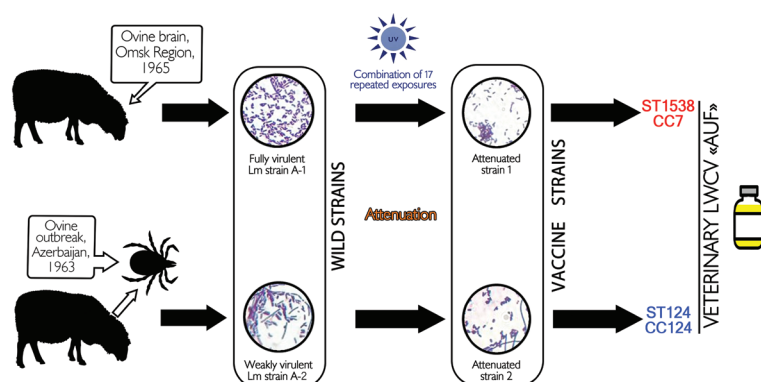




ORIGINAL ARTICLE

MLST Evidence of Two Different Sequence Types of *Listeria Monocytogenes* Strains Used for Commercial Veterinary Listeriosis Vaccines

Graphical abstract



Highlights

- This study investigated the live whole-cell vaccine AUF, which has been successfully used for almost 70 years for listeriosis prevention in farm animals in the Russian Federation.
- This commercial vaccine is based on two ovine strains of *Listeria monocytogenes* attenuated from wild Lm isolates through either multiple ultraviolet irradiation (UFR) or routine techniques with multiple passages on bacteriological media.
- Both vaccine Lm strains belonged to a single phylogenetic lineage (II), but to different STs (ST124 and ST1538), and CCs (CC124 and CC7).
- The Lm strains of ST124 associated with one of the vaccine strains have been routinely isolated widely in countries in Europe, Asia, South America, and North America, whereas only a single Lm strain assigned as ST1538, the parent of the second vaccine strain, was found in ovine specimens in the Russian Federation.
- The vaccine Lm strains demonstrated morphological differences when grown on standard bacterial media for Lm.

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Brief statement

This study investigated the main genetic and morphological characteristics of two Lm strains used for manufacturing the live whole-cell vaccine AUF against listeriosis in farm animals. According to MLST analysis, both attenuated Lm strains belonged to a single phylogenetic lineage, lineage II, but to different STs (ST124 and ST1538) and CCs (CC124 and CC7). Additionally, both Lm strains differed morphologically when grown on routine bacterial media. These findings indicated that the veterinary vaccine AUF is formulated on two manufactured attenuated Lm strains belonging to two genetically different lineages.

MLST Evidence of Two Different Sequence Types of *Listeria Monocytogenes* Strains Used for Commercial Veterinary Listeriosis Vaccines

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Abstract

Objective: Listeriosis is among the most severe food-borne diseases affecting both animals and humans, and it presents a serious threat to public health, animal welfare, and the food industry. Food products contaminated with *Listeria monocytogenes* (Lm), the causative agent of listeriosis, are recognized as the primary source of infection. Since the late 1960s, the live whole-cell vaccine AUF (LWCV AUF) has been successfully used for the prevention of listeriosis in farm animals in Russian regions affected by the disease. This commercial vaccine is based on manufactured batches of the two ovine parental Lm strains isolated in various regions of the former USSR, Siberia, and Azerbaijan, from distinct animals in different years. This study was aimed at investigating whether these strains might belong to the same phylogenetic lineage and possess identical sequence types (STs).

Methods: One Lm vaccine strain was attenuated by multiple ultraviolet irradiation (UFR), whereas the other was obtained through routine techniques involving multiple passages on bacteriological media. Multilocus sequence typing (MLST) analysis was performed with amplification and sequencing of internal fragments of seven housekeeping genes (*abcZ*, *bglA*, *cat*, *dapE*, *dat*, *ldh*, and *lhkA*). For morphological comparison, both Lm strains were grown on Chromocult Listeria Agar Ottaviani and Agosti bacterial media.

Results: Both attenuated Lm strains belonged to a single phylogenetic lineage (lineage II), and were assigned to different STs (ST124 and ST1538), and clonal complexes (CCs), (CC124 and CC7). In addition, these strains demonstrated different morphological characteristics when grown on bacterial media standard for Lm.

Conclusion: The LWCV AUF is formulated on two manufactured attenuated Lm strains belonging to two genetically different lineages.

Key words: *Listeria monocytogenes*, listeriosis, vaccine, farm animals, AUF, ultraviolet irradiation

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INTRODUCTION

Listeria monocytogenes (Lm), the causative agent of listeriosis, is a leading food-borne pathogen [1]. Although the disease is primarily sporadic, the mortality rate during listeriosis outbreaks can reach 30%; consequently, this infection poses a major burden for both animal and human health services, particularly among newborns, older people, pregnant women, and immunocompromised individuals [2,3]. Lm, a Gram-positive, rod-shaped bacterium, is a facultative intracellular pathogen that induces septicemia, and neurological and reproductive diseases in a wide range of hosts. Lm strains are resistant to diverse environmental conditions, including temperatures (1–45°C) and pH values (4.0–9.5), and can tolerate NaCl concentrations as high as 10% [w/v] [4]. These characteristics contribute to the ubiquitous distribution of Lm strains. Therefore, this organism is an extremely dangerous pathogen that substantially affects human and animal welfare, environmental health (One Health) [5], and food safety [6]. Several laboratory diagnostic methods have been used for inter- or intra-specific discrimination in investigations of the diversity of Lm strains [7]. Initially, a serotyping scheme for the discrimination of Lm isolates into serotypes according to the specific structure of Lm somatic (O) and flagellar (H) antigens was successfully developed [8]. Although this method was generally accepted to study the distribution, epidemiology, and virulence of Lm isolates, no detailed information on their clonal structure has been reported [9]. Multilocus sequence typing (MLST) is a powerful and portable approach for identifying individual clonal lineages of pathogenic microorganisms with high resolution and specificity. With this technique, the analysis of single nucleotide polymorphisms (SNPs) in housekeeping genes facilitates rapid interlaboratory comparisons through open accessible databases [10]. MLST has substantial advantages over other methods, because of its ability to reveal the evolutionary relationships among bacterial strains, including Lm representatives [10–16]. According to MLST, Lm strains are currently divided into four major phylogenetic lineages (I–IV) [14], 526 clonal complexes (CCs), and 1289 sequence types (STs) with differing virulence [1,16,17]. Lm isolates of lineage I are associated with cases of human and ruminant encephalitis [18,19]. Lm strains belonging to lineage II are usually isolated from foods, humans, animals, and natural and production environments, whereas Lm representatives of lineages III and IV are rare and are isolated primarily from wild and live farm animals (S1 Table). Most Lm strains of lineage I and some isolates of lineage II are hypervirulent clones associated with outbreaks of human and animal (bovine and small ruminant) listeriosis [18,20–23]. In contrast, Lm strains of lineage II are primarily hypovirulent [15,24].

According to the World Organization for Animal Health (WOAH, founded as OIE, Office International des Epizooties in French), multiple veterinary, sanitary, and zoological hygienic rules, including control of feed quality

and vaccination of target farm animals, are essential for preventing listeriosis in animal husbandry [25]. Several experimental vaccines are under development, such as *Listeria* attenuated live whole-cell vaccines (LWCV), live vector vaccines, inactivated vaccines, killed but metabolically active vaccines, bacterial ghost-based vaccines, subunit vaccines, nano-vaccines, peptide-loaded dendritic-cell vaccines, and bivalent vaccines [26–33]. However, most of these vaccines have been reported to be unable to elicit a reliable level of protective immunity in target farm animals [26,34–37].

In the early 1960s, the LWC listeriosis vaccine AUF (LWCV AUF) was developed and successfully used for listeriosis prevention in farm animals in countries of the former Soviet Union [38]. During laboratory and manufacturing trials, both LWCV AUF strains demonstrated harmlessness, relatively low virulence, and pronounced immunogenicity in animal models such as mice, rabbits, chinchillas, minks, pigs, cattle, and small ruminants, such as sheep and goats [38,39].

The residual virulence value did not increase after 20-fold passages through the brain in white mice; 3-fold passages in white mice and rabbits; and 5-fold passages in guinea pigs. These findings provided strong evidence of the absence of substantial risk of LWCV AUF strains eventually reverting to virulence. According to Selivanov et al. [39], single and repeated vaccination of laboratory animals with the LWCV AUF elicited immunity lasting as many as 10 months.

Extended manufacturing trials in 1971 in animal farms in various regions, such as the Tatar ASSR, Altai Krai, Voronezh, Ulyanovsk, Saratov, and Rostov, demonstrated a 10-fold decrease in the listeriosis-associated case-fatality rate among animals in affected farms, from 2.1% before vaccination to no more than 0.2% after vaccination [38]. Overall, further active preventive immunization of animals in affected farms with LWCV AUF during the subsequent 5–7 years markedly decreased listeriosis-associated mortality. Moreover, outbreaks of the disease in some farms were completely eliminated.

Currently, the commercial LWCV AUF is a lyophilizate of microbial cells of AUF strain. The vaccine is designed for injection in cattle, sheep, goats, pigs, and rabbits in farms at risk of listeriosis. Initial vaccination of livestock is recommended for calves from 2 months of age; lambs and kids from 1 month of age; piglets from 14 days of age; and rabbits from 2 months of age. Adult animals can also be vaccinated. The vaccine is administered intramuscularly into the thigh area twice, with an interval 10 days, in doses of 0.5–2.0 ml for the first immunization and 0.5–5.0 ml for the second and any subsequent injections. A single vaccination dose contains 7.5 billion bacterial cells. Immunity against listeriosis in immunized livestock develops within 10–14 days and lasts as many as 12 months. After repeated immunization, specific immunity to listeriosis is prolonged 12 further months.

TABLE 1 | Allele profiles and MLST characteristics of the Lm strains AUF-1 and AUF-2, and the relevant parental Lm strains A-1 and A-2.

Lm strain ID	Allele (locus)							ST	CC	Phylogenetic Lineage	Specimen Source	Isolation Year	Reference
	<i>abcZ</i>	<i>bglA</i>	<i>cat</i>	<i>dapE</i>	<i>dat</i>	<i>ldh</i>	<i>lhkA</i>						
AUF-1	5	8	5	7	6	65	1	1538	CC7	II	Attenuated from A-1	1965	[38-40]
A-1	5	8	5	7	6	65	1	1538	CC7	II	Wild ovine Lm isolate	1965	[38,39]
AUF-2	23	5	10	30	6	6	1	124	CC124	II	Attenuated from A-2	1963	[38]
A-2	23	5	10	30	6	6	1	124	CC124	II	Pasture tick isolate	1963	[38]

This commercial vaccine was based on manufactured batches of the two Lm strains that were attenuated through either multiple ultraviolet radiation (UVR) or routine techniques with multiple passages on bacteriological media from two ovine parental Lm strains isolated in different regions of the former USSR, such as Siberia and Azerbaijan, from distinct animals in different years (Table 1). One of these LWCV AUF strains, AUF-1, was attenuated from the wild field Lm strain A (A-1) isolated from clinical samples from sheep with neurolisteriosis in the Omsk Region in 1965 [38]. The other LWCV AUF strain, AUF-2, was attenuated from a weakly virulent Lm strain A (A-2) isolated from pasture ticks during an outbreak of listeriosis in livestock in 1963 in Azerbaijan [38]. Currently, both strains are available and used for the production of veterinary LWCV AUF against listeriosis in the Russian Federation. Unfortunately, the phylogenetic lineage, CC, and ST of each Lm strain remain unknown. However, knowledge of mechanisms of attenuation of Lm virulent strains must critically be to develop the next generation of effective, safe, and highly immunogenic veterinary listeriosis vaccines.

The aim of this study was to investigate the MLST profiles and bacterial morphology of the two ovine Lm strains, AUF-1 and AUF-2, that have been successfully used for the production of commercial LWCV AUF in the Russian Federation, and to compare their relevant characteristics with those of the two parental Lm isolates, A-1 and A-2.

METHODS

Vaccine strains

The Lm strains AUF-1 (ID 104334 in the *Listeria* PasteurMLST database) and AUF-2 (ID 104335) were attenuated from the parental Lm strains, the wild fully virulent Lm strain A-1 (ID 104332) and the weakly virulent Lm strain A-2 (ID 104333), respectively. Both parental Lm strains originated from biomaterials from sheep with listeriosis (Table 1, S1 Table) [38]. For the attenuation of AUF-1, 17 repeated UVR exposures of the related parental wild fully virulent Lm strain A-1 were applied [38,40].

AUF-2 was attenuated from the weakly virulent Lm strain A-2 through routine techniques used worldwide [38,39]. Before the experiments, all strains were

kept lyophilized with either bovine serum or gelatine agar medium as a stabilizer, as previously described [41]. Lyophilized Lm cultures were restored with 1 ml tryptone soya yeast extract broth (HiMedia, India), then plated on tryptone soya yeast extract agar (HiMedia, India) and Chromocult *Listeria* Agar Ottaviani and Agosti (ALOA) (HiMedia, India). The plates were cultivated at $37 \pm 2^\circ\text{C}$ for 24 h. Typical colonies were carefully verified according to Gram staining, bacterial cell morphology, and motility at $25 \pm 2^\circ\text{C}$ (*Listeria* Motility Medium, HiMedia, India), as previously reported [41], and used for experiments. In some experiments, the bacterial cultures were plated on brain-heart infusion agar (BD Bioscience, EU) and grown overnight before the experiment [42,43].

DNA isolation

Total DNA was isolated from bacterial culture samples with a DNeasy Blood and Tissue Kit (Qiagen, USA) according to the manufacturer's instructions, as previously described [42,43]. The DNA concentration was measured with a spectrophotometer (Bio-Rad Laboratories, Redmond, WA, USA) according to the manufacturer's instructions.

Multilocus sequence typing

MLST of the Lm strains AUF-1, AUF-2, A-1, and A-2 was performed through amplification and sequencing of internal fragments of seven housekeeping genes (*abcZ*, *bglA*, *cat*, *dapE*, *dat*, *ldh*, and *lhkA*) with a scheme by Ragon et al. [44], as previously reported [42,43]. The PCR amplification conditions were as follows: an initial cycle of 94°C for 4 min; 35 amplification cycles, each consisting of 94°C for 30 s, 52°C for 30 s (except for *bglA* which has an annealing temperature of 45°C), and 72°C for 2 min; and a final incubation at 72°C for 10 min. PCR products were evaluated with 1% agarose gel electrophoresis. Amplified DNA was eluted from the reaction mixture with a Cleanup Mini kit (Evrogen, Russia) after sequencing (<https://evrogen.ru/>). The ST, CC, and phylogenetic lineage for each investigated Lm strain were determined by comparison of the relevant concatenated nucleotide sequences of AUF-1, AUF-2, A-1, and A-2 with the corresponding allelic profiles of *Listeria* isolates available in the BIGSdb-Lm database (<https://bigsd.b.pasteur.fr/listeria/>). MLST profiles were classified into STs and grouped into CCs according

to the *Listeria* sequence typing database and its tools available on the BIGSdb-Lm platform [44,45]. To demonstrate the phylogenetic relationships among AUF-1, AUF-2, A-1, A-2, and Lm strains isolated worldwide, we generated a minimum-spanning tree based on an MLST locus scheme, as visualized with GrapeTree [46]. Esri ArcGis Desktop 10.6.1 (www.esri.com; accessed on May 8, 2024) was used for cartographical and spatiotemporal analysis of Lm cases worldwide. All Lm strains used for construction of the GrapeTree are listed in S1 Table.

RESULTS

MLST analysis

According to the MLST analysis, all four Lm strains represented a single phylogenetic lineage, lineage II. However, AUF-1 and A-1 belonged to ST1538 and CC7, whereas AUF-2 and A-2 were assigned to ST124 and CC124 (Table 1). In fact, AUF-1 had an allelic profile identical to that of the parent Lm strain A-1, and AUF-2 demonstrated identical allelic characteristics to those of Lm strain A-2 (Table 1, S1 Table). No additional polymorphisms were found in the nucleotide sequences of the seven relevant housekeeping genes of either AUF-1 or AUF-2. The relevant individual allele profiles were typical for these STs, either ST1538 or ST124, respectively (S1 Fig).

The minimum spanning tree demonstrated genetic relatedness among AUF-1, AUF-2, A-1, A-2, and all Lm strains belonging to either the relevant STs and CCs (ST1538, CC7, and ST124, CC124, respectively) or to other STs and CCs isolated worldwide (Fig 1). A single possible ancestor recognized as ST155, CC155, was identified for both AUF-1 and AUF-2, whereas AUF-1 and

AUF-2 were evolutionary descendants of two distinct genetic lineages formed via ST7, CC7 and ST26, CC26, respectively (Fig 1).

The group founder for ST1538 was ST7, belonging to CC7 – the third large clonal complexes in Lm after CC1 and CC2, which comprised 358 Lm strains available in the BIGSdb-Lm database (<https://bigsdb.pasteur.fr/listeria/>). Three additional STs, ST659, ST1514, and ST1211, were revealed between ST155 and ST7. Thus, ST1538 was recognized as the descendant of ST155 in the fourth generation. Importantly, ST1538, the descendant of the first generation of ST7, was a likely ancestor of ST126, the descendant of the second generation of ST7.

ST124 evolved from ST26 as the descendant of the third generation via ST1368 and ST635. ST26 belonging to CC26, was also a possible ancestor and the group founder for a number of descendants of the first and the second generation, such as ST27, ST93, ST380, ST436, ST501, ST706, ST750, ST754, ST1277, ST1350, ST1386, ST1515, ST2196 and ST229, ST635 and ST1503, respectively. Furthermore, ST124 was a group founder for at least five STs, ST452, ST1409, ST1550, ST1551, and ST1552, the descendants of the first generation of ST124 (Fig 1).

The alleles of two of the seven housekeeping genes, *lhcA* and *dat*, were identical in AUF-1, AUF-2, A-1, and A-2; Lm strains ST1211, ST26, ST1368, ST635, ST452, ST1409, ST1409, ST1550, ST1551, and ST1552; and large ST groups such as ST7 ($n = 234$) (S1 and S2 Tables). The identical alleles of six of the seven housekeeping genes (*abcZ*, *bglA*, *cat*, *dapE*, *dat*, and *lhcA*) were present in the genetic lineage of AUF-1 and A-1 as well as in Lm

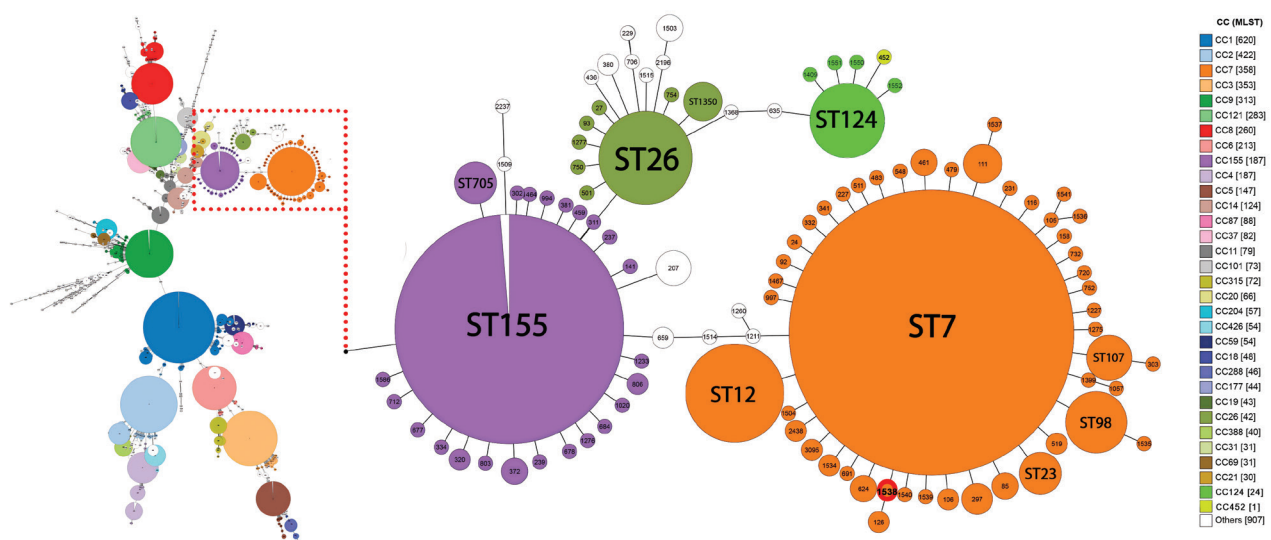


FIGURE 1 | MLST minimum spanning tree demonstrating the genetic relatedness among AUF-1 (ST1538), A-1, AUF-2 (ST124), A-2, and all Lm strains ($n = 5326$) available in the BIGSdb-Pasteur MLST database (<https://bigsdb.pasteur.fr/listeria/>, accessed on September 3, 2024). Each individual circle represents one sequence type (ST). The circle sizes are proportional to the number of strains. Links between circles represent the number of allelic mismatches between individual STs (S1 Table). The positions of ST1538 and ST124 with respect to all STs are shown in yellow square.

representatives of their closest ancestor, ST7 ($n = 234$). Three of five descendants of the genetic lineage similar to AUF-2 and A-2 belonging to CC124, ST1550, ST1551, and ST1552 demonstrated identical alleles in six of the seven housekeeping genes with AUF-2 and A-2 lineage (*abcZ*, *bglA*, *cat*, *dapE*, and *lhkA*). Similarly, another descendant of the same AUF-2/A-2-associated genetic lineage, ST1409, had identical alleles for six of the seven housekeeping genes (*abcZ*, *cat*, *dapE*, *dat*, *ldh*, and *lhkA*). However, only five of those genes (*abcZ*, *cat*, *dapE*, *dat*, and *lhkA*) were identical among ST124, ST1409, ST1550, ST1551, and ST1552.

The identical alleles of only five of the seven housekeeping genes were identified in AUF-2 and A-2, their descendant, ST452 (*bglA*, *dapE*, *dat*, *ldh*, and *lhkA*), and their closest ancestor, ST635 (*abcZ*, *dapE*, *dat*, *ldh*, and *lhkA*). The possible ancestor of both ST1538 and ST124 (ST155, CC155) had a single common allele in only one of the seven housekeeping genes (*lhkA*).

In fact, the Lm strains assigned to ST7 (CC7), the possible closest ancestor of AUF-1 and A-1 (ST1538, CC7) have been sporadically isolated from various sources (human, animal, food, natural environment, production environment, feed, and farm environment) for almost a century worldwide. The closest possible ancestors in the lineage of AUF-2 and A-2, ST635 (CC635) and ST1368 (CC906), were isolated from humans and animals in Canada and the USA less than a decade ago, whereas Lm strains represented their possible common ancestor, ST26 (CC26) and, at least since the beginning of 1960s, were also widely distributed in various sources, primarily in European countries (S2 Table).

Notably, at least 24 Lm strains of ST124 typical of both A-2 and its attenuated derivative, AUF-2, are currently available in the BIGSdb-Lm database (<https://bigsd.b.pasteur.fr/listeria/>, S1 Table), whereas only two isolates assigned to ST1538 were identified over nearly 70 years: (i) A-1, the parental strain of the attenuated AUF-1 (Table 1), and (ii) the bovine Lm strain 211 isolated in 1992 in the Kursk Region, Russia [43].

A total of 13 SNPs with respect to the group founder, ST155 (A6G, C108T, T672C, T705C, A795T, A849G, C852T, T1032A, C1170T, A1345G, G2190T, A2325G, and G2717A) were identified in the concatenated nucleotide sequences of seven housekeeping genes of ST1538, to which AUF-1 and A-1 were assigned (Table 1, S2A Fig). These alterations resulted in a single histidine → arginine amino acid substitution, G2717A, in the protein encoded by one of seven housekeeping genes, *ldh*. The additional glycine → serine substitution S1008G was found in *lhkA* for ST126, the descendant of ST1538 (S3 Table, S3A Fig). In the concatenated nucleotide sequences of housekeeping genes of ST124, the relevant ST for both AUF-2 and A-2, we identified 21 SNPs with respect to the representative ancestor ST155, including two nonsynonymous SNPs, A1519G and T1661C (Table 1), affecting two of the seven housekeeping genes, *bglA*

and *dapE* (S4 Table, S2B Fig). These mutations led to two substitutions, K507E and V554A, which were essential for ST1368, the closest ancestor of ST124 and all available ST124 descendants, such as ST452, ST1409, ST1550, ST1551, and ST1552 (S4 Table, S3B Fig), while ST1409, ST1550, ST1551 and ST1552 demonstrated the presence of several nonsynonymous SNPs in *lhkA*, similarly to the *lhkA* gene in ST126.

The representatives of ST7, CC7, the closest ancestor of AUF-1 and A-1 (ST1538, CC7), and those of the common group founder, ST155, CC155, which were isolated from a variety of sources (such as animals and humans; feed and food; and natural, farm, and production environments), appeared to be the most globally distributed STs (S1 Table, Fig 2). However, other related STs were also identified in certain regions of the world. Thus, ST26 originated primarily from animal specimens (cattle and rabbits), food (meat and vegetables), and production environments in Europe (S1 Table); ST124 was derived from feed (silage), animals, food, and natural and production environments in Europe (Finland, Denmark, Belgium, Spain, and Switzerland), Asia (Kazakhstan), South America (Chile), and North America (Mexico); and ST1409, CC124 was found in food in only Europe (Finland).

Comparative bacterial morphology of the Lm parental and vaccine strains

Overall, the Lm vaccine strains AUF-1 and AUF-2, and the parental strains A-1 and A-2, had morphology typical of Lm bacteria, and were Gram-positive, motile, non-encapsulated, non-spore-forming short rods (Fig 3, S2 Table). The Lm strains A-2 and AUF-2 grown on ALOA had typical Lm morphology, and formed small round, translucent, low-convexity colonies. In contrast, the Lm strains A-1 and AUF-1 showed pronounced polymorphism in bacterial shape, with predominant filamentous forms and less abundant growth on ALOA.

DISCUSSION

In this study, the MLST and basic morphological characteristics of two Lm strains, AUF-1 and AUF-2, which have been successfully used as live veterinary vaccines against listeriosis [38], were carefully investigated to clarify whether both strains belonged to a single lineage or two different genetic lineages. Both strains were found to belong to the same genetic lineage, lineage II, to which hypervirulent and hypovirulent Lm strains widely found in humans, animals, food, feed, and farm environments are assigned (S1 and S2 Tables). However, we found differences in the allele profiles for the seven relevant housekeeping genes in each of the strains, thus demonstrating their attribution to different CCs and STs: CC7 and ST1538 for AUF-1, and CC124 and ST124 for AUF-2 (Table 1, S1 and S2 Tables). In fact, each of the relevant parental Lm strains, A-1 and A-2, was isolated independently in different years, in various animal households,

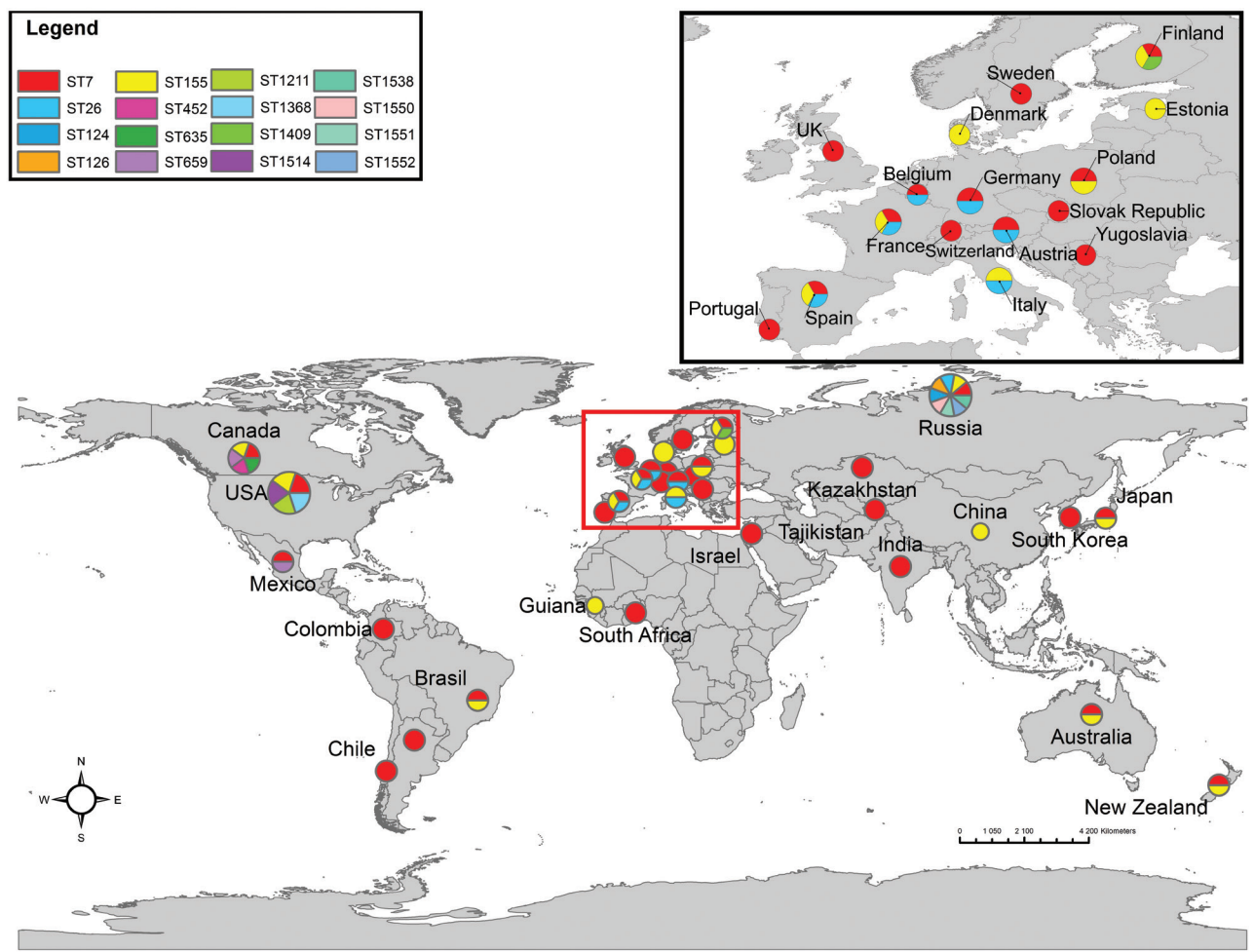


FIGURE 2 | Global distribution and biodiversity of Lm in various regions of the world. The different Lm STs available in the BIGSdb-Lm database (<https://bigsdb.pasteur.fr/listeria/>) are colored in individual circles according to the legend.

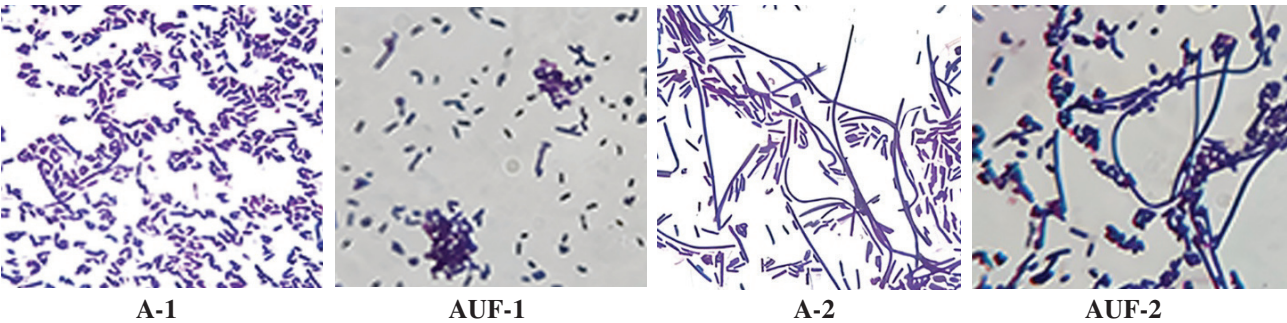


FIGURE 3 | Comparative morphology of Lm strains: AUF-1, A-1, AUF-2, A-1, and A-2 (Gram staining, magnification ×1000).

at distinct regional locations, namely in Siberia and Azerbaijan, respectively, by two different research groups. Each of these Lm strains demonstrated MLST characteristics identical to those of the relevant descendant, AUF-1 (CC7, ST1538) and AUF-2 (CC124, ST124), respectively (Table 1, S1 and S2 Tables). Remarkably, a single potential ancestor belonging to CC155, ST155, was identified for both AUF-1 and AUF-2 and the relevant parental Lm

strains, A-1 and A-2, whereas AUF-1 along with A-1, as well as AUF-2 simultaneously with A-2 were evolutionary descendants of two distinct genetic lineages formed via CC7 and CC26, respectively. Moreover, both AUF-1 and AUF-2, the strains used for LWCV AUF, have been attenuated from wild virulent Lm clinical isolates derived from this common ancestor, ST155, CC155, comprising primarily hypervirulent Lm strains [25].

Importantly, UVR attenuation did not affect the housekeeping genes used for MLST analysis of both AUF strains belonging to two different genetic lineages, CC7 and CC124. The absolute identity of the relevant concatenated nucleotide sequences of either AUF-1 or AUF-2 and their respective parental strains, A-1 and A-2 (Fig 2), strongly supported this conclusion. However, we observed markedly differing bacterial cell morphology for the AUF-1/A-1 pair and the AUF-2/A-2 pair, including pronounced polymorphism in bacterial shape, with predominant filamentous forms in the latter two strains (Table 1, Fig 1). These observations might indicate that the UVR treatment has been quite mild for the Lm strains, and has affected mutations in Lm genes other than the housekeeping genes, as recently demonstrated for AUF-1 [40]. In contrast, the marked phenotypic difference between A-2 and its attenuated descendant, AUF-2, might be a specific feature of the relevant MLST profile, which differed from the MLST characteristics of A-1 and AUF-1; however, an influence of UVR on the vaccine strain AUF-2 cannot be entirely excluded.

DATA AVAILABILITY STATEMENT

The concatenated sequences of the Lm housekeeping genes for the Lm strains AUF-1, AUF-2, A-1, and A-2 are available in the Listeria Pasteur MLST database (<https://bigsd.b.pasteur.fr/listeria/>) under accession numbers ID 104334, ID 104335, ID 104332, and ID 104333, respectively.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIAL

Supplementary Material can be downloaded from https://zoones-journal.org/wp-content/uploads/2025/02/zoones20240050_Suppl.zip.

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