | 37 | 1 | CC121 | II |
| ST145 | 1 | 1 | 11 | 11 | 2 | 12 | 5 | CC2 | I |
| ST328 | 3 | 1 | 1 | 1 | 3 | 131 | 3 | CC1 | I |
| ST399 | 8 | 6 | 15 | 6 | 6 | 2 | 1 | CC14 | II |
| ST520 | 3 | 18 | 27 | 3 | 4 | 106 | 23 | CC315 | I |
| ST580 | 6 | 37 | 6 | 4 | 1 | 4 | 1 | CC9 | II |
| ST710 | 3 | 1 | 1 | 1 | 3 | 1 | 87 | CC1 | I |

### *4.2.14 Allele templates*

*abcZ* (537 bp):

AAATCGACGAACAGAATGCGTATAGGACTTTTCCGCAAGATGGAAAAACTATCAATCCGT
TTCTTCGATAGTCGCAATGATGGCGAAATGCTTAGTCGTTTCACTAGTGACTTAGATAAT
ATTTCCAACACACTAAACCAAGCATTGATCCAAGTACTATCCAACGTCGCGCTAATGATT
GGTGTTATCATCATGATGTTCCAACAAAACGTGGAACTAGCCTTCGTTACTCTAATATCT
GCTCCATTTGCGATTATTATTGCGACAGTGATTATTCGAAAAGCCCGCAAATTCGTTGAT
ATTCAACAAGATGAACTAGGCGTACTTAACGGCTACATTGACGAAAAAATCTCTGGTCAA
AAAATTATTATCACAAATGGCTTAGAAGAAGAAACAATTGACGGCTTTGTTAAACAAAAC
AATATCGTTAAAAACGCAACTTACAAAGGTCAAGTTTACTCCGGTTTACTTTTCCCAATG
ATGCAAGGTATTTCCCTATTAAATACAGCTATTGTTATCTTCTTCGGTGGATGGTTA

*bglA* (399 bp):

AACCAATTCGAAGGCGCTTACAACGTCGATGGAAAAGGACTTTCCGTTCAAGATGTTACT
CCAAAAGGCGGATTTGGTCACATTACTGACGGTCCAACACCAGATAACTTAAAATTAGAA
GGAATTGACTTCTATCATCGCTACAAAGATGACGTGAAACTTTTTGCCGAAATGGGCTTC
AAGGTTTTCCGTACTTCCATCGCTTGGTCCCGTATCTTCCCAAATGGTGACGAAACTGAG
CCAAACGAAGCAGGATTACAATTTTACGATGATTTATTCGATGAACTTCTAGCACATAAT
ATCGAACCACTGATTACTTTATCTCACTATGAAACACCACTTCACTTATCGAAAACTTAC
GACGGCTGGGTAAATAGAAAAATGATCGACTTCTATGAA

*cat* (486 bp):

GCTCGTGGTGCTGGTGCTCACGGGAAATTTGTCACTAAGAAAAGCATGAAAAAATATACA
ATGGCTAAATTTTTGCAAGAAGAAGGAACGGAAACGGAGGTTTTTGCTCGTTTTTCAACA
GTAATTCATGGGCAACATTCTCCAGAAACATTACGTGATCCACGCGGTTTCTCCGTTAAG
TTTTATACAGAAGAAGGGAATTATGATTTTGTCGGAAATAATTTGCCGGTATTCTTCATT
CGTGATGCGATTAAGTTTCCGGATGTTATTCATTCCTTGAAGCCTGATCCACGCACAAAT
ATTCAAGATGGCAACCGTTACTGGGATTTCTTTAGCCTTACACCGGAAGCTACGACGATG
ATTATGTACTTATTCAGTGATGAAGGAACGCCGGCTTCTTACCGGGAAATACGTGGCTCT

AGTGTTCATGCGTTCAAATGGATTAATGAAGAAGGCAAAACAGTTTATGTAAAACTGCGC
TGGATT

*dapE* (462 bp):

TTGCAAAAGTTGTTAGCTGAACACGGTATTGAGTCCGAAAAGGTAAAATACGACGTAGAC
AGAGCCAGCCTAGTTAGCGAAATTGGTTCCAGTGACGAGAAAGTTTTGGCGTTTTCAGGG
CATATGGATGTCGTTGATGCGGGTGATGTCTCGAAGTGGAAGTTCCCACCTTTTGAAGCA
ACAGAGCATGAAGGGAAAATATACGGACGTGGCGCGACGGATATGAAGTCAGGTCTAGCG
GCGATGATTATTGCAATGATTGAGCTTCATGAAGAAAAACAAAAACTAAATGGCAAAATT
AGATTATTAGCAACGGTTGGTGAAGAAGTCGGTGAACTTGGAGCCGAACAACTAACGCAA
AAAGGTTACGCAGATGATTTAGATGGCTTGATTATCGGCGAACCGAGTGGACACCGGATT
GTTTATGCGCATAAAGGTTCCATTAATTATACCGTTAAATCC

*dat* (471 bp):

GAAGTAGTTCGTCTATATAATGGAAAATTCTTCACTTATAATGAACACATTGATCGTTTA
TATGCGAGTGCAGCAAAAATTGACTTAGTTATTCCTTATTCTAAAGAAGAGTTACGAGCG
TTACTTGAAAAATTAGTTGCTGAAAATAATATTAATACAGGAAATGTCTATTTACAAGTG
ACTCGAGGTGTTCAAAACCCGCGTAATCACGTTATGCCAGATGATTTCCCGCTGGAAGGC
GTTTTAACAGCAGCAGCTCGTGAAGTACCAAGAAATGAACAACAATTTGTGCAAGGTGGA
CCAGTAATTACAGAAGAAGATGTTCGTTGGTTACGCTGTGACATCAAGAGTTTGAATTTA
CTTGGAAACATTTTAGCAAAAAACAAAGCACATCAACAAAATGCGTTAGAAGCTGTTTTA
CACCGCGGAGAGCAAGTAACTGAGTGTTCAGCTTCCAATATTTCTATTATT

*ldh* (453 bp):

TATAGCGACTGCCACGATGCGGACTTAGTTGTTGTAACTGCCGGGACTGCACAAAAACCT
GGTGAAACTCGTTTAGATTTAGTAAATCGTAATATTAAAATCATGAAAGGCATCGTGGAT
GAAGTAATGGCTAGCGGATTTGACGGTATCTTCTTAATCGCTTCTAACCCAGTAGATATC
TTAACTTACGCTACATGGAAATTCTCAGGTCTTCCAAAAGAACGTGTTATCGGTTCTGGA
ACAAGCCTTGATACAGCACGTTTCCGTATGTCAATTGCCGACTATCTAAAAGTAGATGCT
CGTAACGTCCATGGTTACATCCTTGGCGAACACGGCGATACAGAATTCCCAGCATGGAGC
CACACAACTGTCGGCGGTCTTCCAATCACTGAATGGATTAGCGAAGATGAACAAGGTGCA
ATGGATACTATTTTCGTAAGTGTTCGTGATGCA

*lhkA* (480 bp):

TATCCAACACAGATGAATCAGCCGTTACCAAAGGATTTCTCTATTTCTTCGGATGATAAG
AAAAAACTTGAAAGTGGCGAAACAGTTAGTAAGAAAATAGATAATCGCTTTAATAAAGAA
ATGACAATTGTGTACGTCCCAATAATGAATGGCGACAAATTTGTCGGTTCTATCGTGCTC
AATTCACCTATTAGCGGTACGGAGCAAGTAATTGGTACGATTAACCGCTATATGTTCTAC
ACTATTTTACTTTCTATAACGGTAGCACTTATTCTTAGCGCAATCTTGTCCAAACTACAA
GTAAATCGAATCAACAAACTACGAGCAGCGACAAAAGACGTTATTCAAGGCAATTACAAA
GCTCGATTGAAGGAAAATAATTTTGATGAAATTGGTGCACTTGCCATTGATTTCAATAAA
ATGACACAAACCCTTGAAACATCTCAAGAAGAAATAGAACGACAAGAGAAGCGGAGACGC

### *4.2.15 Antimicrobial resistance AMR*

The antibiogram signatures of *L. monocytogenes* isolates were evaluated according to the liquid microdilution method reported by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, ECOFF). Antibiotic susceptibility testing was performed using Sensititre$^{TM}$ GPN3F plates (Thermo Scientific, Rockville, MD, USA) which are specially manufactured for Gram-positive bacteria (Figure 23). These specific antibiotics were selected for susceptibility testing because they are commonly used to treat infections caused by Gram-positive bacteria, including *L. monocytogenes*.

These plates contained a range of 18 antibiotics at varying concentrations: erythromycin ERY (0.25–4 mg/L), clindamycin CLI (0.12–2 mg/L), gentamicin GEN (500 mg/L), streptomycin STR (1000 mg/L), quinupristin/dalfopristin SYN (0.12–4 mg/L), daptomycin DAP (0.25–8 mg/L), vancomycin VAN (1–128 mg/L), tetracycline TET (2–16 mg/L), ampicillin AMP (0.12–16 mg/L), gentamicin GEN (2–16 mg/L), rifampin RIF (0.5–4 mg/L), levofloxacin LEVO (0.25–8 mg/L), linezolid LZD (0.5–8 mg/L), penicillin PEN (0.06–8 mg/L), ciprofloxacin CIP (0.5–2 mg/L), trimethoprim/sulfamethoxazole SXT (0.5/9.5–4/76 mg/L), ceftriaxone AXO (8–64 mg/L), gatifloxacin GAT (1–8 mg/L), and oxacillin + 2% NaCl OXA+ (0.25–8 mg/L) (Lachtara et al., 2023, Wieczorek & Osek, 2017).

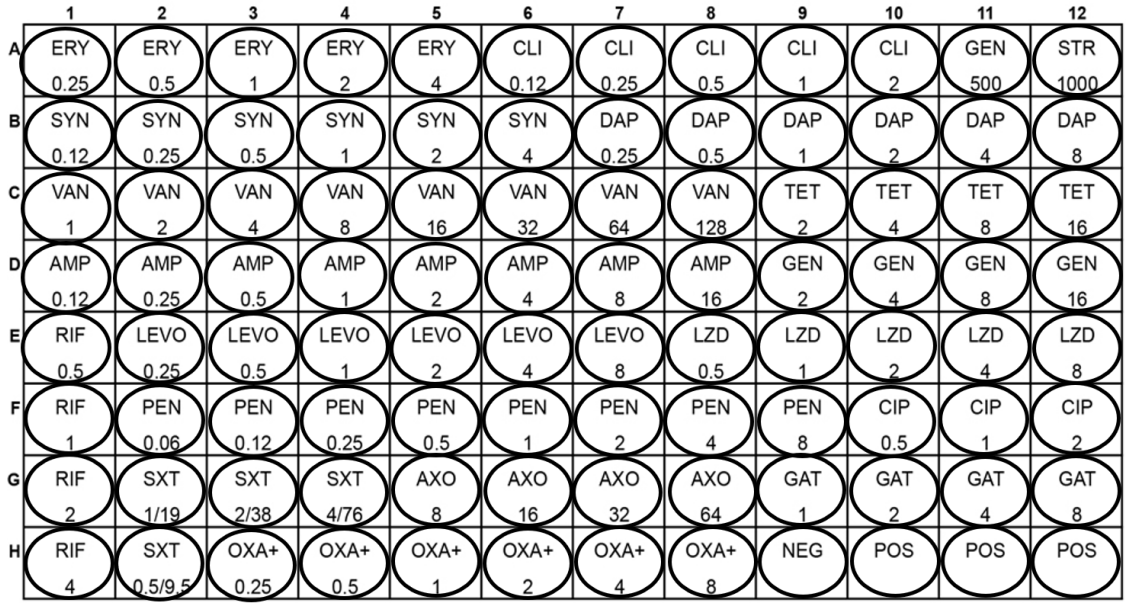



Figure 23. Antimicrobial susceptibility plate for testing *L. monocytogenes* isolates.

The determination of the minimum inhibitory concentration (MIC) in this study followed the broth microdilution method in accordance with ISO 20776-1 (EN ISO 20776-1/2019). First, tryptic soy agar was used as a solid medium for the cultivation of *L. monocytogenes* isolates from which colonies were standardized to the McFarland equivalent of 0.5 McF, and then verified by OD measurement using UV-1800 spectrophotometer (Shimandzu, Cole-Parmer, Durham, NC, USA) (Figure 24). Next, 30 µL of inoculum was transferred to 11 mL of Mueller–Hinton broth (MHB, Merck, Darmstadt, Germany) and 50 µL was pipetted into microdilution panels wells which were sealed and incubated at 37°C for 18–24 h.


Figure 24. Spectrophotometer OD measurement.

The optical density (OD) at 600 nm was measured using a UV-1800 spectrophotometer (Shimandzu, Cole-Parmer, Durham, NC, USA). The values obtained are presented in Table 17.

Table 17. The obtained OD values of the 114 isolates.

| No. | $OD_{600}$ | No. | $OD_{600}$ | No. | $OD_{600}$ |
|---|---|---|---|---|---|
| 1 | *0.268* | 39 | *0.215* | 77 | *0.291* |
| 2 | *0.184* | 40 | *0.298* | 78 | *0.233* |
| 3 | *0.191* | 41 | *0.217* | 79 | *0.291* |
| 4 | *0.193* | 42 | *0.198* | 80 | *0.296* |
| 5 | *0.205* | 43 | *0.247* | 81 | *0.287* |
| 6 | *0.283* | 44 | *0.223* | 82 | *0.293* |
| 7 | *0.219* | 45 | *0.275* | 83 | *0.299* |
| 8 | *0.188* | 46 | *0.232* | 84 | *0.294* |
| 9 | *0.266* | 47 | *0.247* | 85 | *0.278* |
| 10 | *0.219* | 48 | *0.261* | 86 | *0.212* |
| 11 | *0.192* | 49 | *0.251* | 87 | *0.258* |
| 12 | *0.205* | 50 | *0.254* | 88 | *0.264* |
| 13 | *0.191* | 51 | *0.232* | 89 | *0.233* |
| 14 | *0.261* | 52 | *0.254* | 90 | *0.298* |
| 15 | *0.233* | 53 | *0.204* | 91 | *0.301* |
| 16 | *0.221* | 54 | *0.277* | 92 | *0.255* |
| 17 | *0.275* | 55 | *0.201* | 93 | *0.265* |
| 18 | *0.279* | 56 | *0.29* | 94 | *0.287* |
| 19 | *0.281* | 57 | *0.226* | 95 | *0.305* |
| 20 | *0.286* | 58 | *0.264* | 96 | *0.258* |
| 21 | *0.289* | 59 | *0.275* | 97 | *0.335* |
| 22 | *0.265* | 60 | *0.242* | 98 | *0.269* |
| 23 | *0.292* | 61 | *0.225* | 99 | *0.252* |

| | | | | | |
|---|---|---|---|---|---|
| 24 | *0.221* | 62 | *0.276* | 100 | *0.259* |
| 25 | *0.224* | 63 | *0.262* | 101 | *0.309* |
| 26 | *0.294* | 64 | *0.281* | 102 | *0.300* |
| 27 | *0.287* | 65 | *0.233* | 103 | *0.288* |
| 28 | *0.223* | 66 | *0.266* | 104 | *0.289* |
| 29 | *0.299* | 67 | *0.284* | 105 | *0.278* |
| 30 | *0.225* | 68 | *0.274* | 106 | *0.254* |
| 31 | *0.294* | 69 | *0.206* | 107 | *0.265* |
| 32 | *0.211* | 70 | *0.258* | 108 | *0.201* |
| 33 | *0.289* | 71 | *0.296* | 109 | *0.266* |
| 34 | *0.193* | 72 | *0.285* | 110 | *0.300* |
| 35 | *0.206* | 73 | *0.273* | 111 | *0.311* |
| 36 | *0.189* | 74 | *0.245* | 112 | *0.288* |
| 37 | *0.211* | 75 | *0.267* | 113 | *0.291* |
| 38 | *0.208* | 76 | *0.189* | 114 | *0.285* |

Sensititre™ GPN3F plates were carefully filled, sealed with foil and incubated. In Figure 25, the plates before sealing and before incubation are shown.

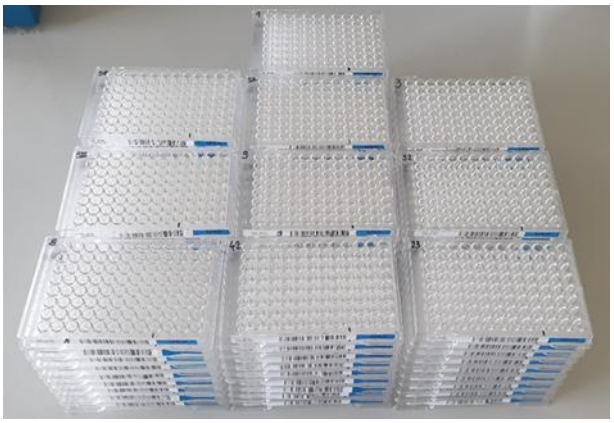


Figure 25. Sensititre™ GPN3F plates (Thermo Scientific, Rockville, MD, USA).

For the evaluation of the quality of the technique, certified strains were used, as a negative control strain, *Staphylococcus aureus* ATCC 29213 was used, while as a positive control of *L. monocytogenes* 1/2a ATCC 13932 and 4b ATCC 35152 were used. The isolate's susceptibility was categorized as susceptible (S) and resistant (R) to each of the antimicrobials, in line with the result obtained from the susceptibility testing using standard reference documents (EUCAST, 2024).

Figure 26 shows the plate after incubation, ready for reading and expression of results. The expression of results was done on pre-prepared forms based on the antimicrobial ranking in order to evaluate the results more accurately.

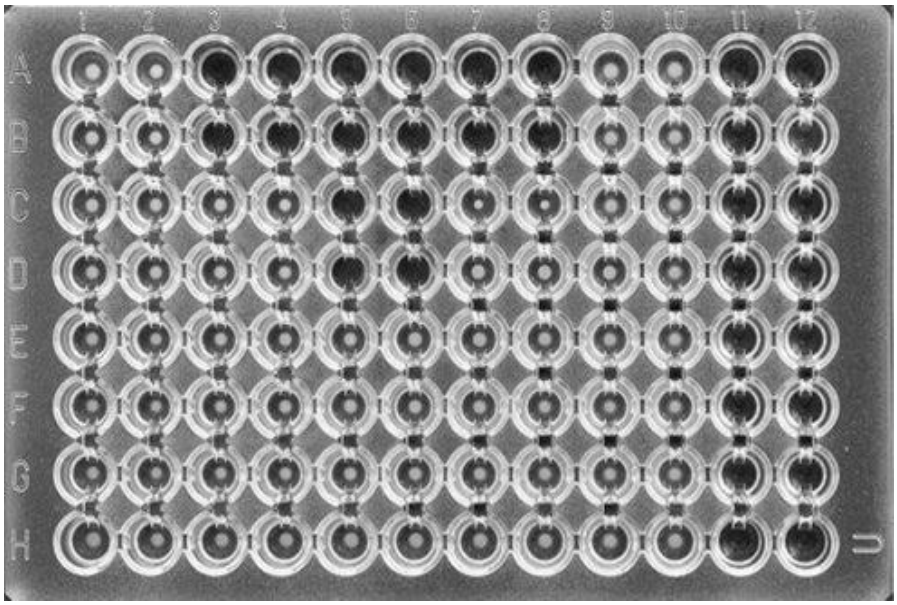


Figure 26. Reading of Sensititre™ GPN3F plates.

### *4.2.16 Calculation and Interpretation of Multiple/Antibiotic Resistance Indices multiple / Antibiotic Resistance Index*

For each isolate of *L. monocytogenes* that showed phenotypic resistance against three or more classes of antibiotics from the panel of 18 antibiotics tested, multiple antibiotic resistance phenotypes (MARPs) were computed. The multiple antibiotic resistance index (MARI) was calculated using the method from Krumperman (Krumperman, 1983).

The calculation and interpretation of the MAR Index for each strain was conducted according to the mathematical formula:

$$MARI = \frac{a}{b}$$

Where:

“a” represents the number of antibiotics to which an isolate was resistant, and

“b” represents the total number of antibiotics tested.

If MARI > 0.2, it is interpreted that those antibiotics are frequently consumed in that area and represent a high risk of antibiotic resistance.

Furthermore, the antimicrobial resistance index was evaluated for each food category according to the formula:

$$ARI = \frac{a}{b}$$

Where:

a = the aggregate antibiotic resistance score of all isolates from a particular sample type,

b = the number of antibiotics × no. of isolates from the sample type.

### *4.2.17 Statistical analysis*

Data related to *L. monocytogenes*, serotypes, complex clones, sequence types, and their antibiotic resistance were analyzed using the statistical methods below. The Chi-squared test was performed to assess the relationship between the variables of interest. P-value < 0.05 was used in testing the statistical significance of all experimental data. To identify the allelic profile of *L. monocytogenes* sequence types, the following software was used: https://bigsdb.pasteur.fr/listeria, accessed on 25 March 2024.

# 5 RESULTS

## 5.1 *L. monocytogenes* in food products

Between 2016 and 2022, a total of 995 samples were analyzed, of which 117 (11.76%) tested positive for Listeria monocytogenes, underscoring the pathogen's significant presence in the food products examined. These isolates were distributed across four categories: ready-to-eat products (RTE), food products consumed cooked (FPCC), raw materials (RM), and environmental samples (ES) (Table 18).

The highest number of isolates was found in food products consumed cooked (35.90%), followed closely by ready-to-eat products (35.04%) and raw materials (28.21%). Only one isolate (0.85%) was detected in environmental samples, recorded in 2021.

The number and distribution of isolates varied across the years. Notably, raw materials showed a peak in 2019 (75.76%), while ready-to-eat products had the highest detection rate in 2021 (31.71%). No *L. monocytogenes* was found in ready-to-eat products in 2020.

Table 18. *L. monocytogenes* isolates from food chain product over the years.

| Years | Ready-to-eat products | | Food products consumed cooked | | Raw materials | | Environmental samples | |
|---|---|---|---|---|---|---|---|---|
| | Positive Cases | % | Positive Cases | % | Positive Cases | % | Positive Cases | % |
| **2016** | 5 | 12.20% | 10 | 23.81% | 0 | 0 | 0 | 0 |
| **2017** | 9 | 21.95% | 2 | 4.76% | 0 | 0 | 0 | 0 |
| **2018** | 7 | 17.07% | 9 | 21.43% | 4 | 12.12% | 0 | 0 |
| **2019** | 5 | 12.20% | 14 | 33.33% | 25 | 75.76% | 0 | 0 |
| **2020** | 0 | 0.00% | 2 | 4.76% | 1 | 3.03% | 0 | 0 |
| **2021** | 13 | 31.71% | 3 | 7.14% | 2 | 6.06% | 1 | 100.0% |
| **2022** | 2 | 4.88% | 2 | 4.76% | 1 | 3.03% | 0 | 0 |
| **Total of positive 117 (100.0%)** | **41** | **35.04%** | **42** | **35.90%** | **33** | **28.21%** | **1** | **0.85%** |

## 5.2 Distribution of *L. monocytogenes* serotypes

The map provides a clear visual representation of the geographic distribution of sampling sites across Kosovo, highlighting the locations of various business operators involved in food production. It offers valuable insights into how food products are distributed and sampled throughout the region (Figure 27).

Each location is marked with a circle that is color-coded to distinguish between different product categories. Brown circles are used to represent sampling sites that focus on meat and meat products, which may include various types of processed meats or fresh cuts. Yellow circles are designated for sites involved in dairy products, such as milk, cheese, and other dairy-based items. Blue circles indicate locations where fish products are produced or processed, covering a range of fish types and related products. Lastly, red circle represents combined food products, where a mix of ingredients from different categories may be processed or sold.

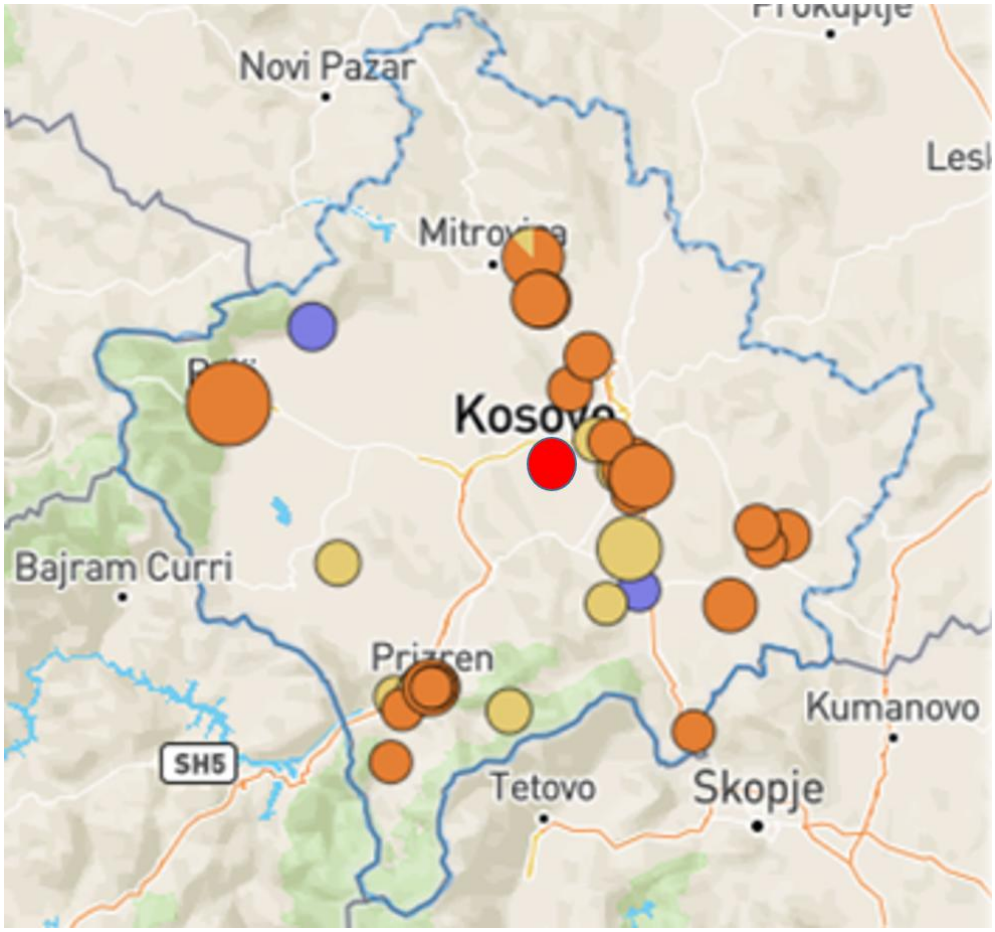



Figure 27. Map showing the geographical location of the sites from which *L. monocytogenes* was isolated.

Table 19 provides detailed information on the lineages and corresponding serotypes identified in *L. monocytogenes* isolated from food products. The data reveal a diverse serotype structure within the *L. monocytogenes* population in the studied samples, with a distribution in four serotypes derived from two lineages.

Table 19. The table presents the distribution of different *L. monocytogenes* lineages and serotypes across various sample types of food categories.

| Lineages | Serotype | Meat and meat products | Milk and milk products | Fish products | Combined food products | Environment samples |
|---|---|---|---|---|---|---|
| Lineage I | 1/2b (4) | 2 | 1 | 1 | | |
| (39) | 4b (35) | 22 | 10 | 2 | 1 | |
| Lineage II | 1/2a (40) | 23 | 12 | 1 | 3 | 1 |
| (78) | 1/2c (38) | 35 | 2 | | 1 | |

Serotype 1/2b was represented by 4 isolates, of which 2 isolates from meat products, and one each from fish and dairy products. These isolates were part of Lineage I, highlighting a relatively small but significant presence of this serotype in the studied samples. In contrast, Serotype 4b, also part of Lineage I, was represented by a larger group of 35 isolates (Figure 28). These isolates were identified from the following food categories; 22 were isolated from meat products, 10 from dairy products, 2 from fish products and one from combined food products.

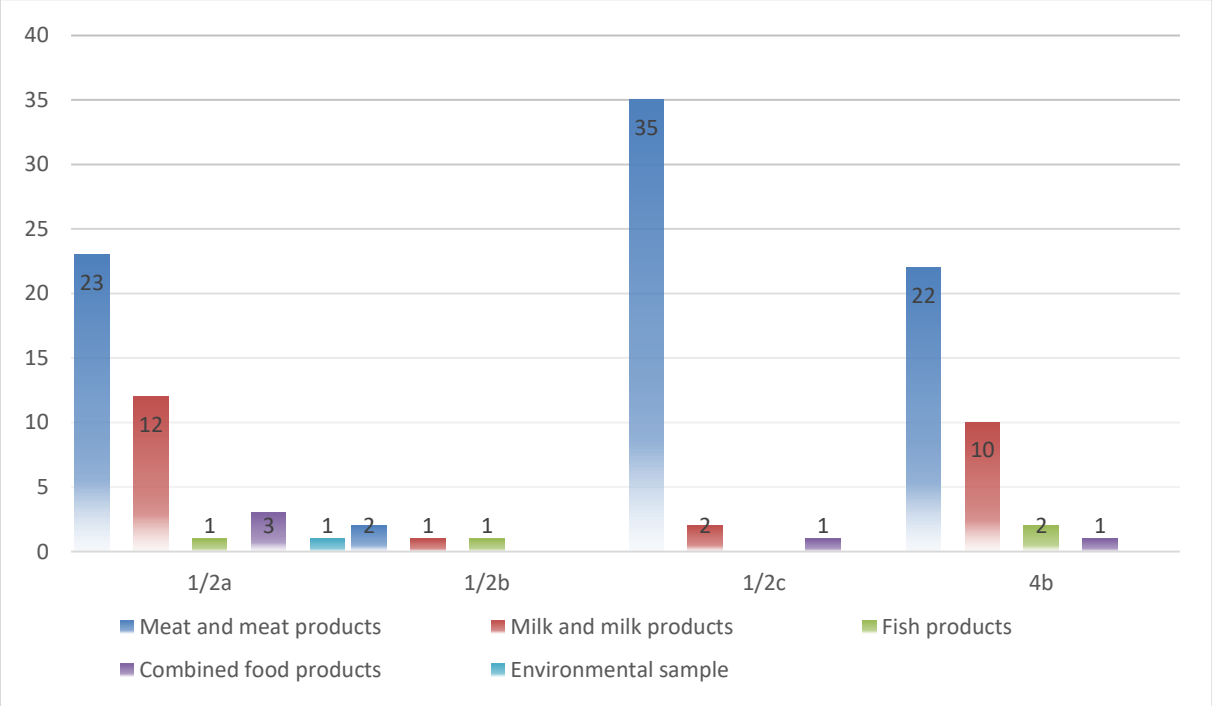



Figure 28. The graphic presentation of the four serotypes of *L. monocytogenes* identified in the food categories.

Together, the isolates from Serotype 1/2b and Serotype 4b comprised a total of 39 isolates within Lineage I.

Lineage II, on the other hand, was represented by Serotype 1/2a and Serotype 1/2c. Serotype 1/2a was identified in a total of 40 strains isolated from 23 meat products, 12 dairy products, 3 isolates from combined food products as well as one isolate each from fish products and environmental samples. This diversity in clonal complexes within Serotype 1/2a further highlights the heterogeneity of *L. monocytogenes* within this lineage. Serotype 1/2c, represented by 38 isolates, was mainly associated with meat products with 35 isolates, dairy products with 2, and combined food products with one isolate.

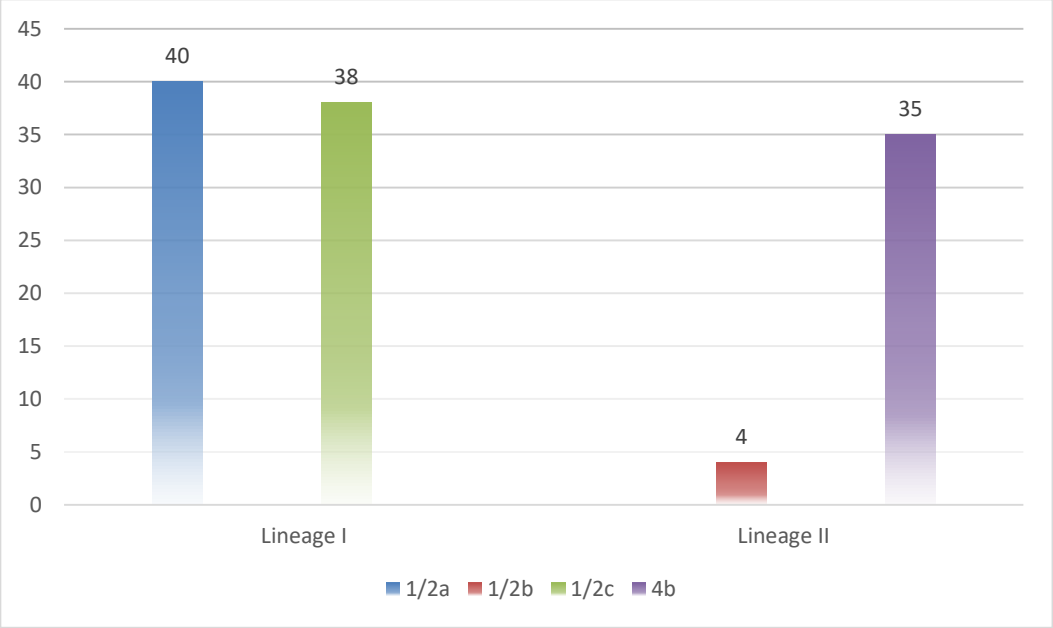



Figure 29. The figure shows bar chart comparing the distribution of *L. monocytogenes* serotypes in Lineage I and II.

The analysis revealed the presence of diverse *L. monocytogenes* lineages and clonal complexes across different food sectors in Kosovo. Serotypes 1/2b and 4b were predominantly associated with Lineage I, while Serotypes 1/2a and 1/2c were mainly linked to Lineage II, indicating notable serotype variability among the isolates (Figure 29).

These categories were sampled extensively, providing statistically robust data for occurrence analysis (Table 20; Figure 30 a, b).

Occurrence varied across food categories. Among processed food types, meat products showed the highest occurrence, with *L. monocytogenes* detected in 14.29% of the tested samples. In dairy products, the occurrence was 6.39%, with cheese being the most commonly affected subcategory. RTE foods exhibited a lower occurrence rate of 6.33% (Table 20).

Food products intended for consumption after cooking (FPCC) demonstrated a occurrence of 14.95%. The highest occurrence was observed in raw materials, where 55.00% of samples tested positive for *L. monocytogenes*. These findings reflect considerable variation in contamination rates depending on food type and processing stage.

Table 20. The number of samples per food category and the number of samples positive for *L. monocytogenes* in food chain and environmental samples in Kosovo between 2016 and 2022.

| Food category | No. of tested samples | No. of positive samples/No. of tested Samples (%) | Ready to-eat food | Food products consumed cooked | Food processing environment | Raw material |
|---|---|---|---|---|---|---|
| Meat and meat products | 574 | 82/574 (14.29%) | 20/286 (6.99%) | 42/249 (16.87%) | - | 20/39 (51.28 %) |
| Milk and milk products | 407 | 26/407 (6.39%) | 21/361 (5.82%) | 0/32 (0) | 1/6 (16.66%) | 4/8 (50.00%) |
| Fish and fishery products | 9 | 4/9 (44.44 %) | 0/1 (0) | - | - | 4/8 (50.00%) |
| Combined food products (meat, dairy) | 5 | 5/5 (100.00%) | - | - | - | 5/5 (100.00%) |
| Total | 995 | 117/995 (11.76%) | 41/648 (6.33%) | 42/281 (14.95%) | 1/6 (16.66%) | 33/60 (55.00%) |

The occurrence of *L. monocytogenes* varied significantly across different ready-to-eat (RTE) food subcategories. Among RTE dairy products, the occurrence was 5.82%. RTE meat products showed a slightly higher occurrence rate of 6.99%. In contrast, fish and fishery products exhibited a notably higher occurrence of 44.44%.

Combined food products, consisting of mixtures of meat and dairy, showed a 100.00% occurrence rate.

Figure 33 a, b presents the distribution of *L. monocytogenes* isolates by type of food product and geographical location of sampling points in Kosovo.

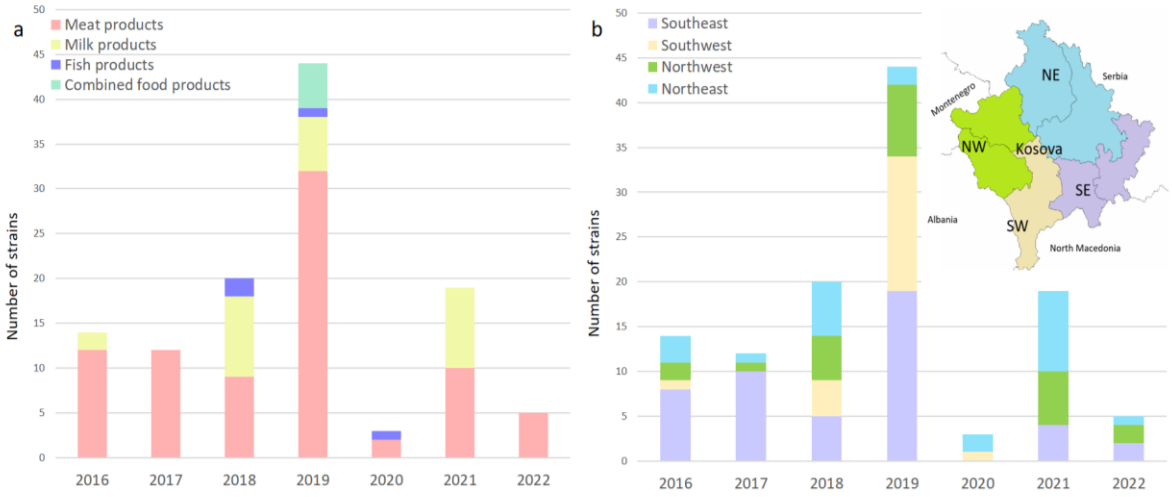



Figure 30. a. Distribution of *L. monocytogenes* over the years in meat products, milk products, fish products, and combined products; b. map showing the geographic location of sampling sites. Regional location of the sampling sites among business operators in Kosovo, with purple bars in the southeast (SE), brown in the southwest (SW), green in the northwest (NW), and blue in the northeast (NE).

## 5.3 Results confirmed by PFGE analysis

Initially, all isolates were confirmed using the end-point PCR method, which enables the specific identification of *Listeria monocytogenes* through the amplification of DNA fragments. Following confirmation, the isolates were subjected to molecular analysis using pulsed-field gel electrophoresis (PFGE), a standardized method widely used for genetic characterization of bacteria in epidemiological investigations. Figure 31 presents the positive results of the 117 obtained isolates, showing distinct genetic profiles based on the DNA fragment patterns generated after digestion with restriction enzymes. These results confirm the presence of *L. monocytogenes* and provide a foundation for further analysis of the genetic diversity and clonal relatedness among the tested isolates.

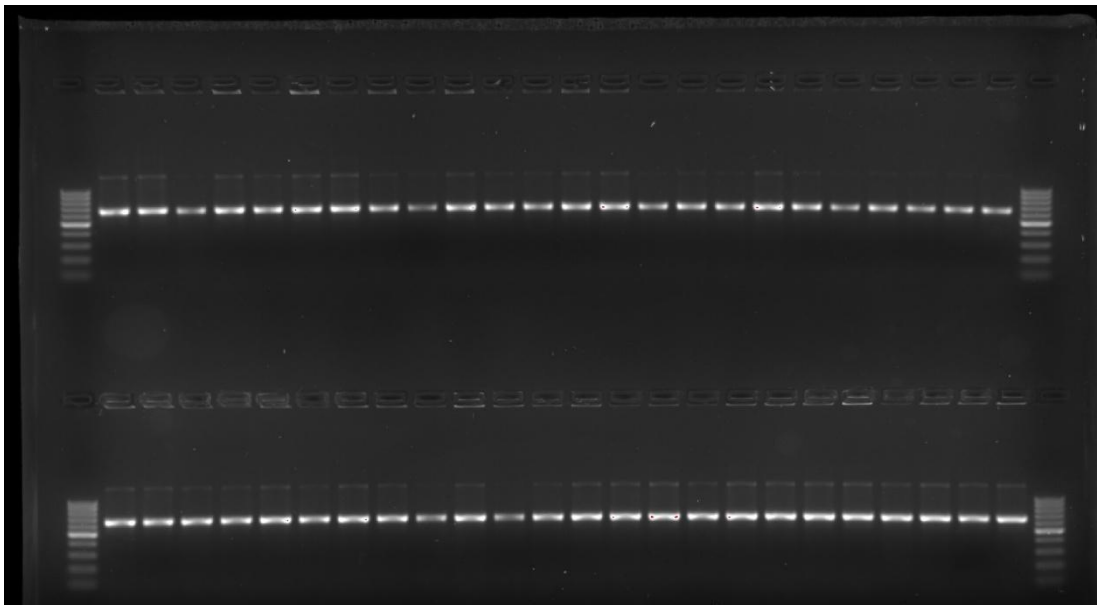


Figure 31. *L. monocytogenes* identified by PFGE.

### 5.4 Results of the identification of molecular groups and clonal complexes by RT-PCR

Figure 32 illustrates the amplification plots of various molecular serogroups using qPCR analysis. The x-axis represents the number of PCR cycles, while the y-axis shows the fluorescence intensity (ΔRn), which correlates with the amount of amplified DNA. Each curve corresponds to a different serogroup, including LMO 0737, ORF 2319, PLCA, LMO 1118, PRS, and ORF 2110. The amplification signals begin to diverge after approximately 18–20 cycles, indicating the exponential phase of PCR where target DNA is being efficiently amplified. Distinct variations in amplification efficiency and threshold cycle (Ct) values among serogroups suggest differences in DNA quantity or quality and primer-probe binding efficiency.

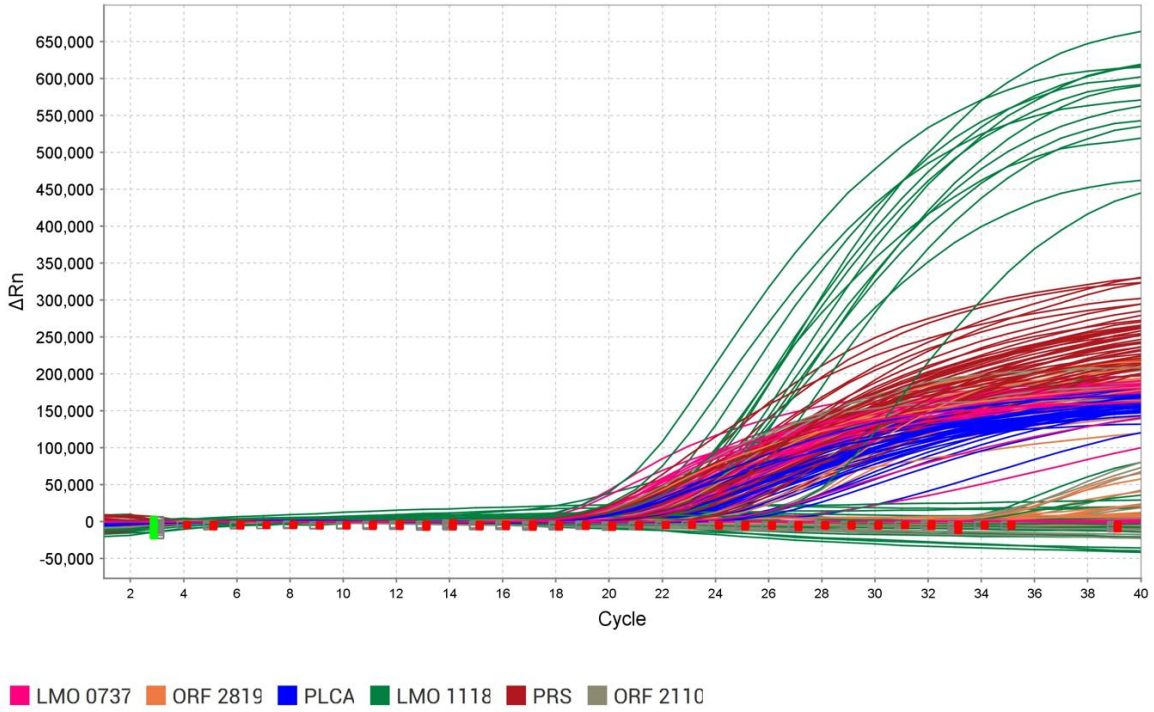



Figure 32. Amplification plots of molecular serogroups.

Figure 33 shows the amplification plots for different Clonal Complexes (CCs). Similar to Figure 32, the amplification curves begin to rise sharply after around 18 cycles. Clonal complexes such as CC6 - IVb, CC122 duplex IIc, CC204 - IIb, CC87 - IIa, CC2 - IVb, CC8_duplex IIa, CC3 - IIa, CC5 - IIa, CC1 - IVb, CC7 - IIb, and CC14 - IIb are represented. The curves exhibit different slopes and Ct values, indicating variable amplification kinetics among the complexes. This suggests genetic variability among CCs and may be useful for differentiation and identification in molecular epidemiology studies.

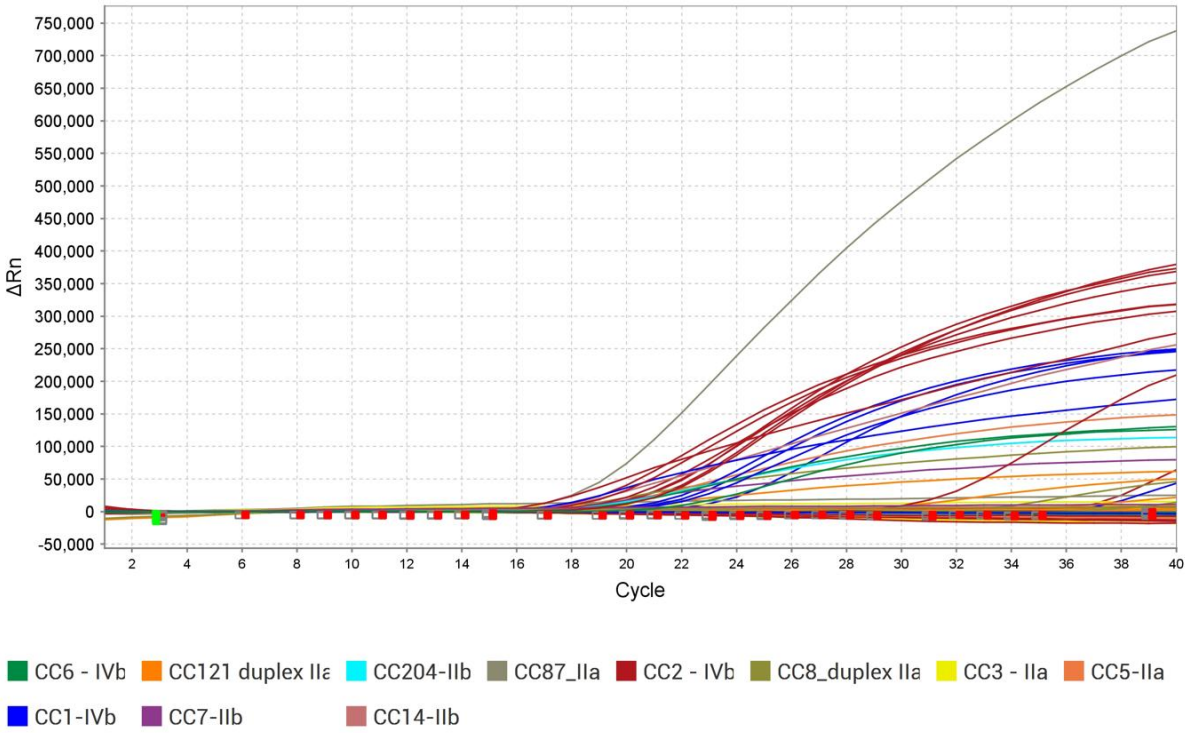



Figure 33. Amplification plots of Clonal Complexes.

Positive controls were provided by the European Reference Laboratory for *L. monocytogenes*.

Positive amplifications were determined when reactions with a detection threshold less than or equal to 30 cycles (Ct ≤ 30) were recorded, while late reactions, over 30 cycles (Ct >30), were considered to be non-specific. Additionally, multilocus sequence typing (MLST) was used to identify ST32, which was not detected by the CC primer panel by real-time PCR.

## 5.5 Genetic Diversity of *L. monocytogenes*

Among the 117 L. monocytogenes isolates analyzed by multiplex PCR, 17 distinct clonal complexes (CCs) were identified, reflecting notable genetic diversity within the population. These CCs were distributed across four molecular serotypes: IIa, IIb, IIc, and IVb.

Serotype IIa was the most prevalent, accounting for 34.19% of the isolates, followed by IIc (32.48%), IVb (29.91%), and IIb (3.48%). This distribution demonstrates a predominance of serotypes IIa and IIc among the sampled isolates.

Based on phylogenetic analysis, the isolates were classified into two major lineages. Lineage II was the most common, comprising 78 isolates (66.66%), while Lineage I included 39 isolates (33.33%). No isolates belonging to Lineages III or IV were detected.

## 5.6 Results of Lineages

Analyzing the distribution of *L. monocytogenes* lineages across different food chain categories, a distinct pattern emerged regarding the occurrenece of Lineages I and II. Among the ready-to-eat (RTE) food isolates, Lineage I was identified in 48.78% of the samples. Conversely, Lineage II was detected in 51.22% of the RTE isolates, making it slightly more prevalent in this category.

In food products consumed cooked (FPCC), the occurrence of Lineage I was lower, identified in 23.81% of the isolates. Lineage II, on the other hand, was detected in a substantial 76.19% of FPCC isolates.

Raw materials presented a notably different pattern, with Lineage I detected in 27.27% of the isolates. Among the raw material isolates, Lineage II was detected in 72.73% of cases, confirming its predominance in this category.

The distribution of Lineages I and II across the three main food chain categories—ready-to-eat (RTE) foods, food products consumed cooked (FPCC), and raw materials—is summarized in Table 21. Lineage II was the dominant lineage across all categories, while Lineage I was detected most frequently in RTE foods.

Table 21. The molecular characteristics of *L. monocytogenes* isolated from food products.

| Food chain | Food category | Lineage | Molecular Serotype | Clonal Complex |
|---|---|---|---|---|
| Ready to eat food (n=41) | Meat and meat product (20) | I (10) | IIb (1) | CC3 (1) |
| | | | IVb (9) | CC2 (1), CC4 (1), CC6 (6), CC315 (1) |
| | | II (10) | IIa (8) | CC7 (1), CC8 (3), CC29 (1), CC37 (3) |
| | | | IIc (2) | CC9 (2) |
| | Milk and milk product (21) | I (10) | IIb (1) | CC87 (1) |
| | | | IVb (9) | CC2 (5), CC4 (2), ST32 (2) |
| | | II (11) | IIa (10) | CC8 (1), CC14 (1), CC26 (1), CC29 (6), CC37 (1) |
| | | | IIc (1) | CC9 (1) |

| | | | | |
|---|---|---|---|---|
| | Total | I (48.78%), II (51.22%) | IIb (2), IVb (18) IIa (18), IIc (3) | CC2 (6), CC3 (1), CC4 (3), CC6 (6), CC7 (1), CC8 (4), CC9 (3), CC14 (1), CC26 (1), CC29 (7), ST32 (2), CC37 (4), CC87 (1), CC315 (1) |
| Food product consumed cooked (n=42) | Meat and meat product (42) | I (10) | IIb (1) | CC5 (1) |
| | | | IVb (9) | CC2 (4), CC4 (1), CC6 (3), CC315 (1) |
| | | II (32) | IIa (11) | CC7 (4), CC8 (1), CC14 (3), CC29 (1), CC37 (2) |
| | | | IIc (21) | CC9 (21) |
| | Total | I (23.81%), II (76.19% ) | IIb (1), IVb (9) IIa (11), IIc (21) | CC2 (4), CC4 (1), CC5 (1), CC6 (3), CC7 (4), CC8 (1), CC9 (21), CC14 (3), CC29 (1), CC37 (2), CC315 (1) |
| Raw material (n=33) | Meat and meat product (20) | I (4) | IVb (4) | CC1 (4) |
| | | II (16) | IIa (4) | CC7 (1), CC8 (3) |
| | | | IIc (12) | CC9 (12) |
| | Milk and milk product (4) | I (1) | IVb (1) | CC315 (1) |
| | | II (3) | IIc (1) | CC9 (1) |
| | | | IIa (2) | CC8 (1), CC26 (1) |
| | Fish meat product (4) | I (3) | IIb (1) | CC87 (1) |
| | | | IVb (2) | CC2 (2) |
| | | II (1) | IIa (1) | CC26 (1) |
| | Combined food products (5) | I (1) | IVb (1) | CC6 (1) |
| | | II (4) | IIa (3) | CC8 (1), CC121 (2) |
| | | | IIc (1) | CC9 (1) |
| | Total | I (27.27%), II (72.73%) | IIb (1), IVb (8) IIa (10), IIc (14) | CC1 (4), CC2 (2), CC6 (1), CC7 (1), CC8 (5), CC9 (14), CC26 (2), CC87 (1), CC121 (2), CC315 (1) |
| Food contact sample (n=1) | Environmental sample (1) | II (1) | IIa (1) | CC7 (1) |
| | Total | II (100.00%) | IIa (1) | CC7 (1) |

When analyzed by food category, the distribution of *L. monocytogenes* lineages varied among the different groups. In the case of meat products, a total of 82 isolates were identified, of which 29.27% were classified as Lineage I, while the remaining 70.73% belonged to Lineage II. This indicates a predominance of Lineage II within this category.

In milk and milk products, 25 isolates were analyzed. Of these, 44.00% were found to belong to Lineage I, whereas 56.00% were attributed to Lineage II.

For combined food products, which include mixtures of ingredients such as meat and dairy, five isolates were obtained. Among these, four isolates, equivalent to 80.00%, were identified as belonging to Lineage II, and one isolate (20.00%) was assigned to Lineage I.

Fish products showed a different distribution pattern. Out of four isolates analyzed in this category, three (75.00%) were categorized as Lineage I, while the remaining one isolate (25.00%) was identified as Lineage II.

Environmental samples yielded a single isolate of *L. monocytogenes,* which was classified as belonging to Lineage II. This isolate represents the only data point from environmental sampling in this study.

The detailed breakdown of the isolates across food categories provides an overview of the lineage distribution and diversity observed in this study, as summarized in Table 19.

## 5.7 Results of Molecular Serotypes

Among the 41 *L. monocytogenes* isolates recovered from ready-to-eat (RTE) foods, the distribution of molecular serotypes demonstrated notable variation. Of these, 18 isolates (43.90%) each belonged to serotypes IIa and IVb, making them the most frequently identified within this category. Three isolates (7.32%) were categorized as serotype IIc, while two isolates (4.88%) were classified as serotype IIb, highlighting the diverse serotype representation in RTE foods.

In food products consumed cooked (FPCC), 42 isolates were analyzed, revealing serotype IIc as the predominant molecular serotype, with 21 isolates (50.00%). This was followed by 11 isolates (26.19%) belonging to serotype IIa, 9 isolates (21.43%) classified as serotype IVb, and 1 isolate (2.38%) identified as serotype IIb.

The 33 isolates obtained from raw materials showed a slightly different distribution. Serotype IIc was the most represented, accounting for 14 isolates (42.42%), followed by serotype IIa, and with 10 isolates (30.30%). Serotype IVb was found in 8 isolates (24.24%), while serotype IIb was identified in only 1 isolate (3.03%). These findings reflect the varied serotype composition in raw material samples.

When the data were divided by food category, distinct patterns emerged. Among the 82 isolates from meat products alone, molecular serotype IIc was the most prevalent, identified in 35 isolates (42.68%). This was followed by serotype IIa, represented in 23 isolates (28.05%); serotype IVb, identified in 22 isolates (22.26%); and serotype IIb, found in 2 isolates (2.44%).

In milk and milk products, 25 isolates were analyzed. Serotype IIa was the most commonly identified, present in 12 isolates (48.00%), followed by serotype IVb, with 10 isolates

(40.00%). Serotype IIc was detected in 2 isolates (8.00%), and serotype IIb was represented in 1 isolate (4.00%).

Isolates from combined food products exhibited a different serotype distribution. Among the five isolates in this category, three (60.00%) belonged to serotype IIa, while one (20.00%) each was identified as serotypes IIc and IVb.

For fish products, which had a smaller sample size of four isolates, molecular serotypes IVb and IIa were each represented by one isolate (25.00%), as was serotype IIb. The remaining isolate in this category belonged to serotype IIc.

The single isolate recovered from an environmental sample was identified as belonging to molecular serotype IIa.

### 5.8 Results of Clonal Complexes

Notably, 14 out of the 17 clonal complexes (CCs) were identified through real-time PCR from the 41 isolates obtained from ready-to-eat (RTE) foods. Among these, CC29, CC2, and CC6 emerged as the most dominant clonal complexes. Specifically, seven isolates were classified as CC29, followed by six isolates for each of the CC2 and CC6 clonal complexes.

In contrast, CC9 was the most frequently detected clonal complex in both food products consumed cooked (FPCC) and raw material (RM) categories. In total, 21 isolates from FPCC and 14 isolates from RM were identified as CC9, underscoring its occurrence in these two food groups.

When focusing on meat product-only isolates, a total of 13 different clonal complexes were identified. Of the 82 meat product isolates, CC9 was by far the most represented clonal complex, with 35 isolates (42.68%) expressing this group. This was followed by CC6, which appeared in 9 isolates (10.98%); CC8, represented by 7 isolates (8.53%); and CC2, expressed by 5 isolates (6.10%).

For the 25 milk product-only isolates, 11 different clonal complexes were identified. In this category, CC29 was identified in six isolates (24%), followed by CC2, which was detected in five isolates (20%). In contrast, CC9 was only detected in two milk product isolates (8%).

In fish products, three out of the four isolates belonged to CC2, with two isolates, while the remaining isolates were classified as CC26 and CC87, each represented by a single sample.

Regarding combined food products, four groups of clonal complexes were identified. Interestingly, CC121, which was not detected in any other food category, was expressed by two isolates (50%). CC6, CC8, and CC9 were each expressed by a single isolate.

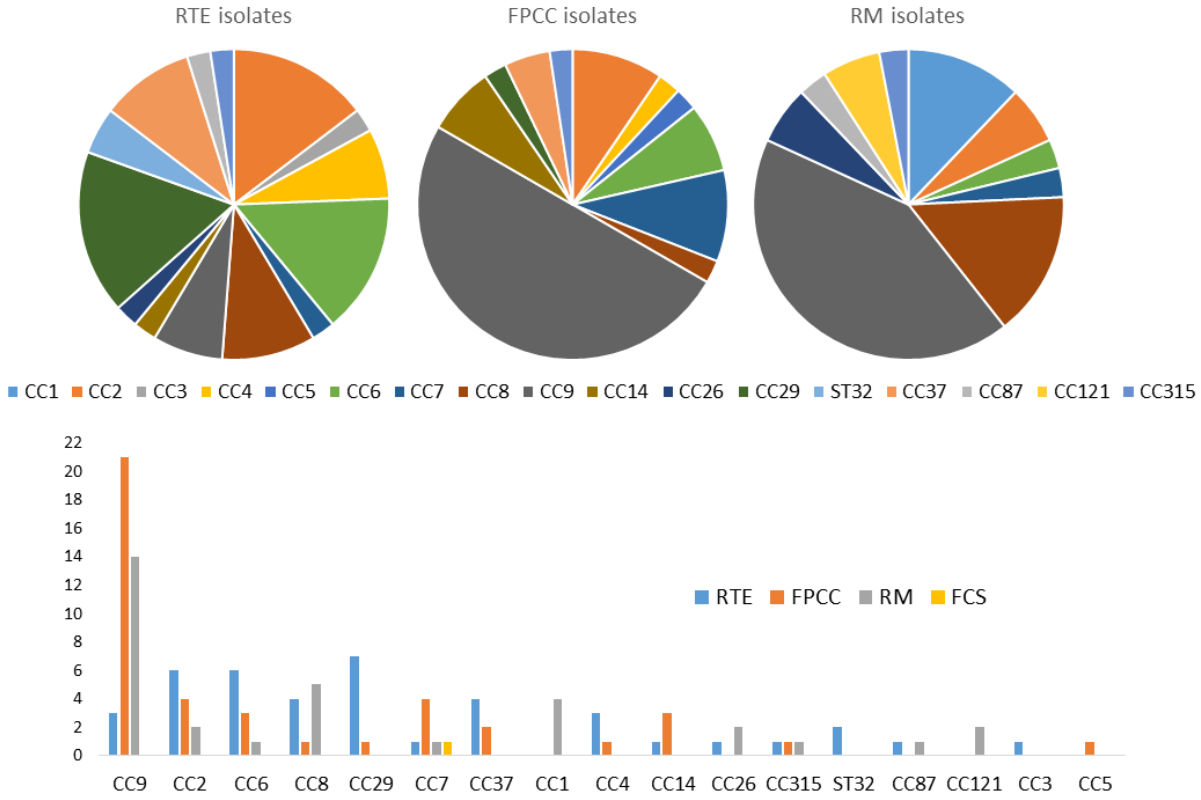



Figure 34. Graphical distribution of *L. monocytogenes* clonal complexes in the food chain.

These findings, summarized and illustrated in Figure 34, offer a detailed picture of the distribution of *L. monocytogenes* clonal complexes across various food categories. They emphasize the diversity of clonal complexes within each food group, with certain complexes like CC9 being more prevalent in specific food types, such as FPCC and meat products, while others like CC29 and CC2 show stronger associations with RTE and milk products.

A minimum spanning tree (MST) was reconstructed based on food chain categories in order to better understand the population structure of *L. monocytogenes* clonal complexes within the Republic of Kosovo.

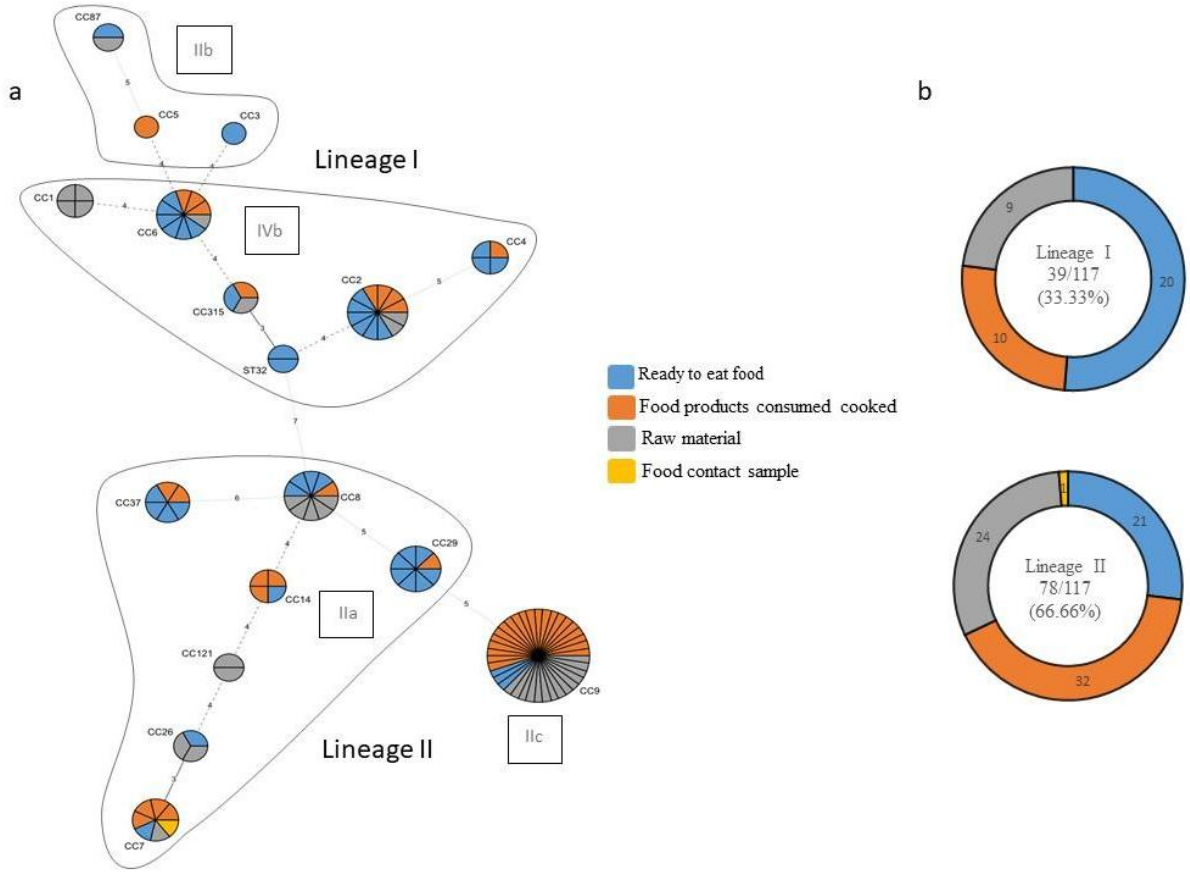



Figure 35. a. The minimum spanning tree (MST) of multilocus sequence typing (MLST) clonal complexes (CCs) of the 117 *L. monocytogenes* strains included in the study panel. Each CC is indicated by a circular node whose size reflects the number of strains. The CCs used to build the MST were obtained by an MLST alternative method, which provides only the CC, to build the MST; the smallest ST allelic code within the CC was used. The numbers along the node connecting the lines indicate the number of allelic differences between them. The color reflects the food chain category: ready to eat food in blue, food products consumed cooked in orange, raw material in grey, and food contact sample in yellow. Each delimited area groups the CCs belonging to the same molecular serotype, indicated in a black frame; b. strain distribution according to the lineage and type of food sector.

The MST, illustrated in Figures 35a and 35b, was specifically designed to map the genetic relationships between various isolates collected from different food sectors. This tool helps to determine the degree of relatedness and genetic diversity among the clonal complexes of *L. monocytogenes*, which is critical for tracing the potential sources of contamination and understanding the spread of the pathogen across the food supply chain.

## 5.9 Results of Multilocus Sequence Typing

A total of 114 strains of *L. monocytogenes* were subjected to molecular analysis to determine their multilocus sequence types (MLST) and assess their antimicrobial susceptibility. Through the use of the BIGSdb-Pasteur MLST database for *L. monocytogenes* (accessible at https://bigsdb.pasteur.fr/listeria, accessed on 25 March 2024), sixteen distinct clonal complex–sequence type (CC–ST) allelic profiles were identified. These included CC1, CC2, CC3, CC4, CC6, CC7, CC8, CC9, CC14, CC26, CC29, ST32, CC37, CC87, CC121, and CC315. Table 22 provide a comprehensive summary of the identified clonal complexes and their corresponding sequence types.

Among the identified clones, CC9–ST9 emerged as the most frequently observed, accounting for 29.8% of all isolates. This was followed by CC8–ST8, which represented 8.77% of the isolates, and CC6–ST6, with 7.89%. Clone CC29–ST29 accounted for 7.02%, while CC2–ST2 represented 6.14% of the isolates. Other clones, including CC1, CC3, CC4, CC7, CC14, CC26, ST32, CC37, CC87, CC121, and CC315, were observed at lower frequencies, each constituting less than 5.26% of the total strains examined.

Table 22 presents a summary of the isolates, including their clonal complex, sequence type, and frequency across the monitored years, offering a clear view of the genetic diversity observed within the studied population of *L. monocytogenes*.

Table 22. Genetic characteristics of *L. monocytogenes* isolates originating from food products.

| Molecular Genotype of Isolates | | | | Strain Origin (No. of Isolates) | | | | |
|---|---|---|---|---|---|---|---|---|
| Lineage | Serotype Group | Clonal Complex | Sequence Type | MP | MDP | FMP | ES | CFP |
| I | IVb | CC1 | ST328 | 3 | | | | |
| | | | ST710 | 1 | | | | |
| | | CC2 | ST2 | 1 | 5 | 1 | | |
| | | | ST145 | 4 | | 1 | | |
| | IIb | CC3 | ST3 | 1 | | | | |
| | IVb | CC4 | ST4 | 1 | 2 | | | |
| | | CC6 | ST6 | 9 | | | | 1 |
| | | ST32 | ST32 | | 2 | | | |
| | IIb | CC87 | ST87 | | 1 | 1 | | |
| | IVb | CC315 | ST520 | 2 | 1 | | | |
| II | IIa | CC7 | ST7 | 5 | | | 1 | |
| | | | ST12 | 1 | | | | |
| | | CC8 | ST8 | 7 | 2 | | | 1 |
| | IIc | CC9 | ST9 | 31 | 2 | | | 1 |
| | | | ST580 | 4 | | | | |
| | | CC14 | ST14 | | 1 | | | |
| | | | ST399 | 3 | | | | |
| | IIa | CC26 | ST26 | | 2 | 1 | | |
| | | CC29 | ST29 | 2 | 6 | | | |
| | | CC37 | ST37 | 5 | 1 | | | |
| | | CC121 | ST121 | | | | | 1 |

MP—Meat and meat products; MDP—Milk and dairy products; FMP—Fish meat products; ES—Environment sample; CFP—Combined food products.

The multilocus sequence typing (MLST) analysis of *L monocytogenes* strains yielded a detailed genetic profile, identifying allelic configurations for 21 distinct sequence types (STs). The sequence types observed included ST2, ST3, ST4, ST6, ST7, ST8, ST9, ST12, ST14, ST26, ST29, ST32, ST37, ST87, ST121, ST145, ST328, ST399, ST520, ST580, and ST710. Tables 22 and Figure 36 outline the distribution of these sequence types across the studied strains.

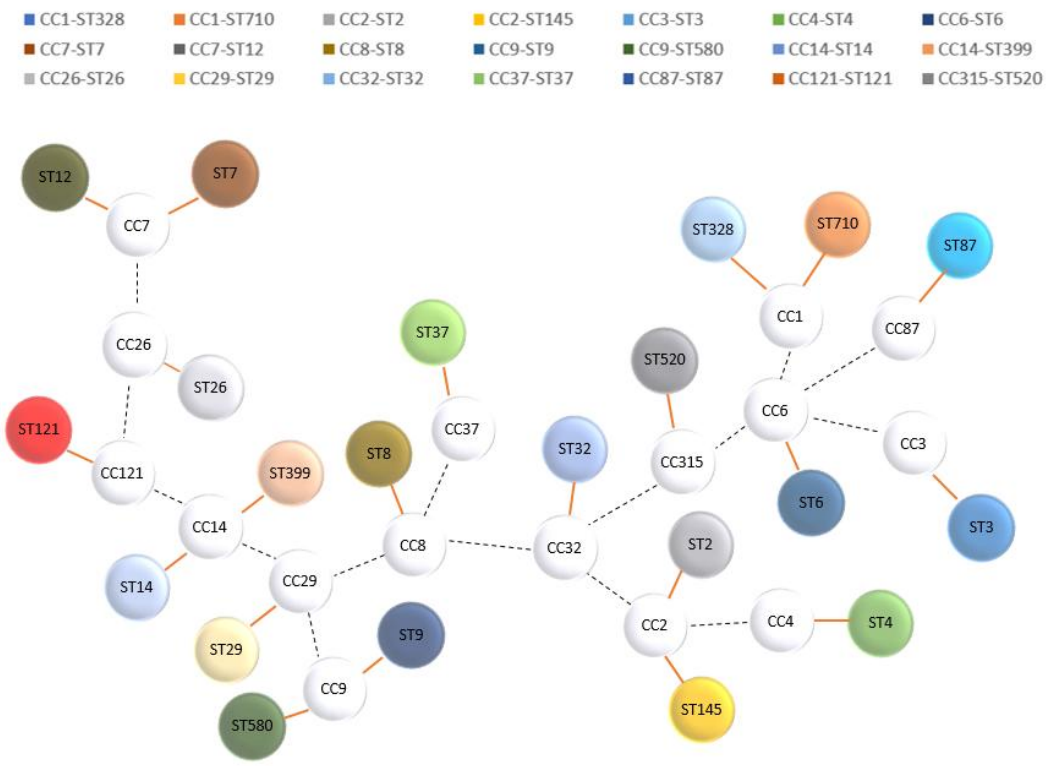



Figure 36. Spanning tree of Sequence typing of isolates.

Within the isolates obtained from meat and meat products, which constituted the largest category of samples (n = 80), 16 unique sequence types were identified. Among these, the least represented sequence types were ST2, ST3, ST4, ST12, and ST710, with each accounting for a single isolate (1.25% of the total meat isolates). ST29 and ST520 were slightly more prevalent, with two isolates each (2.5%). Three isolates (3.75%) were identified as ST328 and ST399. Sequence types ST145 and ST580 each accounted for four isolates (5.0%), while ST7 and ST37 were detected in five isolates (6.25%). Sequence type ST8 was represented by seven isolates (8.75%), and ST6 was observed in nine isolates (11.25%). The most prevalent sequence type in meat products was ST9, identified in 31 isolates, representing 38.75% of the total.

For dairy products, a total of 25 isolates were analyzed, and 11 sequence types were detected. Four sequence types, ST14, ST37, ST87, and ST520, were identified in single isolates (4.0% each). Sequence types ST4, ST8, ST9, ST26, and ST32 were each detected in two isolates (8.0% each). ST145 was found in five isolates (20.0%), while ST29 was the most frequently identified sequence type in this category, with six isolates (24.0%).

Fish products yielded a smaller number of isolates, with only four analyzed in total. These isolates were evenly distributed among four sequence types: ST2, ST26, ST87, and ST145, with each sequence type represented by one isolate (25.0%). Similarly, for combined food products, four isolates were identified, each corresponding to a different sequence type: ST6, ST8, ST9, and ST121, also with one isolate (25.0%) for each sequence type.

Environmental samples were represented by a single isolate in this study. This isolate was identified as ST7, adding another distinct sequence type to the dataset and contributing to the genetic diversity observed among the strains analyzed.

### 5.10 Antimicrobial Susceptibility

Among all the *L. monocytogenes* isolates tested against a panel of 18 antibiotics at varying concentrations, varying levels of resistance and susceptibility were observed across different sample types (Table 23).

Table 23.Antimicrobial susceptibility of *L. monocytogenes* distributed across phylogenetic lineages and food categories.

| Phylogenetic Lineage | Food Category | Resistant | Intrinsic Resistance | Sensitive |
|---|---|---|---|---|
| *Lineage I (n = 37)* | MP (*n* = 22) | GEN (5), STR (1), ERY (4), TET (1), SYN (3), RIF (4), CIP (6), PEN (1), LEVO (6) | AXO (15), OXA+ (4), CLI (22), DAP (22) | VAN, AMP, LZD, GAT, SXT all by (22) |
| | MMP (*n* = 11) | PEN (1), LEVO (1) | AXO (4), CLI (11), DAP (11) | VAN, AMP, LZD, GAT, SXT all by (11) |
| | FMP (*n* = 3) | GEN (1), ERY (1), TET (1), SYN (1), RIF (1), PEN (1), LEVO (1) | AXO (2), OXA+ (2), CLI (3), DAP (3) | VAN, AMP, LZD, GAT, SXT all by (3) |
| | CFP (*n* = 1) | - | AXO (1), CLI (1), DAP (1) | VAN, AMP, LZD, GAT, SXT all by (1) |
| *Lineage II (n = 77)* | MP (*n* = 58) | GEN (13), ERY (8), SYN (13), RIF (14), CIP (1), PEN (6), LEVO (16) | AXO (18), OXA+ (22), CLI (58), DAP (56) | VAN, AMP, LZD, GAT, SXT all by (58) |

| | | | | |
|---|---|---|---|---|
| | MMP (*n* = 14) | LEVO (1) | CLI (14), DAP (12) | VAN, AMP, LZD, GAT, SXT all by (14) |
| | FMP (*n* = 1) | - | CLI (1), DAP (1) | VAN, AMP, LZD, GAT, SXT all by (1) |
| | ES (*n* = 1) | LEVO (1) | CLI (1), DAP (1) | VAN, AMP, LZD, GAT, SXT all by (1) |
| | CFP (*n* = 3) | GEN (1), RIF (1) | OXA+ (2), CLI (3), DAP (3) | VAN, AMP, LZD, GAT, SXT all by (3) |

Isolates from meat products demonstrated the highest resistance profile, showing resistance to 13 out of the 18 antibiotics tested, equivalent to 72.2% of the total antimicrobials. The remaining 5 antibiotics, representing 27.8%, were effective, with the isolates being susceptible to these treatments.

Dairy product isolates exhibited a contrasting pattern, with resistance observed against 5 of the 18 antibiotics tested, accounting for 27.8% of the antimicrobial panel.

For isolates obtained from fish products, resistance was observed against 11 of the 18 antibiotics tested, representing 61.1% of the antimicrobial panel. Conversely, 7 antibiotics (38.9%) were effective in inhibiting these isolates.

The single isolate from an environmental sample demonstrated resistance against 3 of the 18 antibiotics tested, accounting for 16.7% of the panel. The remaining 15 antibiotics (83.3%) were effective, showing a lower resistance profile compared to the other categories.

Isolates derived from combined food products displayed resistance to 6 of the 18 antibiotics tested, representing 33.3%. Meanwhile, susceptibility to 12 antibiotics (66.7%) was observed, indicating a mixed response in this category.

The susceptibility of *L. monocytogenes* isolates to a panel of antimicrobials evaluated in this study demonstrated varying resistance levels across different antibiotics, as shown in Figure 37 a, b. Resistance was observed in all 114 isolates (100%) to daptomycin, making it the most universally resisted antibiotic in the study. Similarly, a high percentage of isolates, 110 out of 114 (96.5%), were resistant to clindamycin. Ceftriaxone showed resistance in 40 isolates (35.1%), highlighting its moderate efficacy against the strains tested.

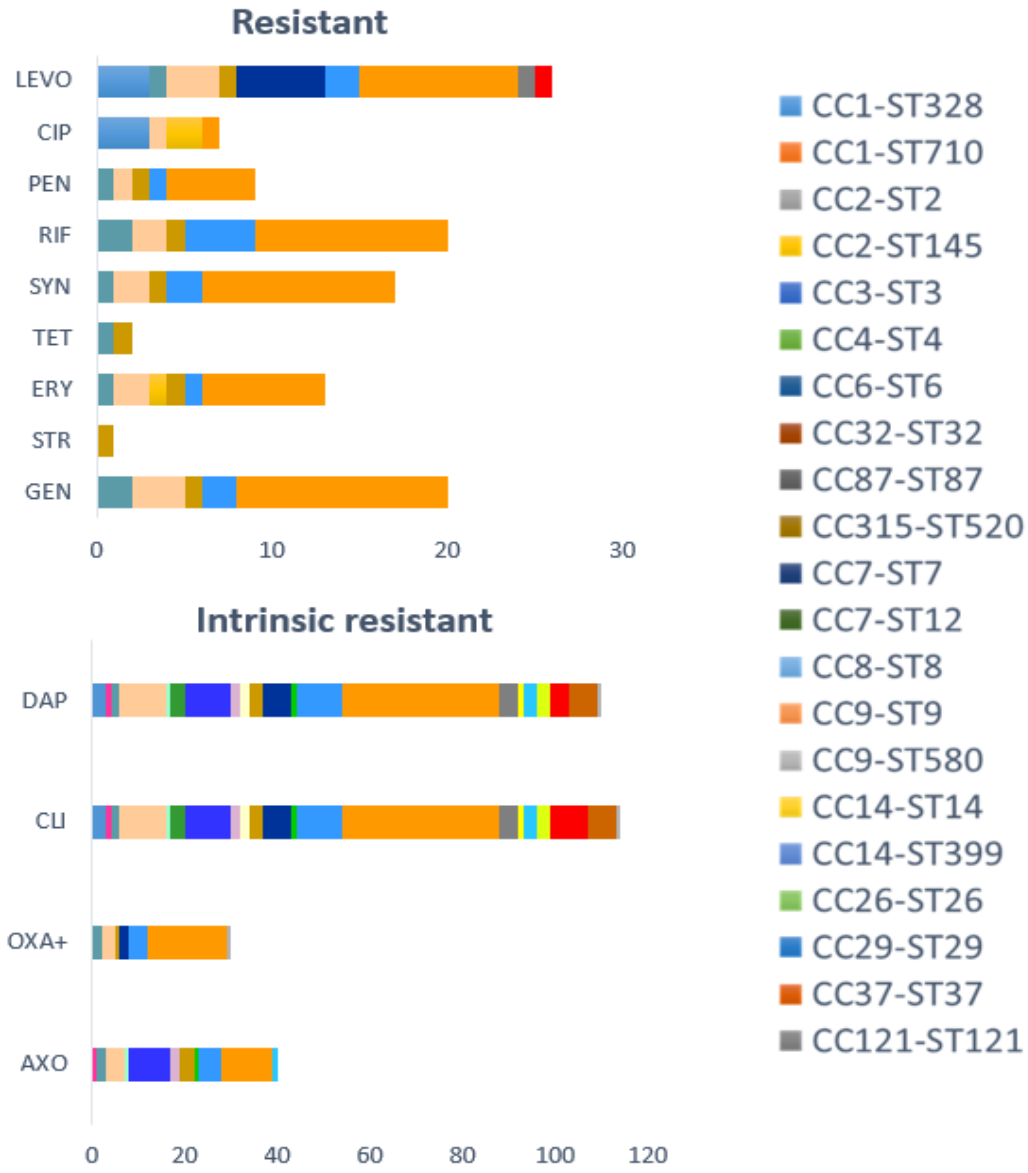



Figure 37. a. Strains resistant against nine emergency antimicrobials LEVO, CIP, PEN, RIF, SYN, TET, ERY, STR, and GEN, shown in respective colors for each CC-STs. b. Four antibiotics, which *L. monocytogenes*, are naturally resistant (intrinsic resistance) to DAP, CLI, OXA+, and AXO, shown in respective colors for each CC-STs.

For oxacillin combined with 2% NaCl, resistance was observed in 30 isolates (26.3%), while levofloxacin showed resistance in 26 isolates (22.8%). Resistance to gentamicin and rifampin was reported in 20 isolates each, accounting for 17.5% of the total isolates tested. A slightly lower resistance level was seen with quinupristin/dalfopristin, where 17 isolates (14.9%) were resistant. Erythromycin resistance was identified in 13 isolates (11.4%), and penicillin resistance was observed in 9 isolates (7.89%).

Lower levels of resistance were noted for ciprofloxacin, with 7 isolates (6.14%) showing resistance, and for tetracycline, with resistance observed in only 2 isolates (1.75%). Streptomycin demonstrated the lowest resistance rate, with just 1 isolate (0.88%) resistant to this antibiotic.

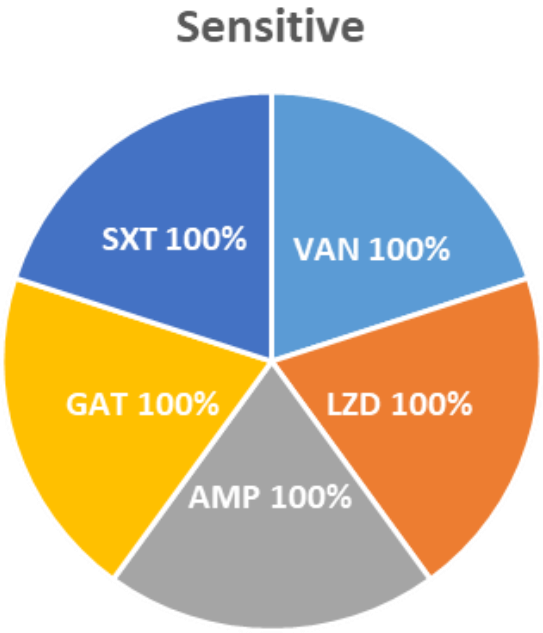



Figure 38. A pie chart displays that all isolates are sensitive to VAN, SXT, LZD, GAT, and AMP.

In contrast, the isolates were entirely susceptible to several antimicrobials tested in this study. These included ampicillin AMP, gatifloxacin GAT, vancomycin VAN, linezolid LIN, and trimethoprim/sulfamethoxazole SXT (Figure 38).

The Sankey plot (Figure 39) represents the flow of relationships between antibiotics used and clones (CC–ST) identified from isolates, further divided by phylogenetic lineage I and II. Each segment represents a measurable relationship between a given antibiotic and a specific cluster, indicating occurrence or resistance in those groups. The colors distinguish phylogenetic lineages and CC–ST with purple and antibiotics and green representing resistant antibiotics. The thickness of the flow (or relationship) reflects the number or percentage of isolates showing that relationship, making it clear which antibiotics are most closely related to certain CC–STs and how they are divided across phylogenetic lineages.

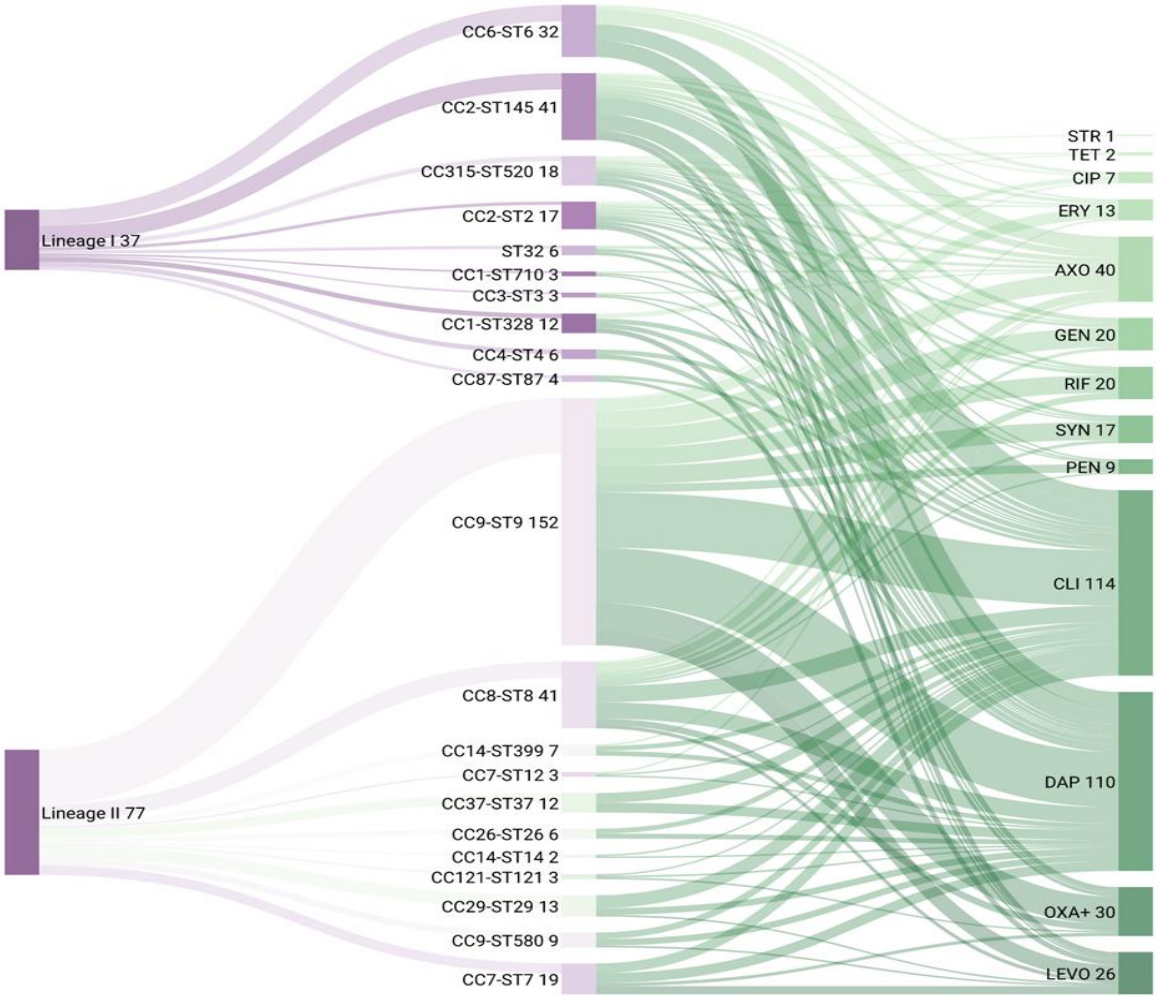



Figure 39. Sankey chart—correlation of antibiotics to CC–ST and phylogenetic lineage I and II.

The analysis of multi-resistant clones against antimicrobials revealed notable variation in the frequency of resistance among the different clonal complexes and sequence types (CC–ST). The most frequently identified multi-resistant clone was CC9–ST9, which accounted for 16.7% of the total multi-resistant isolates. This was followed by CC6–ST6, representing 8.77% of the multi-resistant isolates. Clonal complexes CC2–ST145 and CC8–ST8 were equally represented, each constituting 6.14% of the total.

Other clonal complexes displayed lower frequencies of multi-resistance. CC7–ST7 accounted for 4.39% of the multi-resistant isolates, while CC1–ST328 made up 2.63%. Clonal complexes such as ST32 and CC315–ST520 were observed at 1.75% each. A range of other clonal complexes, including CC1–ST710, CC3–ST3, CC7–ST12, CC9–ST580, CC14–ST399, CC29–ST29, and CC121–ST121, were each represented by 0.88% of the multi-resistant isolates.

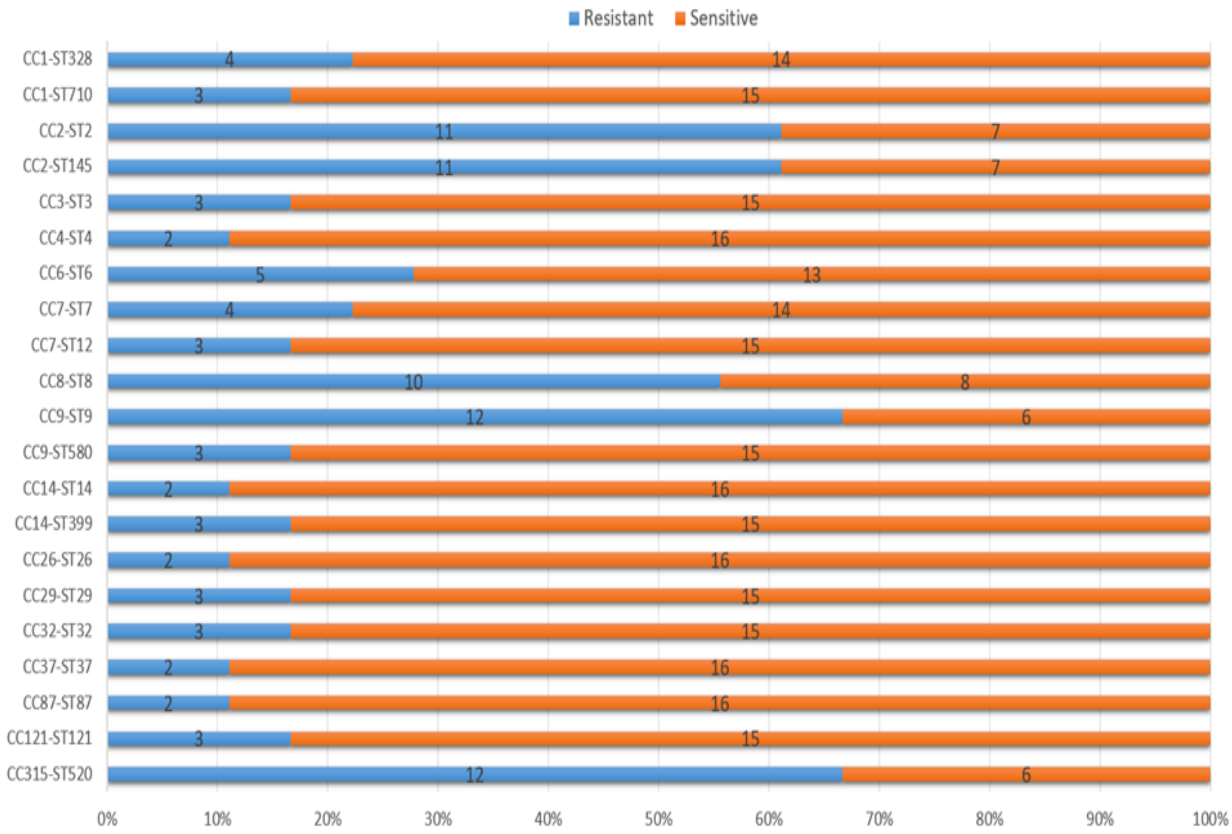



Figure 40. Graph showing the ratio of resistant and sensitive isolates.

In contrast, several clonal complexes did not exhibit multi-resistance to the antimicrobials tested. These included CC4–ST4, CC14–ST14, CC26–ST26, CC37–ST37, and CC87–ST87. These clones were entirely susceptible to the panel of antimicrobials evaluated in the study.

Figure 40 provide detailed visual representations and data supporting the observed distribution of antimicrobial resistant (red ribbons) and susceptible (blue ribbons) clones. These findings illustrate the diversity in antimicrobial resistance profiles across the clonal complexes and sequence types analyzed.

### *5.10.1 Results of Minimum Inhibitory Concentration*

Table 24 provides a detailed overview of the antimicrobial resistance profiles of *L. monocytogenes* isolates, accompanied by the minimum inhibitory concentration (MIC) values determined for each antibiotic tested.

The MIC values presented in Table 24 offer a quantitative measure of the concentrations required to inhibit bacterial growth for each antibiotic, revealing the levels at which the isolates displayed resistance or susceptibility. For antibiotics where resistance was observed, the MIC values were typically elevated, indicating a reduced efficacy of these agents against the isolates. Conversely, lower MIC values for certain antibiotics indicated higher susceptibility among the isolates, which may point to potential treatment options.

Table 24. The minimum inhibitory concentration (MIC) distribution of the 114 *L. monocytogenes* strains.

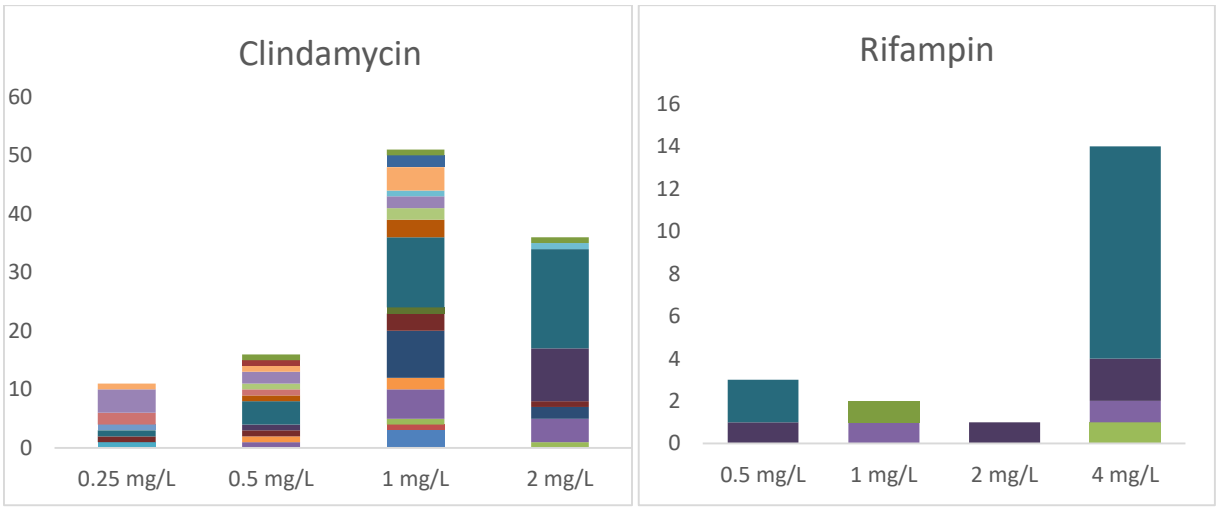
| Antimicrobials | MIC (mg/L) | | | | | | | | | | | | | | Interpretation | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.06 | 0.12 | 0.25 | 0.50 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 500 | 1000 | S | R |
| Gentamicin | | | | | | 2 | 1 | 3 | 16 | | | | 1 | | ≤2 | >2 |
| Streptomycin | | | | | | | | | | | | | | 1 | <1000 | ≥1000 |
| Erythromycin | | | 31 | 6 | 3 | 9 | 4 | | | | | | | | ≤1 | >1 |
| Tetracycline | | | | | | 1 | 0 | 0 | 1 | | | | | | ≤1 | >1 |
| Quinupristin/ Dalfopristin | | 5 | 36 | 54 | 2 | 0 | 17 | | | | | | | | ≤1 | >1 |
| Rifampin | 0 | 0 | 0 | 3 | 2 | 1 | 14 | | | | | | | | ≤0.06 | >0.06 |
| Ciprofloxacin | | | | 81 | 24 | 7 | | | | | | | | | ≤0.001 | >2 |
| Penicillin | 7 | 50 | 26 | 2 | 9 | 9 | 0 | 0 | | | | | | | ≤1 | >1 |
| Levofloxacin | | | 0 | 88 | 25 | 0 | 1 | | | | | | | | ≤0.001 | >1 |
| Ceftriaxone | | | | | | | | 32 | 24 | 9 | 31 | | | | <32 | ≥32 |
| Oxacillin+2% NaCl | | | 4 | 7 | 33 | 40 | 3 | 27 | | | | | | | <2 | ≥4 |
| Clindamycin | | 0 | 11 | 16 | 51 | 36 | | | | | | | | | <0.25 | ≥0.25 |
| Daptomycin | | | 0 | 0 | 4 | 29 | 62 | 19 | | | | | | | ≤1 | >1 |
| Vancomycin | | | | | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | ≤2 | >2 |
| Ampicillin | | 51 | 15 | 8 | 1 | 0 | 0 | 0 | 0 | | | | | | ≤1 | >1 |
| Linezolid | | | | 9 | 62 | 42 | 0 | 0 | | | | | | | ≤4 | >4 |
| Gatifloxacin | | | | | 0 | 0 | 0 | 0 | | | | | | | ≤1 | >1 |
| Trimethoprim/Sulfamethoxazole | | | | 0 | 0 | 0 | 0 | | | | | | | | ≤4/76 | >4/76 |

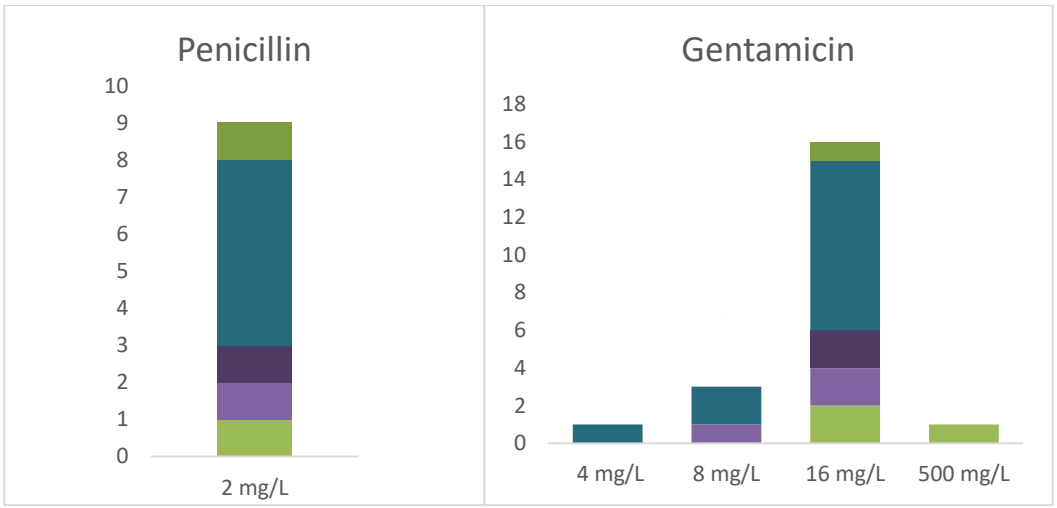
Note: Thick line breakpoints were used for interpretation of the antimicrobial susceptibility of *L. monocytogenes* based on the EUCAST (erythromycin, penicillin, ampicillin and trimethoprim/sulfamethoxazole); breakpoints for *Staphylococcus aureus* were adopted for the following antibiotics: gentamicin, tetracycline, quinupristin/dalfopristin, rifampin, ciprofloxacin, levofloxacin, clindamycin, daptomycin, vancomycin, linezolid, and gatifloxacin; intrinsic resistance (INT) antibiotics ceftriaxone, oxacillin + 2% NaCl, and clindamycin; streptomycin with a concentration of 1000 mg/L; and (S) Sensitive and (R) Resistant. The grey zone represents the concentration range for each antimicrobial from the GPN3F plate.

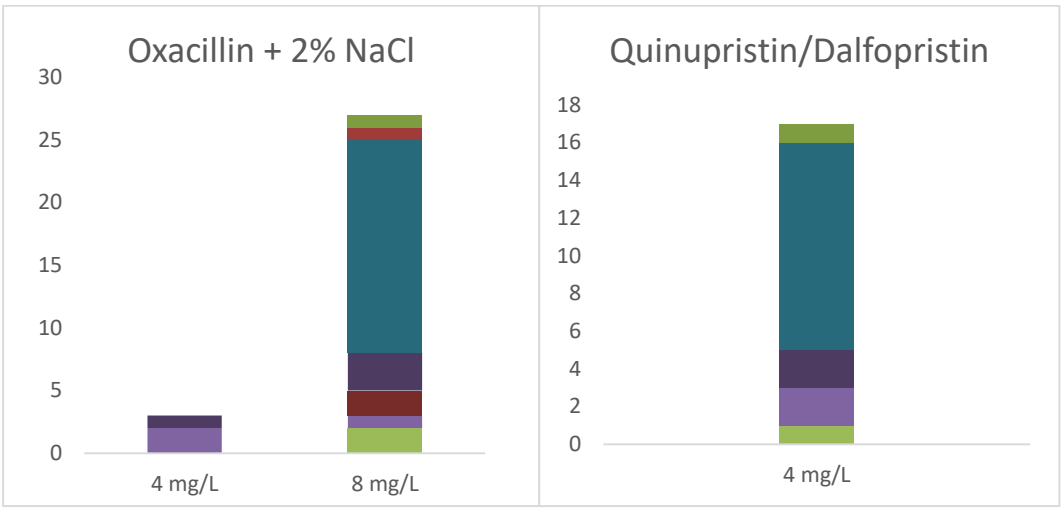
The data presented in the table show variability in antimicrobial susceptibility among the tested isolates. Daptomycin exhibited consistently high minimum inhibitory concentration (MIC) values across all isolates, indicating uniform resistance. Clindamycin also showed elevated MIC values in the majority of isolates, corresponding with a high overall resistance rate.

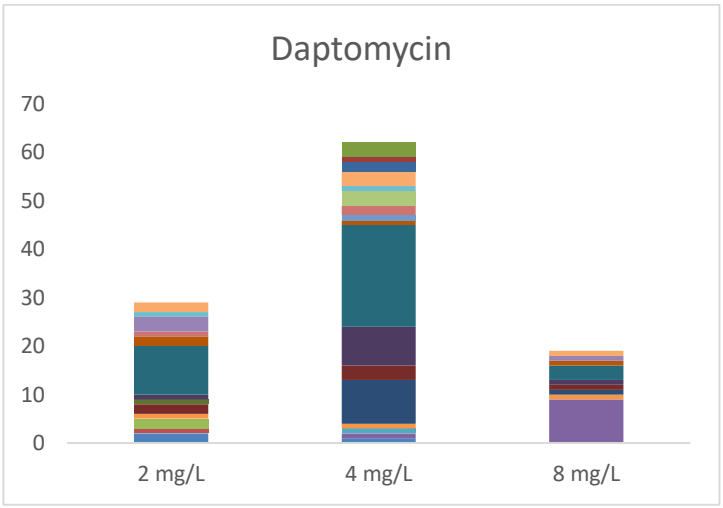
In contrast, antibiotics like ampicillin, vancomycin, and linezolid demonstrated low MIC values across all isolates, consistent with the complete susceptibility observed for these agents.

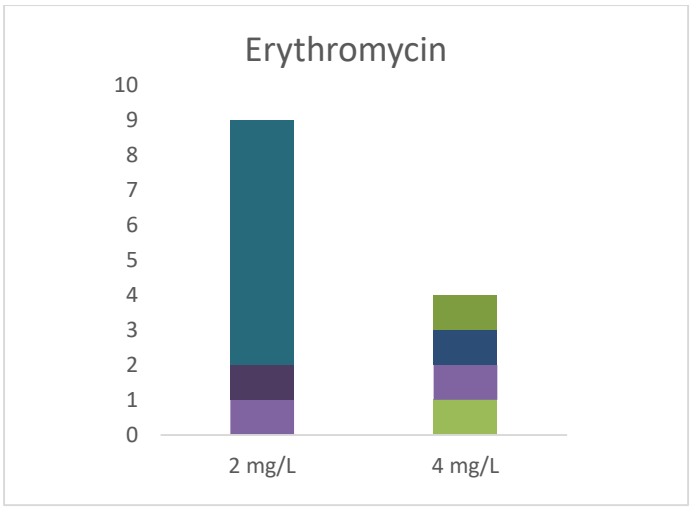
In Figure 41 were represented the Minimum inhibitory concentration (MIC) values of isolates in this study to the panel of thirteen antibiotics: GEN – gentamicin; STR – streptomycin; ERY – erythromycin; TET – tetracycline; SYN - quinupristin/dalfopristin; RIF – rifampin; PEN – penicillin; CIP – ciprofloxacin; LEVO – levofloxacin; OXA+ - oxacillin + 2% NaCl; AXO – ceftriaxone; CLI - clindamycin; DAP – daptomycin.

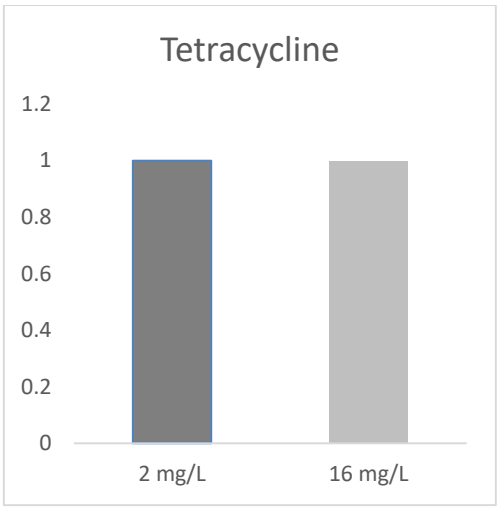


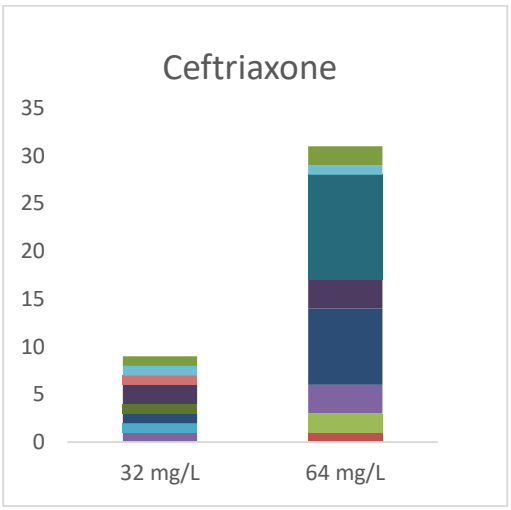

Penicillin
10
9
8
7
6
5
4
3
2
1
0
2 mg/L
Gentamicin
18
16
14
12
10
8
6
4
2
0
4 mg/L
8 mg/L
16 mg/L
500 mg/L

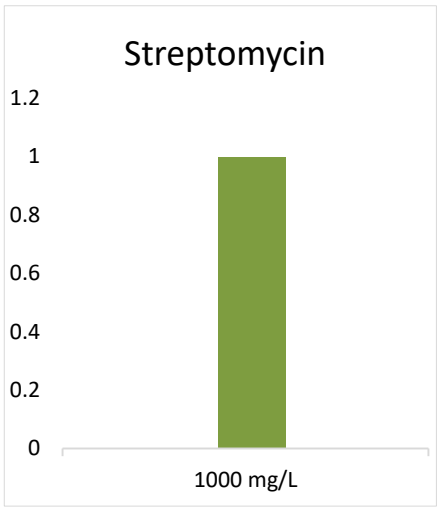

Oxacillin + 2% NaCl
30
25
20
15
10
5
0
4 mg/L
8 mg/L
Quinupristin/Dalfopristin
18
16
14
12
10
8
6
4
2
0
4 mg/L

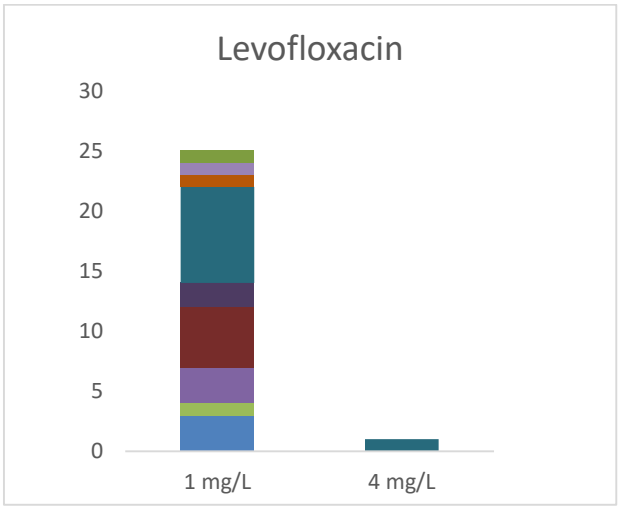

Daptomycin
70
60
50
40
30
20
10
0
2 mg/L
4 mg/L
8 mg/L

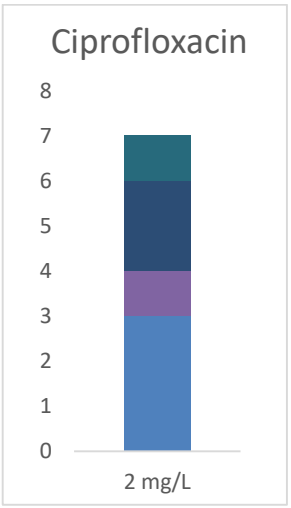

Erythromycin
10
9
8
7
6
5
4
3
2
1
0
2 mg/L
4 mg/L


Tetracycline
1.2
1
0.8
0.6
0.4
0.2
0
2 mg/L
16 mg/L


Ceftriaxone
35
30
25
20
15
10
5
0
32 mg/L
64 mg/L


Streptomycin
1.2
1
0.8
0.6
0.4
0.2
0
1000 mg/L


Levofloxacin
30
25
20
15
10
5
0
1 mg/L
4 mg/L


Ciprofloxacin
8
7
6
5
4
3
2
1
0
2 mg/L

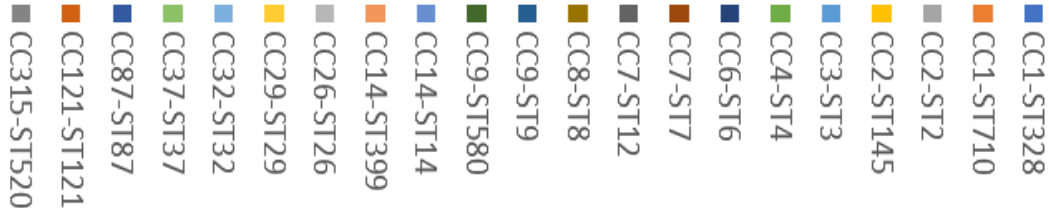



Figure 41. Minimum inhibitory concentration (MIC) values of isolates.

### *5.10.2 Results of Multiple Antibiotic Resistance*

The antibiotic resistance patterns and multiple antibiotic resistance (MAR) index of the *L. monocytogenes* isolates are detailed in Table 25. A total of 27 distinct resistance patterns were identified across the 18 antimicrobials tested, with the number of antibiotics to which isolates exhibited resistance ranging from 3 to as many as 12. These resistance patterns provide a comprehensive depiction of the varying degrees of resistance among the isolates, highlighting the complexity of antimicrobial interactions within this bacterial population.

Among the identified resistance phenotypes, CLI–DAP (clindamycin–daptomycin) and CLI–DAP–AXO (clindamycin–daptomycin–ceftriaxone) were the most frequently observed, with 45 and 19 occurrences, respectively.

All isolates demonstrated resistance to at least one antibiotic in the panel, reflecting a universal baseline resistance across the population. Of these, four isolates (3.5%) were resistant to a single antibiotic, indicating a minimal resistance profile in this subset. A larger portion of isolates, 45 in total (39.5%), exhibited resistance to two antibiotics. Resistance to three antibiotics was observed in 32 isolates (28.1%), illustrating an intermediate resistance level within this group.

### *5.10.3 Results of Multidrug-resistance - MDR*

Multidrug-resistant (MDR) phenotypes, defined as resistance to three or more antibiotics, were observed in a significant majority of isolates, with 65 (57.0%) demonstrating resistance to multiple antimicrobials. This occurrence of MDR phenotypes underscores the adaptability of *L. monocytogenes* to withstand diverse antimicrobial agents.

The ARI values ranged from 0.06 to 0.67, with lower values indicating minimal resistance and higher values reflecting broader resistance across the antibiotic panel. This range highlights the variation in resistance profiles within the isolate population.

Table 25 complements the findings presented in by providing additional data on the distribution of resistance patterns and ARI values across the isolates. This combined dataset offers valuable insights into the resistance landscape of *L. monocytogenes* isolates and serves as a critical resource for understanding their antimicrobial resistance profiles.

Table 25. Genetic profiles of antibiotic resistant of *L. monocytogenes* isolates and antimicrobial-resistance phenotypes/indices of *L. monocytogenes* strains from food samples.

| S/N | Food types | Year | Line-ages | CC-STs | Antibiotic resistance phenotypes (M)ARPs patterns | No. of antibiotics | MARI |
|---|---|---|---|---|---|---|---|
| 1 | MP | 2016 | I | ST32 | CLI-DAP-AXO | 3 | 0.17 |
| 2 | MP | 2016 | II | CC14-ST14 | CLI-DAP | 2 | 0.11 |
| 3 | MP | 2018 | II | CC26-ST26 | CLI-DAP | 2 | 0.11 |
| 4 | MP | 2018 | II | CC8-ST8 | CLI-DAP | 2 | 0.11 |
| 5 | MP | 2018 | I | CC2-ST145 | CLI-DAP | 2 | 0.11 |
| 6 | MP | 2018 | I | CC87-ST87 | CLI-DAP | 2 | 0.11 |
| 7 | MP | 2018 | II | CC29-ST29 | CLI | 1 | 0.06 |
| 8 | MP | 2018 | I | CC2-ST145 | PEN-LEVO-CLI-DAP | 4 | 0.22 |
| 9 | MP | 2018 | I | CC2-ST145 | CLI-DAP | 2 | 0.11 |
| 10 | MP | 2018 | I | CC2-ST145 | CLI-DAP | 2 | 0.11 |
| 11 | MP | 2018 | II | CC8-ST8 | CLI-DAP | 2 | 0.11 |
| 12 | MP | 2019 | II | CC9-ST9 | CLI-DAP | 2 | 0.11 |
| 13 | MP | 2019 | I | ST32 | CLI-DAP-AXO | 3 | 0.17 |
| 14 | MP | 2019 | II | CC9-ST9 | CLI-DAP | 2 | 0.11 |
| 15 | MP | 2019 | I | CC315-ST520 | CLI-DAP-AXO | 3 | 0.17 |
| 16 | MP | 2019 | I | CC2-ST145 | CLI-DAP-AXO | 3 | 0.17 |
| 17 | MP | 2019 | II | CC26-ST26 | CLI-DAP | 2 | 0.11 |
| 18 | MP | 2021 | II | CC37-ST37 | CLI-DAP | 2 | 0.11 |
| 19 | MP | 2021 | II | CC29-ST29 | CLI-DAP | 2 | 0.11 |
| 20 | MP | 2021 | I | CC4-ST4 | CLI-DAP | 2 | 0.11 |
| 21 | MP | 2021 | I | CC4-ST4 | CLI-DAP | 2 | 0.11 |
| 22 | MP | 2021 | II | CC29-ST29 | CLI-DAP | 2 | 0.11 |
| 23 | MP | 2021 | II | CC29-ST29 | CLI-DAP | 2 | 0.11 |
| 24 | MP | 2021 | II | CC29-ST29 | CLI | 1 | 0.06 |
| 25 | MP | 2021 | II | CC29-ST29 | LEVO-CLI-DAP | 3 | 0.17 |
| ARI | | | | | | | 0.12 |
| 1 | MMP | 2016 | II | CC9-ST580 | CLI-DAP | 2 | 0.11 |
| 2 | MMP | 2016 | II | CC9-ST580 | CLI-DAP | 2 | 0.11 |
| 3 | MMP | 2016 | II | CC9-ST9 | CLI-DAP | 2 | 0.11 |
| 4 | MMP | 2016 | II | CC14-ST399 | CLI-DAP | 2 | 0.11 |
| 5 | MMP | 2016 | I | CC6-ST6 | CIP-CLI-DAP | 3 | 0.17 |
| 6 | MMP | 2016 | II | CC8-ST8 | OXA+-LEVO-GEN-CLI-SYN-DAP-AXO-RIF | 8 | 0.44 |
| 7 | MMP | 2016 | I | CC6-ST6 | CLI-DAP-AXO | 3 | 0.17 |
| 8 | MMP | 2016 | II | CC8-ST8 | CLI-DAP-AXO | 3 | 0.17 |
| 9 | MMP | 2016 | II | CC14-ST399 | CLI-DAP | 2 | 0.11 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 10 | MMP | 2016 | II | CC8-ST8 | CLI-DAP-AXO | 3 | 0.17 |
| 11 | MMP | 2016 | I | CC6-ST6 | CLI-DAP-AXO | 3 | 0.17 |
| 12 | MMP | 2016 | II | CC14-ST399 | CLI-DAP-AXO | 3 | 0.17 |
| 13 | MMP | 2017 | II | CC9-ST9 | CLI-DAP | 2 | 0.11 |
| 14 | MMP | 2017 | I | CC6-ST6 | CLI-DAP-AXO | 3 | 0.17 |
| 15 | MMP | 2017 | I | CC6-ST6 | CIP-CLI-DAP-AXO | 4 | 0.22 |
| 16 | MMP | 2017 | I | CC6-ST6 | CLI-DAP-AXO | 3 | 0.17 |
| 17 | MMP | 2017 | II | CC37-ST37 | CLI-DAP | 2 | 0.11 |
| 18 | MMP | 2017 | I | CC6-ST6 | ERY-CLI-DAP-AXO | 4 | 0.22 |
| 19 | MMP | 2017 | I | CC6-ST6 | CLI-DAP-AXO | 3 | 0.17 |
| 20 | MMP | 2017 | II | CC37-ST37 | CLI-DAP | 2 | 0.11 |
| 21 | MMP | 2017 | II | CC9-ST9 | CLI-DAP | 2 | 0.11 |
| 22 | MMP | 2017 | I | CC6-ST6 | CLI-DAP-AXO | 3 | 0.17 |
| 23 | MMP | 2017 | II | CC37-ST37 | CLI-DAP | 2 | 0.11 |
| 24 | MMP | 2018 | II | CC9-ST9 | CLI-DAP | 2 | 0.11 |
| 25 | MMP | 2018 | II | CC9-ST9 | CLI-DAP | 2 | 0.11 |
| 26 | MMP | 2018 | II | CC9-ST9 | PEN-OXA+-GEN-ERY-CLI-SYN-DAP-AXO-RIF | 9 | 0.5 |
| 27 | MMP | 2018 | II | CC29-ST29 | CLI | 1 | 0.06 |
| 28 | MMP | 2018 | II | CC9-ST9 | CLI-DAP | 2 | 0.11 |
| 29 | MMP | 2018 | II | CC8-ST8 | CLI-DAP | 2 | 0.11 |
| 30 | MMP | 2018 | II | CC7-ST7 | CLI-DAP | 2 | 0.11 |
| 31 | MMP | 2018 | II | CC37-ST37 | CLI-DAP | 2 | 0.11 |
| 32 | MMP | 2018 | II | CC9-ST9 | CLI-DAP | 2 | 0.11 |
| 33 | MMP | 2019 | I | CC315-ST520 | PEN-OXA+-LEVO-GEN-STR-ERY-CLI-SYN-DAP-TET-AXO-RIF | 12 | 0.67 |
| 34 | MMP | 2019 | II | CC9-ST9 | CLI-DAP | 2 | 0.11 |
| 35 | MMP | 2019 | II | CC9-ST9 | CLI-DAP | 2 | 0.11 |
| 36 | MMP | 2019 | II | CC7-ST7 | LEVO-CLI-DAP | 3 | 0.17 |
| 37 | MMP | 2019 | II | CC9-ST580 | LEVO-CLI-DAP | 3 | 0.17 |
| 38 | MMP | 2019 | II | CC9-ST9 | CLI-DAP | 2 | 0.11 |
| 39 | MMP | 2019 | II | CC9-ST9 | OXA+-GEN-ERY-CLI-SYN-DAP-AXO-RIF | 8 | 0.44 |
| 40 | MMP | 2019 | II | CC9-ST9 | CLI-DAP | 2 | 0.11 |
| 41 | MMP | 2019 | II | CC9-ST9 | LEVO-CLI-DAP | 3 | 0.17 |
| 42 | MMP | 2019 | I | CC1-ST328 | LEVO-CIP-CLI-DAP | 4 | 0.22 |
| 43 | MMP | 2019 | II | CC9-ST9 | CLI-DAP | 2 | 0.11 |
| 44 | MMP | 2019 | II | CC8-ST8 | CLI-DAP-AXO | 3 | 0.17 |
| 45 | MMP | 2019 | II | CC9-ST9 | OXA+-LEVO-GEN-ERY-CLI-SYN-DAP-AXO-RIF | 9 | 0.50 |
| 46 | MMP | 2019 | II | CC9-ST9 | PEN-OXA+-GEN-CLI-SYN-DAP-AXO-RIF | 8 | 0.44 |
| 47 | MMP | 2019 | II | CC9-ST9 | PEN-OXA+-LEVO-GEN-CLI-SYN-DAP-AXO-RIF | 9 | 0.50 |
| 48 | MMP | 2019 | II | CC9-ST9 | PEN-OXA+-GEN-ERY-CLI-SYN-DAP-AXO-RIF | 9 | 0.50 |
| 49 | MMP | 2019 | II | CC9-ST9 | PEN-OXA+-LEVO-GEN-ERY-CLI-SYN-DAP-AXO-RIF | 10 | 0.56 |
| 50 | MMP | 2019 | II | CC8-ST8 | PEN-OXA+-GEN-ERY-CLI-SYN-DAP-AXO-RIF | 9 | 0.50 |
| 51 | MMP | 2019 | II | CC8-ST8 | OXA+-LEVO-CLI-DAP-RIF | 5 | 0.28 |
| 52 | MMP | 2019 | II | CC9-ST9 | OXA+-LEVO-GEN-ERY-CLI-SYN-DAP-AXO-RIF | 9 | 0.50 |
| 53 | MMP | 2019 | II | CC9-ST9 | OXA+-LEVO-CLI-DAP | 4 | 0.22 |
| 54 | MMP | 2019 | II | CC9-ST9 | OXA+-CIP-CLI-DAP | 4 | 0.22 |
| 55 | MMP | 2019 | II | CC9-ST9 | OXA+-LEVO-CLI-DAP | 4 | 0.22 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 56 | MMP | 2019 | II | CC9-ST9 | OXA+-LEVO-GEN-CLI-SYN-DAP-AXO-RIF | 8 | 0.44 |
| 57 | MMP | 2019 | II | CC9-ST9 | OXA+-LEVO-GEN-ERY-CLI-SYN-DAP-AXO-RIF | 9 | 0.50 |
| 58 | MMP | 2019 | II | CC9-ST9 | OXA+-CLI-DAP | 3 | 0.17 |
| 59 | MMP | 2019 | I | CC2-ST2 | OXA+-GEN-CLI-DAP-AXO-RIF | 6 | 0.33 |
| 60 | MMP | 2019 | II | CC9-ST9 | OXA+-GEN-CLI-SYN-DAP-AXO-RIF | 7 | 0.39 |
| 61 | MMP | 2019 | II | CC9-ST9 | OXA+-CLI-DAP | 3 | 0.17 |
| 62 | MMP | 2019 | I | CC2-ST145 | OXA+-LEVO-CIP-GEN-ERY-CLI-DAP | 7 | 0.39 |
| 63 | MMP | 2019 | I | CC2-ST145 | OXA+-GEN-ERY-CLI-SYN-DAP-AXO-RIF | 8 | 0.44 |
| 64 | MMP | 2019 | I | CC2-ST145 | GEN-CLI-SYN-DAP-AXO-RIF | 6 | 0.33 |
| 65 | MMP | 2020 | II | CC9-ST9 | OXA+-CLI-DAP | 3 | 0.17 |
| 66 | MMP | 2020 | II | CC7-ST7 | OXA+-LEVO-CLI-DAP | 4 | 0.22 |
| 67 | MMP | 2021 | II | CC7-ST7 | OXA+-LEVO-CLI-DAP | 4 | 0.22 |
| 68 | MMP | 2021 | II | CC29-ST29 | CLI | 1 | 0.06 |
| 69 | MMP | 2021 | II | CC37-ST37 | CLI-DAP | 2 | 0.11 |
| 70 | MMP | 2021 | I | CC4-ST4 | CLI-DAP | 2 | 0.11 |
| 71 | MMP | 2021 | II | CC9-ST580 | CLI-DAP | 2 | 0.11 |
| 72 | MMP | 2021 | II | CC9-ST9 | CLI-DAP | 2 | 0.11 |
| 73 | MMP | 2021 | I | CC1-ST328 | LEVO-CIP-CLI-DAP | 4 | 0.22 |
| 74 | MMP | 2021 | I | CC1-ST328 | LEVO-CIP-CLI-DAP | 4 | 0.22 |
| 75 | MMP | 2021 | I | CC315-ST520 | CLI-DAP-AXO | 3 | 0.17 |
| 76 | MMP | 2022 | I | CC1-ST710 | CLI-DAP-AXO | 3 | 0.17 |
| 77 | MMP | 2022 | II | CC7-ST7 | LEVO-CLI-DAP | 3 | 0.17 |
| 78 | MMP | 2022 | I | CC3-ST3 | CLI-DAP-AXO | 3 | 0.17 |
| 79 | MMP | 2022 | II | CC7-ST12 | CLI-DAP-AXO | 3 | 0.17 |
| 80 | MMP | 2022 | I | CC2-ST145 | LEVO-CLI-DAP | 3 | 0.17 |
| ARI | | | | | | | 0.22 |
| 1 | FMP | 2018 | I | CC87-ST87 | CLI-DAP | 2 | 0.11 |
| 2 | FMP | 2018 | II | CC26-ST26 | CLI-DAP | 2 | 0.11 |
| 3 | FMP | 2019 | I | CC2-ST145 | OXA+-CLI-DAP-AXO | 4 | 0.22 |
| 4 | FMP | 2020 | I | CC2-ST2 | PEN-OXA+-LEVO-GEN-ERY-CLI-SYN-DAP-TET-AXO-RIF | 11 | 0.61 |
| ARI | | | | | | | 0.26 |
| 1 | CFP | 2019 | II | CC6-ST6 | CLI-DAP-AXO | 3 | 0.17 |
| 2 | CFP | 2019 | II | CC9-ST9 | GEN-CLI-DAP | 3 | 0.17 |
| 3 | CFP | 2019 | II | CC121-ST121 | OXA+-CLI-DAP | 3 | 0.17 |
| 4 | CFP | 2019 | I | CC8-ST8 | OXA+-CLI-DAP-RIF | 4 | 0.22 |
| ARI | | | | | | | 0.18 |
| 1 | ES | 2021 | II | CC7-ST7 | LEVO-CLI-DAP | 3 | 0.17 |
| ARI | | | | | | | 0.05 |

Abbreviations: CC-ST – clonal complex-sequence type; (M)ARP – Multi antibiotic resistance phenotypes patterns; MARI – Multiple antibiotic resistance index; ARI- Antibiotic resistance index; MP – Meat products; MMP – Milk and milk products, FMP – Fish meat products; CFP – Combined food products; ES – Environmental sample; GEN – gentamicin; STR – streptomycin; ERY – erythromycin; TET – tetracycline; SYN - quinupristin/dalfopristin; RIF – rifampin; PEN – penicillin; CIP – ciprofloxacin; LEVO – levofloxacin; OXA+ - oxacillin + 2% NaCl; AXO – ceftriaxone; CLI - clindamycin; DAP – daptomycin.

Among the *L. monocytogenes* strains tested, 34 out of 114 isolates (29.8%) demonstrated multiple antibiotic resistance (MAR) values exceeding 0.2, indicating a significant proportion of strains with high resistance potential. This threshold is a widely recognized benchmark for assessing the risk associated with antibiotic resistance in bacterial populations.

The antibiotic resistance index (ARI) was calculated for each category of food and environmental sample to provide a detailed assessment of resistance levels (Table 26). In the case of milk and milk products (MMP), the ARI value was measured at 0.22, reflecting a higher resistance potential in isolates from this category. Similarly, isolates from fish meat products (FMP) demonstrated an ARI value of 0.26, the highest among the categories analyzed, indicating a pronounced resistance level in this subset.

Conversely, isolates from the environmental sample (ES) had an ARI value of 0.05, the lowest observed across all categories, suggesting minimal resistance in this category. Meat and meat products (MP) exhibited an intermediate ARI value of 0.12, while combined food products (CFP), which included a mix of different food types, showed an ARI value of 0.18. Both MP and CFP isolates fell within the permissible Krumperman threshold, indicating that their resistance levels were less concerning compared to MMP and FMP categories.

These ARI values provide a quantitative measure of resistance across different categories of samples, highlighting the variability in resistance profiles between food types and the environmental isolate. By exceeding the 0.2 threshold in specific categories like MMP and FMP, the data underscore the need for a closer examination of resistance dynamics in these subsets.

Table 26. Multiple antibiotic resistance phenotypes of *L. monocytogenes* (MARPs) strains.

| S/N | Antibiotic Resistance Phenotypes MARPs Patterns | No. of Antibiotics | MARI | Freq |
|---|---|---|---|---|
| 1 | CLI–DAP–AXO | 3 | 0.17 | 19 |
| 2 | LEVO–CLI–DAP | 3 | 0.17 | 7 |
| 3 | OXA+–CLI–DAP | 3 | 0.17 | 4 |
| 4 | GEN–CLI–DAP | 3 | 0.17 | 1 |
| 5 | CIP–CLI–DAP | 3 | 0.17 | 1 |
| 6 | OXA+–LEVO–CLI–DAP | 4 | 0.22 | 4 |
| 7 | LEVO–CIP–CLI–DAP | 4 | 0.22 | 3 |
| 8 | CIP–CLI–DAP–AXO | 4 | 0.22 | 1 |
| 9 | ERY–CLI–DAP–AXO | 4 | 0.22 | 1 |
| 10 | OXA+–CLI–DAP–RIF | 4 | 0.22 | 1 |
| 11 | OXA+–CIP–CLI–DAP | 4 | 0.22 | 1 |
| 12 | OXA+–CLI–DAP–AXO | 4 | 0.22 | 1 |
| 13 | PEN–LEVO–CLI–DAP | 4 | 0.22 | 1 |

| | | | | |
|---|---|---|---|---|
| 14 | OXA+–LEVO–CLI–DAP–RIF | 5 | 0.28 | 1 |
| 15 | GEN–CLI–SYN–DAP–AXO–RIF | 6 | 0.33 | 1 |
| 16 | OXA+–GEN–CLI–DAP–AXO–RIF | 6 | 0.33 | 1 |
| 17 | OXA+–GEN–CLI–SYN–DAP–AXO–RIF | 7 | 0.39 | 1 |
| 18 | OXA+–LEVO–CIP–GEN–ERY–CLI–DAP | 7 | 0.39 | 1 |
| 19 | OXA+–LEVO–GEN–CLI–SYN–DAP–AXO–RIF | 8 | 0.44 | 2 |
| 20 | OXA+–GEN–ERY–CLI–SYN–DAP–AXO–RIF | 8 | 0.44 | 2 |
| 21 | PEN–OXA+–GEN–CLI–SYN–DAP–AXO–RIF | 8 | 0.44 | 1 |
| 22 | PEN–OXA+–GEN–ERY–CLI–SYN–DAP–AXO–RIF | 9 | 0.50 | 3 |
| 23 | OXA+–LEVO–GEN–ERY–CLI–SYN–DAP–AXO–RIF | 9 | 0.50 | 3 |
| 24 | PEN–OXA+–LEVO–GEN–CLI–SYN–DAP–AXO–RIF | 9 | 0.50 | 1 |
| 25 | PEN–OXA+–LEVO–GEN–ERY–CLI–SYN–DAP–AXO–RIF | 10 | 0.56 | 1 |
| 26 | PEN–OXA+–LEVO–GEN–ERY–CLI–SYN–DAP–TET–AXO–RIF | 11 | 0.61 | 1 |
| 27 | PEN–OXA+–LEVO–GEN–STR–ERY–CLI–SYN–DAP–TET–AXO–RIF | 12 | 0.67 | 1 |

GEN—gentamicin; STR—streptomycin; ERY—erythromycin; TET—tetracycline; SYN—quinupristin/dalfopristin; RIF—rifampin; PEN—penicillin; CIP—ciprofloxacin; LEVO—levofloxacin; OXA+—oxacillin+ 2% NaCl; AXO—ceftriaxone; CLI—clindamycin; DAP—daptomycin.

# 6 DISCUSSION

## 6.1 Occurrence of *L. monocytogenes*

This is the first study of this kind carried out in the RKS. The study is novel because it analyzed large numbers and types of samples over extended periods of time and provided the first instance of the molecular characterization of *L. monocytogenes* from a variety of matrices in the country.

The sampling targeted mainly RTE meat and milk products or food products intended for consumption as cooked. *L. monocytogenes* was isolated from 117 out of a total of 995 samples examined (11.76%). In comparison with the findings of the present study, where *Listeria monocytogenes* was detected in 11.76% the EFSA and ECDC (2023) European Union One Health 2022 Zoonoses Report reported considerably lower contamination rates in food products across the EU. According to the report, the overall EU-level prevalence of *L. monocytogenes* in ready-to-eat (RTE) food samples remained below 2%, with most member states reporting contamination rates ranging between 0.1% and 1.5%, depending on the product category and sampling context (EFSA & EDC, 2023).

The highest contamination rates were found in raw materials (55.00%) and FPCC (14.95%), whereas RTE foods had the lowest occurrence (6.33%). This distribution highlights the risk associated with raw and minimally processed food products, which are more susceptible to contamination due to factors such as handling and storage conditions (EFSA & ECDC, 2023).

Within RTE products, the occurrence of *L. monocytogenes* was 5.82% for milk products (mostly cheese) and 6.99% for meat products. These figures are higher than the average occurrence reported in EU countries for both product categories, which stand at 0.37% and 2.1%, respectively (EFSA & ECDC, 2023). Previous studies conducted in the Republic of Kosovo on RTE products reported a higher occurrence, with 6.9% for cheese (Studenica et al., 2022) and 10.1% for meat products (Kukleci et al., 2019).

For meat products consumed cooked, the occurrence was 16.95%, higher than in a similar study conducted on raw sausages and raw meat in France, where the prevalence was 10% and 12%, respectively (Roussel et al., 2014, Felix et al., 2018).

Due to the relatively small number of combined food products (meat and dairy) and fish product samples examined in this study, the calculated prevalence of *L. monocytogenes* cannot

be taken as an accurate statistic, so it was not possible to assess the significance of the different distributions of serotypes between environmental and food strains.

## 6.2 Molecular typing and Lineages

The genetic diversity of the strains observed in Kosovo was compared with studies conducted either at the European level (Painset et al., 2019) or at the national level, in France (Felix et al., 2018, Maury et al., 2016), Switzerland (Ebner et al., 2015), and Slovakia (Kubicova et al., 2021).

Only Lineage I (33.33%) and Lineage II (66.66%) strains were identified in the present study. This distribution is in agreement with findings reported by other researchers, where Lineage I strains typically account for 22–37% and Lineage II strains for 63–78% of *L. monocytogenes* isolates in food products and environmental sources (Wang et al., 2012; Moura et al., 2016). The predominance of Lineage II observed in our study is consistent with its association with food and food-processing environments, while Lineage I, although more frequently linked to human clinical cases, was less prevalent.

In the present study, four main molecular serotypes were identified: IIa (34.19%), IIb (3.48%), IIc (32.48%), and IVb (29.91%). The predominance of molecular serotypes IIa and IIc, accounting for over two-thirds of the isolates, aligns with previous studies reporting these molecular serotypes as prevalent in various geographical regions (Korsak et al., 2012, Mackiw et al., 2020, Daza, P.B et al., 2024). The diversity of molecular serotypes is indicative of the various sources and potential reservoirs of contamination within the food chain. Regarding the IIc (or 1/2c) strains, they have largely been reported in meat products in previous studies (Thevenot et al., 2005, Meloni et al., 2013, Kramarenko et al., 2013).

Conversely, they are rare in milk products (Wagner et al., 2006, Kiss et al., 2006). These results show the large distribution of the IIc (or 1/2c) strain in European countries, including the RKS, during a long period of time. Regarding the IVb (or 4b) strain, this molecular serotype includes the CCs associated with severe human infections (Maury et al., 2016) (CC1, CC2, CC4, and CC6) and is considered hypervirulent. Overall, 26.83% of the meat samples and 40.00% of the milk product samples belonged to this molecular serotype.

### 6.3 Clonal Complexes of *L. monocytogenes*

Overall, 14 CCs were identified among the 41 RTE isolates, with CC29, CC2, and CC6 being the most dominant. Conversely, CC9 emerged as the most prevalent in FPCC and raw materials. This clonal complex is known for its presence in various environmental and food sources but is less commonly associated with severe human cases (Maury et al., 2019). The high occurrence of CC9 in raw materials could suggest that it is a persistent environmental strain, possibly contributing to cross-contamination during food processing.

The most frequent CCs in meat products only were CC9, CC8, CC6, and CC7, whereas CC2 and CC29 were more common in milk products. In all the above studies, CC9 and CC8 were the most frequent CCs for meat, and although CC6 and CC7 were observed, they were not predominant. Surprisingly, CC121 was absent from meat products in the present study, although it was frequently associated with meat products elsewhere (Maury et al., 2016).

For milk products, CC2 was the second most frequent CC according to (Painset et al., 2019). For the other studies, CC2 was observed in milk products but was not predominant (Maury et al., 2016). The CC29 clone was observed in milk products in all the studies above but was not predominant and less abundant than CC2. All 30 isolates expressing the CCs associated with severe human infections (CC1, CC2, CC4, or CC6) belonged to the molecular serotype IVb. In the context of the dynamic development of the Kosovo agri-food sector, *L. monocytogenes* typing will become a crucial tool for food safety management. The data-base created in this study made it possible to investigate outbreaks based on CC front-line screening, drawing links between human clinical cases and food contaminants. This step is essential for selecting strains before confirming outbreaks (Moura et al., 2017) with a sequence-based method such as core genome MLST (cgMLST), which is currently the reference method for molecular surveillance (EFSA&ECDC, 2023, EFSA, 2022). This is the method that would be applied in the RKS in the event of an outbreak through EU-supportive actions, potentially through the EURL's program.

### 6.4 Multilocus Sequence typing of *L. monocytogenes*

Although there have been some studies regarding the occurrence of *L. monocytogenes* from foods and the characterization of isolates (Jashari et al., 2024), data regarding the antimicrobial resistance are limited in Kosovo and so far have been described in isolates from sporadic ovine

listeria meningoencephalitis cases (Hamidi et al., 2020). Several studies have reported on the prevalence, antimicrobial resistance, and molecular characteristics of Listeria monocytogenes isolates from the global food chain (Olaimat et al., 2018; Caruso et al., 2020; Escolar et al., 2017; Jorgensen et al., 2021; Kayode & Okoh, 2022, 2023; Noll et al., 2018; Roedel et al., 2018; Sanlibaba et al., 2018; Wisniewski et al., 2022).

This study provides valuable insight into the occurrence and antimicrobial resistance of Listeria monocytogenes circulating in the food chain in Kosovo between 2016 and 2022. The molecular characterization, including clonal complex and sequence type identification, revealed a diverse population structure consistent with findings from previous studies (Felix et al., 2023; Jashari et al., 2024). These results underscore the presence of multiple resistant strains and highlight the importance of continued surveillance and control measures to mitigate potential public health risks.

From all isolates of *L. monocytogenes* grouped into 16 clonal complexes in our study by MLST, 21 different ST groups were identified. Similar CC–STs were found among isolates in studies carried out in many countries around the world in the food chain. Thus, CC14–ST399, CC7–ST7, CC8–ST8, and CC1–ST328, were isolated from Danish ready-to-eat food samples (Takeuchi-Storm et al., 2023). In China, the study conducted from 2010 to 2019 highlights that in meat products the most predominant isolates belonged to CC9–ST9 clones (Cheng et al., 2022), while the most frequent strains isolated in Poland from meat processing plant environments were CC2–ST2, CC2–ST145, CC3–ST3, CC6–ST6 and CC87–ST87 (Kurpas et al., 2020). In another study carried out on foodstuffs, the environment and clinical samples in Italy, CC14–ST14, CC26–ST26, CC29–ST29, ST32, CC37–ST37 and CC315–ST520 were reported (Caruso et al., 2020). In Poland, the most frequent isolates recovered from food products were CC9–ST9, CC121–ST121 and CC9–ST580 (Sosnowski et al., 2019), while CC7–ST12 and CC1–ST328 were among the most frequent isolates recovered from the food production chain in Australia (Wilson et al., 2018). However, CC1–ST710, which belongs to Lineage I and Serogroup IVb, was not isolated from food products except from clinical cases from a study conducted in northern Taiwan (Liu et al., 2024). Similar findings were reported by Daza Prieto B. et al. who characterized 160 isolates of *L. monocytogenes* obtained from different food sources in Montenegro from 2014 to 2022. Similar distributions of ST8, ST9, and ST121 were observed except for ST 155 (Daza Prieto et al., 2024).

In our study, the clone CC9–ST9 is the most represented, which originates mainly from meat products with an occurrence of 27.2%. A similar result was found in studies carried out on 300 genotypes from five continents (Chenal-Francisque et al., 2011). Another study in France reported CC9 as the most frequently observed isolate recovered from meat products (Maury et al., 2019). Furthermore, ST9 was also reported as the most widespread strain observed in a study on meat products in Norway (Fagerlund et al., 2020).

### *6.5* Antimicrobial resistance of *L. monocytogenes*

Our study indicated that *L. monocytogenes* exhibited the highest resistance to clindamycin (100%) and daptomycin. Resistance was lower for tetracycline and streptomycin, while no resistance was observed against vancomycin, ampicillin, linezolid, gatifloxacin, and trimethoprim-sulfamethoxazole. Similar data in the previous studies performed earlier in Pacific Northwest, Canada, Jordan, and Asia have been reported (Olaimat et al., 2018, Jorgensen et al., 2021). The antibiogram profile of *L. monocytogenes* isolates in our study showed high sensitivity (100%) to vancomycin, ampicillin, linezolid, gatifloxacin, and trimethoprim–sulfamethoxazole. A similar sensitivity of *L. monocytogenes* isolates to these antibiotics except gatifloxacin was observed in a study of food production factories in Germany (Roedel et al., 2019). High resistance has also been reported against clindamycin (100%) (Escolar et al., 2017, Jorgensen et al., 2021), daptomycin (96.5%) (Roedel et al., 2019), amoxicillin (35.1%), (Kayode & Okoh, 2023) and oxacillin+ 2% NaCl (24.6%) (Caruso et al., 2020).

In many previous studies, lower resistance of *L. monocytogenes* isolates levofloxacin (22.8%) (Sanlibaba et al., 2018), gentamicin, and rifampin (Noll et al., 2018) from (17.5%), to quinupristin/dalfopristin (14.9%) (Caruso et al., 2020), erythromycin (11.4%) (Kayode & Okoh, 2022), penicillin (7.89%), ciprofloxacin (6.14%), and tetracycline (1.75%) (Sanlibaba et al., 2018) have been reported. Otherwise, in the conducted studies, streptomycin was sensitive (Adeoye & Okoh, 2022, Sanlibaba et al., 2018), while in our study, one isolate (0.88%) was resistant to streptomycin.

## 6.6 Multiple Antibiotic Resistance Index - MARI

The MARI indices indicate the number of phenotypic resistances observed against the tested antimicrobial agents and vary among the isolates. A high MARI score for an isolate suggests it exhibits resistance against multiple antibiotics, highlighting its significant level of resistance. The ARI of the food category, including meat and meat products and fish meat products were >0.2 of the permissible Krumperman threshold. A study carried out by Kayode & Okoh, 2023 in ready-to-eat products, including meat and fish products also exceeded the ARI threshold of >0.2 and a similarity with the values of our study is observed (Kayode & Okoh, 2023).

## 6.7 Multidrug-resistance

In addition, 57.0% of the isolates displayed multidrug-resistant (MDR) phenotypes against the tested antibiotics. Other authors have noted the varying prevalence of MDR, in North Africa (41.86%), in Spain (54.0%), and in Turkey (73.91%) (Escolar et al., 2017, Manyi-Loh et al., 2023, Sanlibaba et al., 2020).

Additionally, stress from food processing can promote the development of resistance to clinically relevant antibiotics (Olaimat & Holley, 2012, Prestinaci et al., 2015, Fagerlund et al., 2016, Gándara et al., 2016). To conclude, this study revealed the profiles of antimicrobial resistance in correlation with the food category and the sequence types of *L. monocytogenes* strains identified and highlighted the possible risks that could arise from the consumption of these foods. It is worth noting that high resistance against clindamycin, ceftriaxone, oxacillin + 2% NaCl, levofloxacin, gentamicin, rifampin, quinupristin/dalfopristin, erythromycin, penicillin, ciprofloxacin, tetracycline, and streptomycin were observed in this study.

Compared to previous studies in different countries around the world, our results suggest differing important trends in *L. monocytogenes* resistance against antimicrobials isolated from food (Escolar et al., 2017, Sanlibaba et al., 2020 Manyi-Loh et al., 2023, Kayode & Okoh, 2023). These results also showed that the CC9–ST9 isolates, which are the most represented, showed over 66% resistance to the panel of antibiotics used in this study. Therefore, the evolution of antimicrobial resistance remains to be assessed on an annual basis in the future.

# 7 CONCLUSION

This study aimed to investigate the prevalence, molecular characterization, clonal complex distribution, and antimicrobial resistance profiles of *L. monocytogenes* in various food products in the Republic of Kosovo. As the first comprehensive research of its kind conducted in the country, it sought to provide foundational data on the occurrence and molecular diversity of *L. monocytogenes* circulating in the food chain.

The study analyzed 995 food samples, identifying *L. monocytogenes* in 11.76% of them substantially higher than EU averages. The highest contamination rates were found in raw materials and food processing environments, while ready-to-eat (RTE) meat and milk products also showed contamination rates exceeding EU levels. Molecular characterization revealed a dominance of Lineage II (66.66%) and a wide distribution of serotypes, particularly IIa, IIc, and IVb. Fourteen distinct clonal complexes (CCs) and 21 sequence types (STs) were identified, with CC9–ST9 emerging as the most prevalent, especially in meat products. In terms of antimicrobial resistance, all isolates were resistant to clindamycin and showed significant multidrug resistance (57.0%), with MARI values exceeding the threshold (>0.2) in several food categories.

This study provides novel and region-specific insights into the occurrence and genetic diversity of *L. monocytogenes* in the Kosovar food chain, as well as the first detailed report of antimicrobial resistance patterns of isolates in this region. These findings fill a critical knowledge gap and establish a molecular and epidemiological baseline that can support food safety surveillance and outbreak investigations in the country.

The results have significant implications for food safety management, public health policy, and future outbreak preparedness. The identification of hypervirulent clonal complexes (e.g., CC2, CC4, and CC6) and the high rate of antimicrobial resistance underscore the importance of incorporating molecular typing and routine antimicrobial susceptibility testing into national monitoring programs.

Limitations of the study include the relatively small number of combined food product and fish samples, which restricted the statistical significance of some subtype distributions. Furthermore, clinical isolates were not included, limiting direct links to human listeriosis cases.

Future research should expand to include clinical isolates, longitudinal monitoring of prevalent clonal complexes, and whole genome sequencing (WGS)-based analyses such as cgMLST to enable high-resolution outbreak tracking. Additionally, assessing the impact of food

processing and environmental stressors on resistance development would provide further insight into the emergence of MDR strains.

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