Veterinary and Animal Science 19 (2023) 100276

Contents lists available at ScienceDirect

Veterinary and Animal Science

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

Article

# Genetic diversity and known virulence genes in *Listeria innocua* strains isolated from cattle abortions and farm environment

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ARTICLE INFO

*Keywords:*
Listeria innocua
Virulence
Abortions
Cattle
Farm

ABSTRACT

***Listeria innocua*** is considered as non-pathogenic bacteria living in an environment although several cases of immunocompromised humans and ruminant listeriosis infections have been reported. Previously, L. *innocua* was identified as a potential pathogen and virulence in association with L. *monocytogenes PrfA* dependent virulence (LIPI-1) gene cluster was demonstrated in hemolytic L. *innocua*. L. *innocua* usually considered non-pathogenic versus pathogenic L. *monocytogenes* and L. *ivanovii* because of the main virulence gene loss. There are limited studies and reports available about L. *innocua*-caused illness in cattle. A total of 18 STs were identified in cattle abortions while 17 STs in the farm environment with majority of STs were present in both abortions and environmental samples. Genome sequencing showed that in one farm identical L. *innocua* clones were represented in water, feed, soil, and faeces sample groups, suggesting that animals most likely through the faecal shedding may remain as the main source of L. *innocua* in a farm environment. Out of all L. *innocua* isolates *PrfA*-dependent virulence genes were not found in aborted foetuses isolates and environmental L. *innocua* isolate groups; however, in 20% of isolates a complete LIPI-3 pathogenicity island encoding listeriolysin S was identified. In this study, we demonstrated that genetically diverse L. *innocua* clones were widely distributed in cattle farm environment and certain isolates had a significant pathogenicity potential for cattle, thus causing adverse health effects, including abortions.

## 1. Introduction

*Listeria* spp. are widely distributed in soil, water, vegetation and among animals, and by 2021 there are at least 27 species described (Carlin et al., 2021). Most species of genus Listeria are previously described as non-pathogenic, however L. *monocytogenes* is one of the most studied food- and feed-borne pathogens affecting humans, mostly the elderly and immunocompromised individuals, and animals, mostly ruminants (Diriba et al., 2021; Orsi & Wiedmann, 2016). Listeriosis can manifest clinically as meningoencephalitis, abortions, sepsis and gastroenteritis in both humans and ruminants. L. *ivanovii* is the other pathogenic species that mainly infects ruminants causing abortions, stillbirth, and sepsis (Orsi & Wiedmann, 2016).

Mechanisms of L. *monocytogenes* pathogenicity have been widely described, and key molecular determinants have been identified (Vázquez-Boland et al., 2001). Six main virulence genes are located in *Listeria* pathogenicity island (LIPI) – 1 (*prfA, plcA, hly, mpl, actA* and *plcB*) and that is regulated by transcriptional regulator *PrfA* (Chen et al., 2018). Processes activated by *PrfA* are crucial for the infection cycle of L. *monocytogenes*. They include phagosome lysis to release bacteria into cytoplasm and actin-dependent intercellular bacteria motility (Freitag et al., 2009). Internalins are another important group of virulence factors displaying leucine-rich repeats. They include *inlAB* operon contributing to invasion of epithelial and other cells, and *inlC* involved in intercellular spread (Cossart, 2011). LIPI-3 contains genes encoding listeriolysin S (LLS) that plays a role in L. *monocytogenes* colonization of gastrointestinal tract (Quereda et al., 2017).

L. *innocua* typically is a non-haemolytic, rod-shaped bacterium, common in various natural and industrial environments including cattle farms and food processing plants (Kaszoni-Rückerl et al., 2020; Klausner & Donnelly, 1991; Orsi & Wiedmann, 2016; Terentjeva et al., 2021). It is considered as non-pathogenic, however listeriosis cases of L. *innocua* associated fatal bacteraemia (Perrin et al., 2003), meningitis (Favaro et al., 2014) and a fatal early-onset sepsis in a neonate (Arumugam et al.,

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https://doi.org/10.1016/j.vas.2022.100276

Available online 1 December 2022

2001) in humans and cerebral listeriosis in beef bull (Rocha et al., 2013) have been reported. There are few cases of atypical haemolytic L. *innocua* isolated from fish and fishery products, swine slaughterhouses (Moreno et al., 2012) and poultry (Milillo et al., 2012). Among atypical strains L. *monocytogenes prfA* virulence gene cluster including *hly* gene responsible for haemolysis was detected (Moreno et al., 2012; Moura et al., 2019). Atypical haemolytic L. *innocua* strains demonstrated pathogenic potential in the mouse model (Bolger et al., 2014) and zebrafish larvae model (Kaszoni-Rückerl et al., 2020) although these strains were less virulent compared with L. *monocytogenes*.

The aim of this study was to characterize the genetic diversity of L. *innocua* isolated from cattle abortion cases and environment of cattle farms and to assess their virulence gene presence. And further to assess the distribution of isolates in the environment and compare abortion isolates with environment isolates by Sequence Type.

## 2. Materials and methods

### 2.1. Collection of cattle abortion samples

In total 806 cattle abortion cases from January of 2016 till June of 2019 were investigated within the annual state surveillance program for the investigation of cattle abortion cases in the Republic of Latvia. Cattle owners were requested to notify the veterinarian of all observed abortion or stillbirth cases. Subsequently, an official veterinarian collected the aborted or stillbirth foetus and transported it in sterile plastic sampling bags in cooling conditions (5 ± 3 °C) to the laboratory. The state surveillance program provided microbiological investigations of an aborted foetus for various abortion agents, including L. *innocua*. The necropsy of the foetus was performed for the purpose to collect the samples for microbiological analyses. Sampling of heart, spleen, liver, kidneys, and lungs from each foetus was carried out aseptically. The tissue samples of each foetus were pooled and investigated as a one combined sample for each abortion case.

### 2.2. Environmental L. innocua isolates

In total 214 L. *innocua* positive enviromental samples were collected from agricultural cattle farms in the period from June 2019 to June 2020. Samples were collected from 27 farms where listeriosis in ruminants was reported in the last three years (2016 – 2019) ($n = 9$) and control farms ($n = 18$) without listeriosis cases in the last three years. In the study, environmental samples were collected from the soil near the dwellings, drinking waterers, from vegetation and cattle feaces. A total of 92 L. *innocua* isolates from the environmental sample collection were subjected for analysis with WGS, but the actual prevalence of L. *innocua* in soil ($n = 31$), feed ($n = 20$), water ($n = 27$) and animal feces ($n = 14$) was reported in study of Terentjeva et al. (2021).

### 2.3. Bacteriological testing of Listeria spp. and confirmation of L. innocua isolates

The isolation of *Listeria* spp. from environmental and aborted foetus tissue samples was performed according to ISO-11,290–1 (2017). For the bacteria isolation procedure, an amount of 25 g or 25 ml of sample was enriched in Half Fraser broth (Biolife, Italy), homogenized by stomaching (BagMixer® 400, Interscience, France) at speed of 6 strokes per second for 60 s and then incubated 24 h at 30 °C. The inoculum was transferred to Fraser broth at 37 °C and incubated for 24 h. After incubation, 100 μL of inoculum from the Half Fraser and the Fraser media was inoculated on selective Agar Listeria Ottavani & Agosti (ALOA) and OXFORD (Biolife, Italy) plates. After 24 h of incubation at 37 °C, Listeria-specific colonies were confirmed by Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) Biotyper (Bruker, Germany).

Small and round (0.5 – 1 mm in diameter) colonies in blue–green color on ALOA medium and olive colonies on OXFORD medium were considered as *Listeria* spp. and were plated onto sheep blood agar (ThermoFisher, USA). Haemolysis on blood agar was only visually assessed. Colonies that were small, round (0.5 – 1 mm in the diameter), in gray or grayish-white color with or without haemolysis on the sheep blood agar medium, were considered as *Listeria* spp.

### 2.4. Whole genome sequencing (WGS) of L. innocua

L. *innocua* isolates, at least one isolate of each sample group from several farms and environments, were selected for whole genome sequencing. DNA from bacterial culture was extracted with QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. Nextera XT library construction kit (Illumina, USA) and Illumina MiSeq with 300 bp paired reads were used for the preparation of libraries and sequencing according to the manufacturer's protocols, respectively.

Sequencing adapters and low-quality bases were trimmed from raw reads using Trimmomatic v0.38 program (Bolger et al., 2014). *De novo* assembly of the trimmed reads was performed with SPAdes v3.14.0 (Prjibelski et al. 2020). The Kmer-finder program was used to confirm the species at the genome level (Hasman et al., 2014). A minimum of 93% k-mer overlap was required for species confirmation. Kraken2 program with the miniKRAKEN database was also used for species confirmation (Wood et al., 2019), because The Kmer-finder database contained a relatively small number of L. *innocua* reference genomes. Quality control of assemblies was done based on several criteria, including N50, genome size and GC%, and subsequently the sequences were included in further analysis. Low quality sequences were not included in the further analysis; therefore, no environmental isolate from Latgale region was represented.

Assembled genomes were uploaded in Ridom SeqSphere+ 7.0.4 (Jünemann et al., 2013). *Ad hoc* cgMLST scheme with 1347 loci covering 35% of L. *innocua* genome was developed using Ridom SeqSphere+ target definer. As publicly available reference genome *Listeria innocua* Clip11262 (NC_003212.1, complete genome) was used and for comparison eight L. *innocua* genomes (NZ_CM001049.1; CM001048.1; AGCN00000000.1; JZCU00000000.1; JZCS00000000.1; JRYX00000000.1; UAST00000000.1; PNRL00000000.1) were used as these were the only ones available in NCBI Genome database at the time of analysis.

L. *innocua* genomes were analyzed for the presence of virulence factors determined by ≥95% amino acid sequence identity in Diamond (Buchfink et al., 2015). The Virulence factor database (VFDB) was used to select virulence genes in *Listeria* (Liu et al., 2019). MLST sequence type was determined with Ridom SeqSphere+. The cluster was defined with distance 0–5 loci.

### 2.5. Statistics

Association of source type and prevalence were estimated using Rstudio Fishers exact test and $p < 0.01$ was considered as significant.

## 3. Results

### 3.1. Prevalence and genetic diversity of L. innocua in cattle abortions

L. *innocua* was isolated in 57 (7.1%) of 806 aborted foetuses. The number of abortion cases associated with L. *innocua* varied among the years (Table 1). The highest prevalence (40.4%) of L. *innocua* associated abortions was observer in the year 2017, but the lowest prevalence (14.0%) was in the year 2018. L. *innocua* isolates were from 31 farms located in different regions in Latvia. The sequence type (ST) was determined by WGS for 55 isolates, but for two isolates ST remained unknown - a new allele type was indicated. Overall, 18 different STs were detected (Table 1). The most prevalent STs were ST1482 and ST1087 that were detected in eight and seven isolates during the study

**Table 1**
Distribution of L. *innocua* associated abortion cases per year and geographical locations.

| ST | Year | | | | Total | Geographical location | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2016 | 2017 | 2018 | 2019 | | Kurzeme | Zemgale | Latgale | Vidzeme |
| 43 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 |
| 448 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| 474 | 1 | 1 | 0 | 0 | 2 | 0 | 1 | 0 | 1 |
| 485 | 0 | 3 | 0 | 0 | 3 | 1 | 1 | 1 | 0 |
| 493 | 2 | 1 | 0 | 1 | 4 | 0 | 3 | 1 | 0 |
| 530 | 1 | 0 | 0 | 2 | 3 | 0 | 2 | 1 | 0 |
| 537 | 0 | 2 | 0 | 0 | 2 | 0 | 1 | 1 | 0 |
| 603 | 1 | 2 | 1 | 0 | 4 | 1 | 0 | 1 | 2 |
| 637 | 0 | 2 | 0 | 1 | 3 | 1 | 1 | 1 | 0 |
| 1008 | 3 | 0 | 0 | 0 | 3 | 0 | 1 | 2 | 0 |
| 1010 | 0 | 3 | 0 | 1 | 4 | 1 | 0 | 3 | 0 |
| 1085 | 0 | 0 | 1 | 2 | 3 | 0 | 3 | 0 | 0 |
| 1087 | 1 | 4 | 2 | 0 | 7 | 3 | 0 | 2 | 2 |
| 1480 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 |
| 1482 | 4 | 3 | 0 | 1 | 8 | 3 | 2 | 2 | 1 |
| 1619 | 0 | 1 | 2 | 1 | 4 | 1 | 1 | 2 | 0 |
| 2074 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| 2694 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 |
| unknown | 0 | 1 | 1 | 0 | 2 | 0 | 1 | 1 | 0 |
| Total | 14 | 23 | 8 | 12 | 57 | 13 | 17 | 19 | 8 |

years, respectively. Furthermore, ST493, ST603, ST1010 and ST1619 were detected each in four isolates between the studied years. Other STs were detected in one to three isolates. L. *innocua* isolates were found throughout the whole territory of Latvia (Table 1).

All the STs, except ST1085, that were detected in two or more isolates, were observed in different geographical regions of Latvia. ST1085 was detected in three isolates and all were from two farms in Zemgale region. The majority of STs observed in cattle abortion cases were also present in environmental samples, but several of them – ST43, ST485, ST530, ST1480, ST2074 and ST 2694 – were detected only in abortion cases and accounted for 17.5% of L. *innocua* isolates of cattle abortions (Fig. 1).

### 3.2. Genetic diversity of L. innocua in environmental samples

In total, 17 different STs were detected among environmental isolates. The three most prevalent were ST493, ST637 and ST1619. L. innocua ST493 dominated in soil, feed and water samples, ST637 in soil and water, but ST1619 prevailed in water and feed samples (Table 2).

L. *innocua* isolates of ST20, ST599, ST1481 and ST2347 accounted for 3.7% of environmental samples and were observed only among environmental samples. However, in 23.9% of the environmental samples ST was unknown (Table 2). The highest ST diversity was observed in soil samples and drinking throughs (Table 3). In the feed, the diversity of L. *innocua* STs was higher among the samples from the feeding tables,

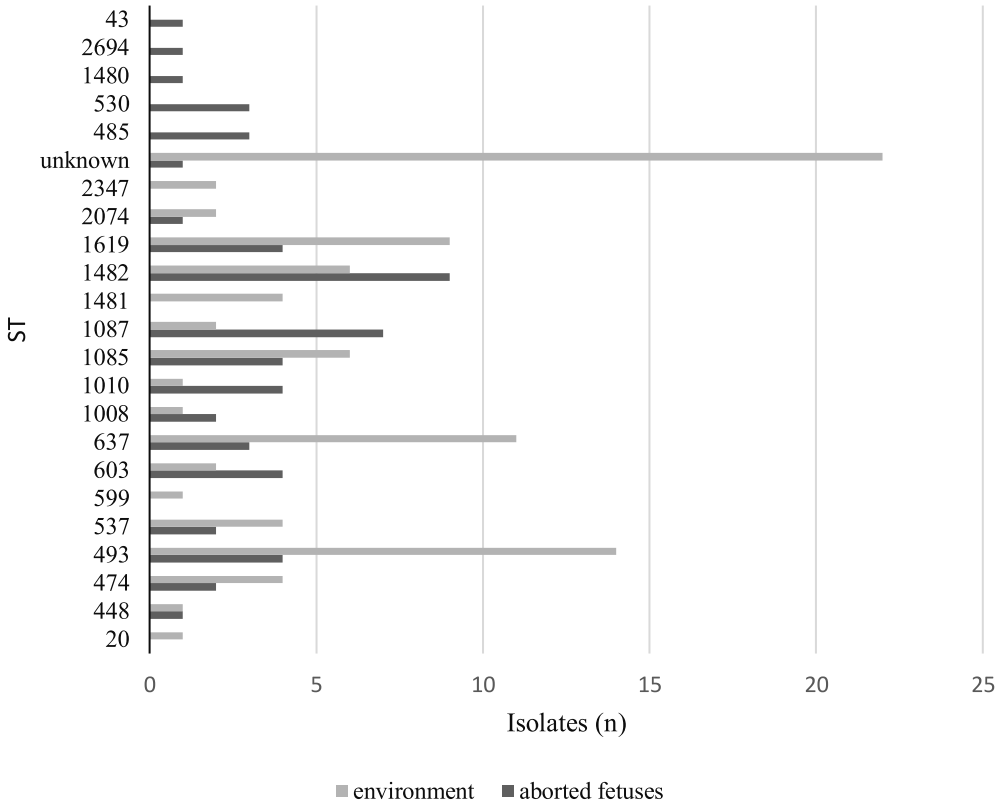


**Fig. 1.** Diversity of L. *innocua* isolates isolated from aborted foetuses and environmental samples.

**Table 2**
Distribution of L. *innocua* STs among environmental sample types and geographical locations.

| ST | Sample type | | | | Total | Geographical location | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Feed | Faeces | Water | Soil | | Kurzeme | Zemgale | Latgale | Vidzeme |
| 20 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 |
| 448 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| 474 | 0 | 1 | 0 | 1 | 2 | 1 | 0 | 0 | 1 |
| 493 | 3 | 0 | 5 | 6 | 14 | 0 | 4 | 0 | 10 |
| 537 | 0 | 0 | 2 | 2 | 4 | 2 | 0 | 0 | 2 |
| 599 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 |
| 603 | 0 | 0 | 2 | 0 | 2 | 0 | 1 | 0 | 1 |
| 637 | 0 | 1 | 4 | 6 | 11 | 5 | 1 | 0 | 5 |
| 1008 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 |
| 1010 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| 1085 | 1 | 1 | 1 | 3 | 6 | 1 | 2 | 0 | 3 |
| 1087 | 0 | 0 | 1 | 1 | 2 | 0 | 0 | 0 | 2 |
| 1481 | 2 | 1 | 0 | 1 | 4 | 0 | 2 | 0 | 2 |
| 1482 | 2 | 1 | 2 | 1 | 6 | 6 | 0 | 0 | 0 |
| 1619 | 3 | 0 | 4 | 2 | 9 | 0 | 1 | 0 | 8 |
| 2074 | 0 | 2 | 0 | 1 | 3 | 0 | 1 | 0 | 2 |
| 2347 | 0 | 2 | 0 | 0 | 2 | 2 | 0 | 0 | 0 |
| unknown | 6 | 4 | 6 | 6 | 22 | 13 | 4 | 0 | 5 |
| Total | 20 | 14 | 27 | 31 | 92 | 31 | 18 | 0 | 43 |

where cattle may ingest contaminated feed.

### 3.3. Cluster analyses

Representative isolates from 15 farms were selected for sequencing with at least two separate sample groups per farm. After MultiQC quality control 92 environmental and 57 samples of aborted foetuses L. *innocua* sequences were included in further analysis such as cluster analysis, virulence gene identification and ST identification. To study the genetic relationships between isolates from different sources and spread of certain clones cgMLST scheme with 1347 loci was developed based on publicly available L. *innocua* whole genome sequences. Minimum spanning tree was used for sequence comparison and 14 clusters were identified including two to eight samples per cluster (Fig. 2, full dataset in Supplementary Table 1). All but one clusters included samples with genetic distance 0–5 cgMLST loci limited to one farm (Fig. 2, Clusters 1,2,3,4,6,7,8,9,10,11,12,13,14) however one cluster (Fig. 2, Cluster 5) included samples from two farms with a genetic distance of only 3 cgMLST loci. Sequences of predetermined sample groups demonstrated high diversity and clustering according to sample groups was not observed.

Analyzing Farm A Cluster 1 included samples from an aborted foetus and environment, that represents potentially contamination from environmental sample to cattle in farm. Other isolates demonstrated high diversity (Fig. 3, Cluster 1).

**Table 3**
Distribution and ST diversity of L. innocua in soil, feeding areas on the farm and drinking water at cattle farms.

| Type of sample | | No. of samples | *No.* of L. *innocua* positive samples (%) * | Sequence types (ST) |
|---|---|---|---|---|
| | Farm | 84 | 48 (58) | 493, 1619, 537, 1482, 599, 637, 1619 |
| Soil** | Pasture | 49 | 24 (49) | 2674, 493, 637, 474, 1085, 537, 1481 |
| | Total | 133 | 72 (54) | |
| | Feeding table | 59 | 30 (51) | 448, 493, 1008, 1619, |
| Feeding area | Pasture | 3 | 1 (33) | 1481 |
| | Storage | 79 | 18 (23) | 1481, 1482 |
| | Total | 141 | 49 (35) | – |
| | Drinking throughs in farm | 73 | 36 (50) | 493, 1619, 637, 537, 603, 1482, 1087 |
| Drinking water | Pasture waterers | 19 | 3 (16) | 603 |
| | Barn bucket | 44 | 14 (32) | 1619, 637, 1482 |
| | Total | 136 | 53 (39) | – |

* The prevalence data were stated by Terentjeva et al. (2021).
** L. innocua prevalence in soil samples was significantly higher than the prevalence in other samples types ($p < 0.01$).

### 3.4. Virulence factor analysis

In total presence/absence of 42 known L. *monocytogenes* virulence factors were determined in 92 genomes of environmental L. *innocua* isolates (Supplementary Table 1). Complete virulence gene cluster LIPI-3 consisting of listeriolysin S encoding genes *llsA, llsX, llsG, llsH, llsB, llsY, llsD, llsP* was detected in 20 isolates (21.73%) and partial– few genes of pathogenity island found in LIPI-3 was in additional 49 isolates (53.26%) but 23 (25.0%) isolates did not contain any LIPI-3 genes. Of 57 isolates from aborted foetuses, complete virulence gene cluster LIPI-3 was detected in 4 (7.01%) isolates, partial – in 30 (52.63%) isolates and in 23 (40.35%) no LIPI-3 virulence genes were found.

Characteristic *Listeria* virulence genes *lap, clpC, clpE, clpP, lplA1, lspA, fbpA, gtcA, iap/cwhA, lpeA, oatA, pdgA, prsA2* were detected in almost all analysed L. *innocua* genomes. Internalin genes – *inlJ, inlK, inlB, inlF* were identified in one to two environmental samples (Supplementary Table 1). Full LIPI-1 island and haemolysis genotype were not found and haemolytic phenotypes were not visually detected.

The significant difference in the presence of virulence genes between clinical isolates and environmental isolates was not identified ($p > 0.05$).

## 4. Discussion

This is one of the first studies where L. *innocua* genetic diversity was described within one and betwefoetusesen several cattle farms and abortion cases. Cattle farm environments are frequently colonized by *Listeria* spp. In our study, the majority of L. *innocua* STs were present in both abortion and environmental samples, indicating that proper farm management and hygiene measures are important tools to prevent the listeriosis in cattle. The high diversity of STs found in drinking throughs in farms could be related to insufficient number of drinking throughs. The use of one drinking through by many cattle facilitates the transmission of pathogens to other cattle. The high diversity of STs was observed among soil samples from farm and pasture, indicating that

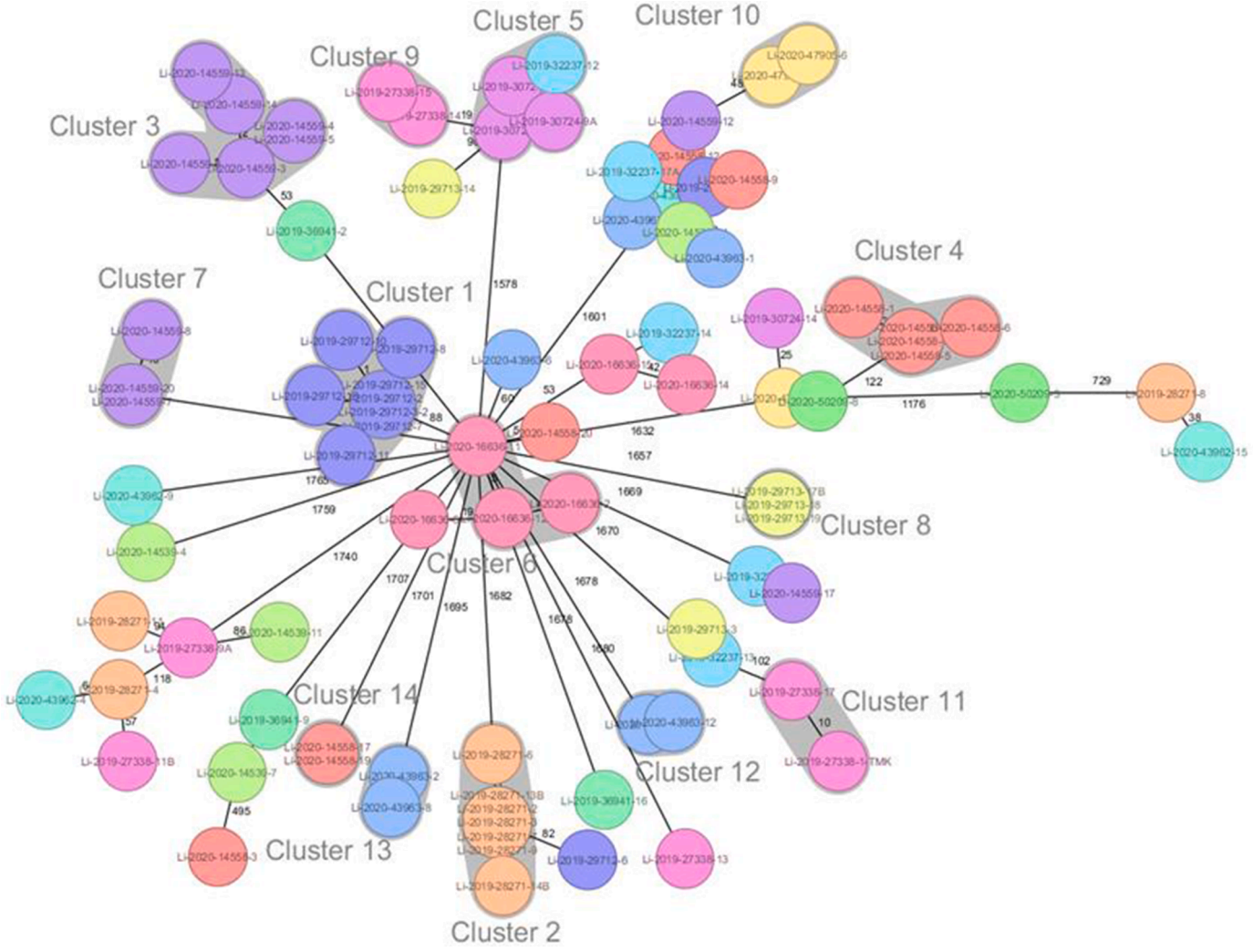


**Fig. 2.** Ridom SeqSphere+ minimum spanning tree based on 1347 loci *ad hoc* cgMLST scheme. MST includes 92 *Listeria innocua* isolates of 15 cattle farms. Each color represents a farm 2019–2020 Lines connect the most similar isolates, numbers on lines indicate the number of different alleles, pairwise ignoring missing values. Colouring according to individual farms named by nearby populated place. Clustering distance – 10, if distance ≤ 10 background is coloured gray, indicating a cluster.

prevention of cattle feed contamination with soil could potentially reduce listeriosis cases in cattle. *Listeria* spp. in cattle can cause high morbidity and mortality, leading to significant economic losses for farmers. Although L. *innocua* is more commonly found compared with L. *monocytogenes* in various natural environments, there are just a few studies characterizing their genetic diversity and virulence potential (Moura et al., 2019; Rocha et al., 2019). Additionally, most studies focused on L. *innocua* in food-producing environment to evaluate the harm of human infection but not the hazard for cattle (Kaszoni-Rückerl et al., 2020; Milillo et al., 2012). This study indicated that L. *innocua* could be a noteworthy pathogen associated with cattle abortion cases.

Previous studies have reported the prevalence of L. *innocua* in 9.7% in cattle and sheep farms and slaughtering environments (Zhao et al., 2021) and 32.5% in poultry abattoirs (Bouayad et al., 2015). Terentjeva et al. (2021) reported that L. *innocua* prevalence in cattle farms environmental samples were significantly higher than the prevalence of other *Listeria* species. Many natural ecological niches were included like ponds, pasture and soil defined as places available to cattle, indicating that L. *innocua* was ubiquitously spread in cattle farm environment. *Listeria* are found in cattle feces, and in the autumn, removing manure in the field fertilizers, they can be a potential source of infection for both humans and cattle through the consumption of contaminated feed (36).

As there is no high-resolution sequencing-based typing scheme publicly available, a L. *innocua* cgMLST scheme with 1347 loci was generated in Ridom SeqSphere+ and used to compare genomes. In our scheme the same distance threshold (10 allelic differences) was used for cluster definition as in L. *monocytogenes* 1701 cgMLST scheme (Ruppitsch et al., 2015), however the suitability of this threshold should be confirmed with experimental data. Results showed that isolates in clusters were mostly limited to one farm and in clusters faecal isolates were included – as evidence that there was a common environmental source and animals were the most likely reservoir and shedders in the farms. Wide genetic diversity was observed among L. *innocua* isolates and most of the detected STs were found in both environmental and aborted foetus isolates. This result suggests a common source of contamination or a pathway for these bacteria in ruminants. MLST typing revealed 22 STs of L. *innocua* in different environments. STs were identified on 149 L. *innocua* isolates. In one farm at least three different L. *innocua* genotypes were isolated from animal feces and this was in line with L. *monocytogenes* studies where diverse genotypes were observed in one location (Ruppitsch et al., 2015).

L. *innocua* genome sequence analysis focused on the presence of virulence genes. Good quality isolates for full genome sequencing were included from each farm and environment. In our study, internalin genes *inlJ, inlK, inlB, inlF* were detected. At least 20% of analysed environmental and 4% of aborted foetus strains contained complete LIPI-3.

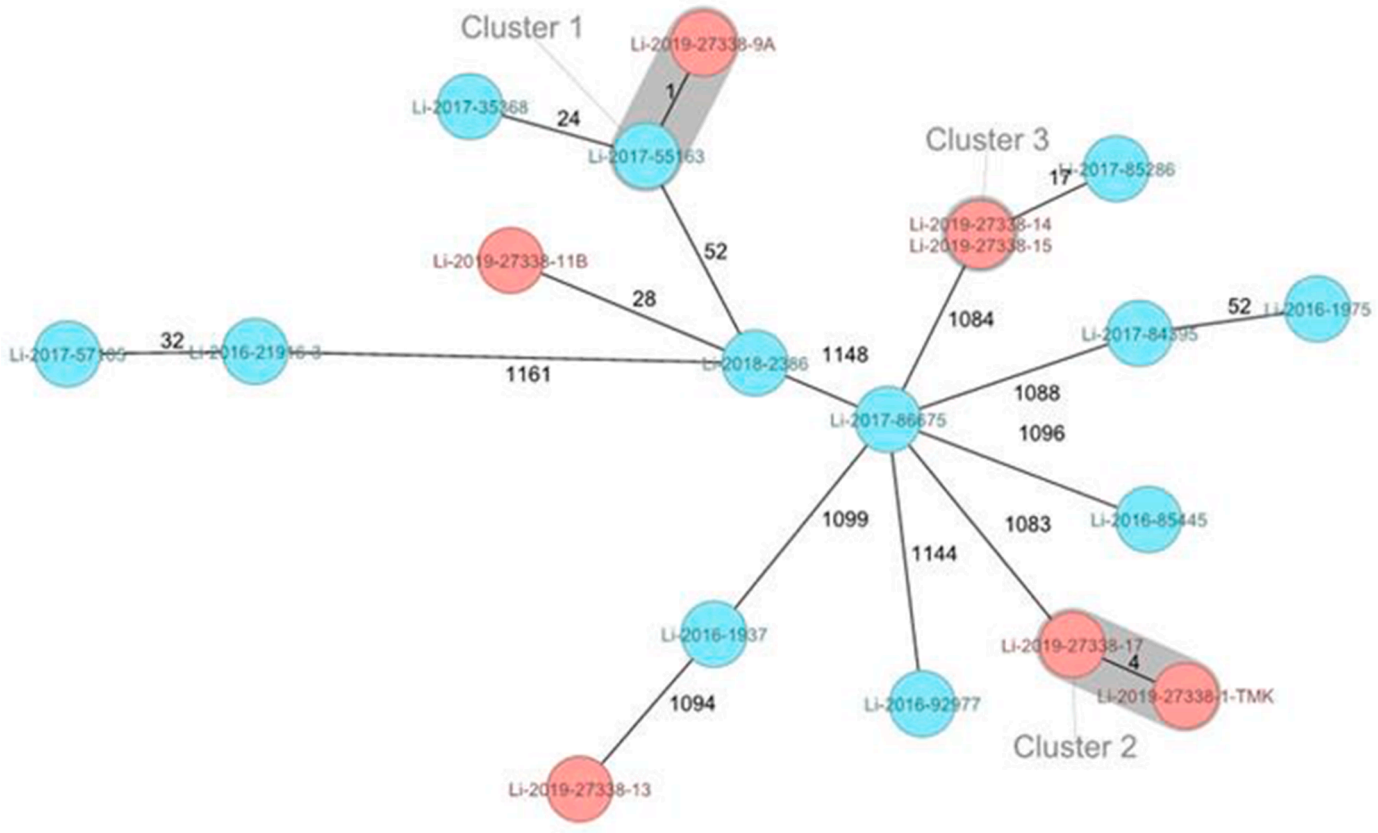


**Fig. 3.** MST includes L. *innocua* isolates of Farm A. Blue represents abortion sample; red – environmental samples such as soil, faeces, feed and water.

Similarly, Clayton *et al*. in 2014 reported 11 of 64 (17%) investigated L. *innocua* strains as LLS positive and observed haemolytic phenotype when llsA was expressed under a constitutive promoter (Klausner & Donnelly, 1991). It has been demonstrated that strains of L. *monocytogenes* can be hypervirulent and hypovirulent and this is due to various virulence factors and their genetic variants (Maury et al., 2016). There are studies of L. *monocytogenes* that LIPI-3 encoded LLS is detected in hypervirulent endemic strains (Cotter et al., 2008). It has a bactericide function; it contributes to colonization of the gut and can modulate host microbiota (Quereda et al., 2017). Further studies of L. *innocua* are necessary to understand the role of LLS and other factors in cattle gut colonization. Our finding could indicate that L. *innocua* has the main virulence factors- *Iap, lpeA, fbpA, lspA, lap, lplA1, gtcA, prsA2* to maintain the pathogenesis and were found in our study in isolates of abortion cases and environment. For example, L. *innocua* isolates also include the *pdgA* and *oatA* genes that protect bacteria against host defenses (Matto et al., 2022). Group of *clpC, clpE* and *clpP* genes are the main stress response mediators and assist with intracellular replication in host cells (Vázquez-Boland et al., 2001). Until now L. *innocua* is assumed to be pathogenic mostly due to presence of L. *monocytogenes* LIPI-1 gene cluster anduntil now, individual risk factors for ruminants are poorly understood. Such atypical strains are rare and can be differentiated by phenotypical haemolysis (Johnson et al., 2004; Moura et al., 2019). However, in our sample set we did not detect LIPI-1 gene cluster or part of it and this was in line with the fact that no haemolytic phenotypes were observed between our isolates. Studying the virulence genes, internalins are surface-exposed virulence factors whose role are recognition of cellular receptors and invasion (Moura et al., 2019).

Moura *et al*. using in vivo model demonstrated virulence and pathogenesis in mice of a *PrfA* cluster and inlA- positive L. *innocua* strain versus negative and L. *monocytogenes* strains (Bolger et al., 2014). They concluded that PrfA-positive strains are virulent but less than L. *monocytogenes*. In that study L. *innocua* contained a partial LIPI-3 cluster. These strains were able to efficiently colonize mice organs such as intestine, spleen and liver and affected survival of zebrafish embryos less than L. *monocytogenes* but more than uninfected groups (Moura et al., 2019). This study focused only on detection of known L. *monocytogenes* virulence factors and further pangenome and genetic characterization in vitro studies between different isolate groups should be continued to identify L. *innocua*-specific virulence genes. Virulencegenes among others detected, could be involved in the ability of L. *innocua* to produce damage in ruminant hosts.

L. *innocua* could be an indicator for unrecognized L. *monocytogenes* contamination and outbreak events. L. *innocua* were more commonly found in environment than L. *monocytogenes* (Terentjeva et al., 2021).

## 5. Conclusions

Our research highlights that L. *innocua* has a pathogenic potential for cattle, indicating its importance in cattle breeding and dairy production chain. L. *innocua* isolates from cattle farm environment and abortion cases showed broad genetic diversity and subsequently the variety in virulence potential.However, more experimental evidence, clinical studies and epidemiological data could support complete understanding of the transmission and pathogenicity of L. innocua.

## Funding

This research was funded by the Latvia Council of Science, grant number Izp-2018/2–0361 “Whole genome-based characterization of environmental Listeria spp. and their role in ruminants listeriosis and public health”.

## Data availability statement

Not applicable

## Supplementary Materials

The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Virulence factors of L. *innocua*

## CRediT authorship contribution statement

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## Declaration of Competing Interest

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

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.vas.2022.100276.

## References


Arumugam, S. K., Govindharaj, K., Subramaniam, A., & Rangasamy, R. (2001). Neonatal Listeria innocua sepsis. *International Journal of Contemporary Pediatrics, 8*(5), 938–940.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics (Oxford, England), 30*(15), 2114–2120.

Bouayad, L., Hamdi, T. M., Naim, M., Leclercq, A., & Lecuit, M. (2015). Prevalence of Listeria spp. and molecular characterization of Listeria monocytogenes isolates from broilers at the abattoir. *Foodborne Pathogens and Disease*, (7), 606–611.

Buchfink, B., Xie, C., & Huson, D. H. (2015). Fast and sensitive protein alignment using diamond. *Nature Methods, 12*(1), 59–60.

Carlin, C. R., Liao, J., Weller, D. L., Guo, X., Orsi, R., & Wiedmann, M. (2021). Listeria cossartiae sp. nov., Listeria farberi sp. nov., Listeria immobilis sp. nov., Listeria portnoyi sp. nov. and Listeria rustica sp. nov., isolated from agricultural water and natural environments. *International Journal of Systematic and Evolutionary Microbiology, 71*(5), Article 004795. Erratum in: Int J Syst Evol Microbiol. 2021 Jun; 71(6).

Chen, M., Cheng, J., Wu, Q., Zhang, J., Chen, Y., Zeng, H., … Ding, Y. (2018). Prevalence, potential virulence, and genetic diversity of Listeria monocytogenes isolates from edible mushrooms in Chinese markets. *Frontiers in Microbiology, 9*, 1711–1722.

Cossart, P. (2011). Illuminating the landscape of host-pathogen interactions with the bacterium Listeria monocytogenes. *Proceedings of the National Academy of Sciences of the United States of America, 108*(49), 19484–19491, 6.

Cotter, P. D., Draper, L. A., Lawton, E. M., Daly, K. M., Groeger, D. S., et al. (2008). Listeriolysin S, a novel peptide haemolysin associated with a subset of lineage I Listeria monocytogenes. *PLoS Pathog, 4*(9). E1000144.

Diriba, K., Awulachew, E., & Diribsa, K. (2021). The prevalence of Listeria species in different food items of animal and plant origin in Ethiopia: A systematic review and meta-analysis. *European Journal of Medical Research, 26*, 60.

Favaro, M., Sarmati, L., Sancesario, G., & Fontana, C. (2014). First case of Listeria innocua meningitis in a patient on steroids and eternecept. *JMM Case Reports, 1*, 1–5.

Freitag, N. E., Port, G. C., & Miner, M. D. (2009). Listeria monocytogenes - from saprophyte to intracellular pathogen. *Nature Reviews Microbiology, 7*(9), 623–628.

Hasman, H., Saputra, D., Sicheritz-Ponten, T., Lund, O., Svendsen, C. A., Frimodt-Moller, N., & Aarestrup, F. M. (2014). Rapid whole-genome sequencing for detection and characterization of microorganisms directly from clinical samples. *Journal of Clinical Microbiology, 52*(1), 139–146.

Johnson, J., Jinneman, K., Stelma, G., Smith, B. G., Lye, D., Ulaszek, J., Evsen, L., Gendel, S., Bennett, R. W., Pruckler, J., Steigerwalt, A., Kathariou, S., Volokhov, D, Rasooly, A., Chizhikov, V., Fortes, E., Duvall, R. E., Hitchins, A. D., Messer, J., et al. (2004). Natural atypical Listeria innocua strains with Listeria monocytogenes pathogenicity island 1 genes. *Applied and Environmental Microbiology, 70*, 4256–4266.

Jünemann, S., Sedlazeck, F. J., Prior, K., Albersmeier, A., John, U., Kalinowski, J., Mellmann, A., Goesmann, A., Von Haeseler, A., Stoye, J., & Harmsen, D. (2013). Updating benchtop sequencing performance comparison, 2013 *Nature Biotechnology, 31*(4), 294–296.

Kaszoni-Rückerl, I., Mustedanagic, A., Muri-Klinger, S., Brugger, K., Wagner, K. H., Wagner, M., & Stessl, B. (2020). Predominance of distinct Listeria innocua and Listeria monocytogenes in recurrent contamination events at dairy processing facilities. *Microorganisms, 8*(2), 234–251.

Klausner, R. B., & Donnelly, C. W. (1991). Environmental sources of Listeria and Yersinia in Vermont dairy plants. *Journal of Food Protection, 54*(8), 607–611.

Liu, B., Zheng, D., Jin, Q., Chen, L., & Yang, J. (2019). VFDB a comparative pathogenomic platform with an interactive web interface. *Nucleic Acids Research, 47* (D1), D687–D692.

Matto, C., D'Alessandro, B., Mota, M. I., Braga, V., Buschiazzo, A., Gianneechini, E., Varela, G., & Rivero, R. (2022). Listeria innocua isolated from diseased ruminants harbour minor virulence genes of L. monocytogenes. *Veterinary Medicine and Science*.

Maury, M. M., Tsai, Y. H., Charlier, C., Touchon, M., Chenal-Francisque, V., Leclercq, A., … Lecuit, M. (2016). Uncovering Listeria monocytogenes hypervirulence by harnessing its biodiversity. *Nature Genetics, 48*(3), 308–313.

Milillo, S. R., Stout, J. C., Hanning, I. B., Clement, A., Fortes, E. D., den Bakker, H. C., Wiedmann, M., & Ricke, S. C. (2012). Listeria monocytogenes and hemolytic Listeria innocua in poultry. *Poultry Science, 91*, 2158–2163.

Moreno, L. Z., Paixão, R., Gobbi, D. D., Raimundo, D. C., Ferreira, T. P., Hofer, E., Matte, M. H., & Moreno, A. M. (2012). Characterization of atypical Listeria innocua isolated from swine slaughterhouses and meat markets. *Research in Microbiology, 163* (4), 268–271.

Moura, A., Disson, O., Lavina, M., et al. (2019). Atypical hemolytic Listeria innocua isolates are virulent, albeit less than Listeria monocytogenes. *Infection and Immunity, 87*(4), E00758–18.

Orsi, R. H., & Wiedmann, M. (2016). Characteristics and distribution of Listeria spp., including Listeria species newly described since 2009. *Applied Microbiology and Biotechnology, 100*(12), 5273–5287.

Perrin, M., Bemer, M., & Delamare, C. (2003). Fatal case of Listeria innocua bacteremia. *Journal of Clinical Microbiology, 41*(11), 5308–5309.

Prjibelski, A., Antipov, D., Meleshko, D., Lapidus, A., & Korobeynikov, A. (2020). Using SPAdes de novo assembler. *Current Protocols in Bioinformatics, 70*(1), 1–29.

Quereda, J. J., Nahori, M. A., Meza-Torres, J., Sachse, M., Titos-Jiménez, P., Gomez-Laguna, J., Dussurget, O., Cossart, P., & Pizarro-Cerdá, J. (2017a). Listeriolysin S Is a streptolysin S-like virulence factor that targets exclusively prokaryotic cells in vivo. *mBio, 8*(2). E00259-17.

Quereda, J. J., Nahori, M. A., Meza-Torres, J., Sachse, M., Titos-Jiménez, P., Gomez-Laguna, J., Dussurget, O., Cossart, P., & Pizarro-Cerdá, J. (2017b). Listeriolysin S is a streptolysin S-like virulence factor that targets exclusively prokaryotic cells in vivo. *mBio, 8*(2). E00259-17.

Rocha, C. E., Mol, J. P. S., Garcia, L. N. N., Costa, L. F., Santos, R. L., & Paixão, T. A. (2019). Comparative experimental infection of Listeria monocytogenes and Listeria ivanovii in bovine trophoblasts. *PloS one, 12*(5), E0176911–21.

Rocha, P. R. D. A., Dalmasso, A., Grattarola, C., Casalone, C., Del Piero, F., Bottero, M. T., & Capucchio, M. T. (2013). Atypical cerebral listeriosis associated with Listeria innocua in a beef bull. *Research in Veterinary Science, 94*, 111–114.

Ruppitsch, W., Pietzka, A., Prior, K., Bletz, S., Fernandez, H. L., Allerberger, F., Harmsen, D., & Mellmann, A. (2015). Defining and evaluating a core genome multilocus sequence typing scheme for whole-genome sequence-based typing of Listeria monocytogenes. *Journal of Clinical Microbiology, 53*(9), 2869–2876.

Terentjeva, M.; Šteingolde, Ž.; Meistere, I.; Elferts, D.; Avsejenko, J.; Streikiša, M.; Gradovska, S.; Alksne, L.; Ķibilds, J. & Bērziņš, A. (2021). Prevalence, genetic diversity and factors associated with distribution of Listeria monocytogenes and other Listeria spp. in cattle farms in Latvia. *Pathogens*.

Vázquez-Boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J., & Kreft, J. (2001). Listeria pathogenesis and molecular virulence determinants. *Clinical Microbiology Reviews*, (3), 584–640.

Wood, D. E., Lu, J., & Langmead, B. (2019). Improved metagenomic analysis with Kraken 2. *Genome Biology, 20*(1), 257–270.

Zhao, Qiang, Hu, Pan, Li, Qianqian, Zhang, Shasha, Li, Hanxiao, Chang, Jiang, Jiang, Qiujie, Zheng, Yu, Li, Yansong, Liu, Zengshan, Ren, Honglin, & Lu, Shiying (2021). Prevalence and transmission characteristics of Listeria species from ruminants in farm and slaughtering environments in China. *Emerging Microbes & Infections, 10*(1), 356–364.