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Occurrence and diversity of *Listeria monocytogenes* in Portuguese dairy farms

Bárbara Nunes [a,b], Ana Rita Barata [a,b,c], Ricardo Oliveira [a,d], Hugo Guedes [a], Carina Almeida [a,d,e], Gabriela Jorge da Silva [f], Teresa Nogueira [a,g], Maria José Saavedra [b,c,h], Gonçalo Almeida [a,i,*]

[a] National Institute for Agricultural and Veterinary Research (INIAV), I.P., Vila do Conde, Portugal
[b] Veterinary Sciences Department, Antimicrobials, Biocides & Biofilms Unit, University of Trás-os-Montes and Alto Douro (UTAD), Vila Real, Portugal
[c] School of Life Science and Environment,Escola de Ciências da Vida e do Ambiente, Universidade de Trás-os-Montes e Alto Douro University of Trás-os-Montes and Alto Douro (UTAD), Vila Real, Portugal
[d] Laboratory for Process Engineering, Environment, Biotechnology and Energy (LEPABE), Porto, Portugal
[e] Centre of Biological Engineering (CEB), University of Minho, Braga, Portugal
[f] Faculty of Pharmacy and Center for Neurosciences and Cell Biology, University of Coimbra, Coimbra, Portugal
[g] Centre for Ecology, Evolution and Environmental Changes (cE3c) & Global Change and Sustainability Institute (CHANGE), Faculdade de Ciências da Universidade de Lisboa, Lisboa, Portugal
[h] Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB) and Institute for Innovation, Capacity building and Sustainability of Agri-Food production (Inov4Agro), University of Trás-os-Montes e Alto Douro, Vila Real, Portugal
[i] Centre for Study in Animal Science (CECA), ICETA, University of Porto, Porto, Portugal

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ABSTRACT

Listeria monocytogenes is a pathogenic microorganism that causes listeriosis, an infection that usually occurs after consumption of contaminated food and is considered particularly dangerous due to its ability to grow and multiply under adverse conditions. In recent years, there has been an increase in the consumption of unprocessed products, such as raw milk and dairy products, by people of all ages, including those with compromised immune systems, which could lead to an increase in foodborne illness. Ruminants play a very important role in the persistence and transmission of *L. monocytogenes* through a continuous oral-faecal cycle. Therefore, farms are considered a reservoir of this microorganism and are involved in the transmission from animals to humans. In this study, samples of faeces, milk, water, silage, feed and teat cups swabs were collected from 8 farms to assess the distribution of the pathogen in the farm environment. Milk samples were also collected from 100 dairy farms to assess the risk associated with the consumption of raw milk. Detection was performed by real-time PCR, while preparation, enrichment and confirmation were performed according to ISO 11290–1, (2017). The prevalence in water was 8.3%, in faeces 12.5% and in feed 12.0%, while in the other samples the microorganism was not detected. It was also observed that this microorganism was more abundant in spring months. The eight isolates were serotyped by real-time PCR and the most frequent serogroup was IVb with 5 isolates (2 of which were IVb-v1) and the remaining 3 were IIb. Two of the clonal complexes (CCs) identified were shared by two isolates (CC 213 and CC 217), the remaining CCs identified (CC 392, CC 554, CC 489, CC 224 and CC 183) were not identified in more than one isolate. This study contributed to a better understanding of the ecology of *L. monocytogenes* in dairy farms, showing that most of the clones found in food were not present in this environment and that genes coding for disinfectants and heavy metals were not detected.

1. Introduction

Listeria monocytogenes is a facultative anaerobic, non-spore forming, Gram-positive bacterium that can grow under unfavourable conditions, resulting in high concentrations in food, making it a public health problem (Evans et al., 2021). Based on somatic (O) and flagellar (H)

* Correspondence to: Instituto Nacional de Investigação Agrária e Veterinária (INIAV), I.P., Pólo de Vairão - Vila do Conde, Portugal.
E-mail address: goncalo.almeida@iniav.pt (G. Almeida).

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antigens, 13 serotypes of *L. monocytogenes* have been described, which can be classified into 4 geno-serogroups, IIa, IIb, IIc and IVb (Varsaki et al., 2022). Atypical strains that do not ferment rhamnose and cannot be recognised by the common Doumith PCR serotyping method were reported as serovar 4 h that is the 14th serotype of *L. monocytogenes* by (Yin et al., 2019). Human listeriosis are caused in 95% of cases by serotypes 1/2a, 1/2b or 4b (Burall et al., 2017). In addition, serotype 4b isolates are responsible for about 50% of human listeriosis cases (Doumith et al., 2005). A variant of serogroup IVb has been discovered and named IVb-v1 (Leclercq et al., 2011). Listeriosis is mainly associated with the consumption of contaminated food and has a high mortality rate (20–30%). It is particularly dangerous for immunocompromised individuals, with symptoms ranging from diarrhoea, vomiting and gastroenteritis to fever, septicaemia and meningitis in more severe cases (Varsaki et al., 2022; Terentjeva et al., 2021; Bashiry et al., 2022; Disson et al., 2021). Since 2008, listeriosis has been a notifiable disease in humans in most EU Member States, but not in animals (International Organization of Standardization ISO, 2017; Magalhães et al., 2015). Animals, especially ruminants, play a very important role in the transmission of *L. monocytogenes*, with transmission via faecal-oral contamination, and farms are therefore considered reservoirs of this microorganism (Šteingolde et al., 2021; Sharifzadeh et al., 2015; Borucki et al., 2004). Most cases of listeriosis on dairy farms are thought to originate from poorly fermented silage, but contamination of raw milk has been linked to faecal contamination during milking or inefficient cleaning of equipment due to the ability of this microorganism to form biofilms (Leclercq et al., 2011; Borucki et al., 2005; Nightingale et al., 2004).

This microorganism does not survive the pasteurisation process, but there is a possibility of contamination after heat treatment, yet the consumption of unpasteurised milk is increasing and, if contaminated, will contribute to an increase in the number of cases of listeriosis (Castro et al., 2018; Lundén et al., 2004). In addition, *Listeria* can enter food processing facilities through raw milk (Almeida et al., 2013).

The general objective of this work was to evaluate the occurrence of *L. monocytogenes* in raw cow's milk samples and to investigate possible transmission routes and sources in the dairy farm environment. Two studies were therefore carried out. The first concerns the epidemiological analysis of *L. monocytogenes* in raw cow's milk collected from dairy farms in the northern region of Portugal. The second study aims to understand the potential impact of climate on the epidemiology of *L. monocytogenes* in the dairy farm environment. Further characterization of isolates will include geno-serotyping, in silico methods to assess antimicrobial resistance (AMR) genes, virulence markers and adaptation genes derived from whole genome sequencing (WGS).

2. Materials and methods

2.1. Sampling

Two parallel studies (A and B) were conducted over 10 months, from November 2020 to September 2021, in randomly selected dairy farms in the main dairy basin of mainland Portugal, known as Entre-Douro e Minho.

In study A, the occurrence of *L. monocytogenes* in raw milk samples was determined by collecting milk from bulk cooling tanks of 100 dairy farms (n = 100).

In study B, different types of samples were collected from eight dairy farms to assess the occurrence/distribution of *L. monocytogenes* in the farm environment and its seasonal variation. Samples included bulk tank milk, untreated tap water, mixed feed and hay from the manger, cattle faeces from the barn floor, teat cup surfaces collected with viscose swab sticks and grass or maize or paston silage from the silos in use, for a total of 158 samples, Table 2. The first set of samples (Round 1) was collected between November 2020 and January 2021, the second (Round 2) from April to May 2021 and the third (Round 3) in early September 2021.

Table 1
List of primers and probes used in the Real Time PCR method for serotyping of *L. monocytogenes*.

Gene	Sequence (3'→5')
prs FWR	CAGGRTTACTCGTTGATTGAATAAC
prs RVR	GCTGAAGAGATTGCGAAAGAAG
prs Probe	CATGACAACCACGGATACTTTCTTCAATGTTAATTTG
plcA FWR	CGGCGCACCTAACCAAGTAA
plcA RVR	CAGTCTGGACAATCTCTTTGAATTTT
plcA Probe	TCAAGATGACTACAATGGTCCGAGTGTGAAAA
Lmo0737 FWR	GCATCTTGTTTAGCAAGTGGATC
Lmo0737 RVR	GAGCACGGAAGTTGCTAGGT
Lmo0737 Probe	CCAACACTTTCTCATCAATACCATCTTCCC
Lmo1118 FWR	CTTAGTATTCCAGGATTTAAGACC
Lmo1118 RVR	CCAAAGAACCAAATTGATCGAATC
Lmo1118 Probe	CCTTTATCTTCTCCTGAGTGTATACGCCTC
ORF2110 FWR	CACTAATCTCATCGACTATAAACTC
ORF2110 RVR	TGCACAAGCAGCAGAGGAAG
ORF2110 Probe	TCTCCGTCATTTGTTACCGTTTCCCCAAC
ORF2819 FWR	ATCACTAAAGCCTCCCATTGAG
ORF2819 RVR	GGAAGATTTCCACGCAATACTC
ORF2819 Probe	CTCGTAAGATCGATATACGTCATGGCAGTTTCC
pUC19 FWR	GCAGCCACTGGTAACAGGAT
pUC19 RVR	GCAGAGCGCAGATACCAAAT
pUC19 Probe	AGAGCGAGGTATGTAGGCGG

All samples were made available by the producers solely for this study. Samples were transported to the laboratory in portable insulated cold-boxes and the presence of *L. monocytogenes* was examined within 24 h. The farmers were asked about the occurrence of cases of bovine listeriosis on the farm, and all of them reported that there had never been a case of bovine listeriosis on the farm.

2.2. Sample preparation and detection of Listeria monocytogenes

Samples were prepared according to ISO 11290–1 (International Organization of Standardization ISO, 2017) and the detection of *L. monocytogenes* was assessed by real-time PCR.

Samples were processed as follows. One litre of water was filtered through sterile, 0.45 μm pore size, 47 mm cellulose acetate filters (VWR International, Vila Nova de Gaia, Portugal) and the filters were transferred to 100 ml Hal-Fraser broth (Bio-Rad, California, USA). Twenty-five grams of milk, feed or faeces were added to 225 ml of Hal-Fraser broth (Bio-Rad, California, USA) and homogenised in a Stomacher (Seward, West Sussex, United Kingdom) for 2 min, while environmental swab sticks (Sarsted, Germany. Ref. 80.1361) were added directly to 10 ml of Hal-Fraser broth (Bio-Rad, California, USA). Inoculated Half-Fraser broths were incubated for 24 h at 30 ± 1 °C.

Detection of *L. monocytogenes* was performed using the commercial iQ-Check *Listeria monocytogenes* II PCR Detection Kit (Bio-Rad, California, USA) according to the manufacturer's instructions. Briefly, 1.5 ml of the enrichment broth was transferred to a microcentrifuge tube and centrifuged at 14,000 rpm for 5 min. The supernatant was discarded and 250 μl of lysis reagent was added to the pellet and incubated at 98 °C for 15 minutes. The lysate was centrifuged at 14,000 rpm for 5 min, and 5 μl of the supernatant was used as DNA template for real-time PCR detection in a final volume reaction of 50 μl. Thermal profiling started with a cycle of 95 °C for 10 min, followed by 50 cycles of 95 °C for 25 s, 58 °C for 30 s and 72 °C for 40 s, with signal readings.

2.3. Isolation and confirmation of Listeria monocytogenes

Positive real-time PCR results were confirmed according to the ISO 11290–1 standard (International Organization of Standardization ISO, 2017). Half-Fraser broth (Bio-Rad, California, USA) was streaked onto Ottaviani and Agosti *Listeria* agar (ALOA, Bio-Rad, California, USA) and incubated at 37 ± 1 °C for a total of 48 ± 2 h, however, if possible the

Table 2
Distribution per round of type of samples collected in each dairy farm for the detection of *L. monocytogenes*.

	No. of positive samples for *Listeria monocytogenes* / total samples collected																							
	Feed (n=25)			Silage (n=42)									Bulk milk (n=24)			Water (n=24)			Swab milking machine (n=19)			Faeces (n=24)		
				Grass (n=16)			Corn (n=24)			Pastone (n=2)														
	Round 1	Round 2	Round 3	Round 1	Round 2	Round 3	Round 1	Round 2	Round 3	Round 1	Round 2	Round 3	Round 1	Round 2	Round 3	Round 1	Round 2	Round 3	Round 1	Round 2	Round 3	Round 1	Round 2	Round 3
Farm 1	0/1	0/1	0/1	0/0	0/0	0/1	0/1	0/1	0/1	0/0	0/0	0/0	0/1	0/1	0/1	0/1	0/1	0/1	0/0	0/1	0/0	0/1	0/1	0/1
Farm 2	0/1	2/2 LMV196 and LMV197	0/1	0/0	0/0	0/1	0/1	0/2	0/0	0/0	0/0	0/0	0/1	0/1	0/1	0/1	1/1 LMV183	0/1	0/1	0/1	0/0	0/1	1/1 LMV198	0/1
Farm 3	0/1	1/1 LMV195	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/0	0/1	0/0	0/1	0/1	0/1	0/1	1/1 LMV194	0/1	0/1	0/1	0/0	0/1	0/1	0/1
Farm 4	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/0	0/0	0/0	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Farm 5	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/0	0/0	0/0	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Farm 6	0/1	0/1	0/1	0/0	0/0	0/0	0/1	0/1	0/1	0/0	0/0	0/0	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/0	0/1	0/1	1/1 LMV199	0/1
Farm 7	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/0	0/0	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Farm 8	0/1	0/1	0/1	0/1	0/0	0/1	0/1	0/1	0/1	0/0	0/0	0/0	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	1/1 LMV181	0/1	0/1
Total	0/8	3/9	0/8	0/5	0/4	0/7	0/8	0/9	0/7	0/1	0/1	0/0	0/8	0/8	0/8	0/8	2/8	0/8	0/7	0/7	0/5	1/8	2/8	0/8
	3/25 (12%)			0/16 (0.0%)			0/24 (0.0%)			0/2 (0.0%)			0/24 (0.0%)			2/24 (8.3%)			0/19 (0.0%)			3/24 (12.5%)		

LMV181, LMV183, LMV194, LMV195, LMV196, LMV197, LMV198 and LMV199 correspond to the isolate code.

incubation was stopped earlier if presumptive colonies were evident. Suspect colonies were plated on tryptone soy yeast extract agar (TSYEA, Bio-Rad, California, USA) and incubated at 37 °C for 18–24 h. Biochemical confirmation was performed by Gram staining, catalase, haemolysis and carbohydrate fermentation.

2.4. Listeria monocytogenes genoserotype identification

Up to five colonies of *L. monocytogenes* grown on TSYEA were used for DNA extraction. The colonies were suspended in a microcentrifuge tube containing 250 µl of lysis reagent buffer (NYZtech, Lisboa, Portugal) and placed at 98 °C for 15 min. It was then centrifuged at 14,000 rpm for 5 min and 5 µl of the supernatant was used for multiplex real-time PCR.

Geno-serotyping was performed by real-time PCR following the method of (Vitullo et al., 2013). A final volume of 25 µl for the reaction mix was prepared using 2.5 µl of supernatant combined with SsoAdvanced Universal Probes Supermix (Bio-Rad, California, USA) (1×) and 0.3 µl of primers and probes (Table 1) with an initial concentration of 20 µM. The PCR conditions were as follows: an initial step at 50 °C for 2 min; 10 min at 95 °C, 39 cycles at 95 °C for 15 sec and 60 °C for 1 min in a thermocycler. The CFX96 Touch DeepWell real-time PCR detection system (Bio-Rad, California, USA) was then used.

2.5. Whole genome sequencing of Listeria monocytogenes isolates

Genomic DNA was extracted and purified using NZY Microbial gDNA Isolation Kit (NZYTech - MB21702, Lisbon, Portugal) and was sent to Eurofins Genomics, Germany (https://eurofinsgenomics.eu/) for whole genome sequencing using the Illumina platform (WGS).

The fastp 0.23.3 software (Chen, 2023; Chen et al., 2018) was used to analyse the quality of the reads and to process the FastQ files of the raw reads. All files had a quality of more than 99% and were passed on to the next step. The genomes were assembled with SPAdes v3.13.1 (Prjibelski et al., 2020). The assembly quality metrics were performed using QUAST v5.2.0 (Mikheenko et al., 2018) and genomes were selected with less than 300 contigs and a genome size between 2.7e6 and 3.2e6. Interspecies contamination was checked using ConFindr 0.8.1 (Low et al., 2019) and refseq.genomes.k21s1000.msh (https://gembox.cbcb.umd.edu/mash/refseq.genomes.k21s1000.msh, accessed on 15–05–2023). All genomes had a contamination status of 'false' and belonged to the genus *Listeria* and were therefore selected. We also searched for the nearest reference genome using Mash 2.2.2 software (Ondov et al., 2016) and the same matrix to verify that it was a *Listeria monocytogenes* genome. Serogroup type prediction was performed using LisSero 0.4.9 (Kwong et al.) and sequence type, clonal complex, and lineage were obtained for each genome by mining the listeria_profile.txt of the stringMLST *Listeria monocytogenes* dataset (https://github.com/jordanlab/stringMLST_datasets/blob/cfa508771d65615216a846c148fc0c40719e1042/Listeria_monocytogenes.zip, accessed on 02–01–2023). Sublineages (SLs) and core genome MLST types (CTs) were determined according to the *Listeria* sequence typing database and its tools available on the BIGSdb-Lm platform (https://bigsdb.pasteur.fr/listeria, accessed on 20–09–2023 and 20–12–2023).

ABRicate 1.0.1 8 (https://github.com/tseemann/abricate) was used to screen the contigs of all genomes for antimicrobial resistance genes using the NCBI AMRFinderPlus (Feldgarden et al., 2019), ARG-ANNOT (Gupta et al., 2014), Resfinder (Zankari et al., 2012), CARD (Jia et al., 2017) and MEGARES 2.00 databases (Doster et al., 2020) (all accessed on 25–07–2023 and 22–11–2023), for virulence genes the VFDB database (Chen et al., 2016) (accessed on 25–07–2023) and BIGSdb-Lm platform (https://bigsdb.pasteur.fr/listeria, accessed on 20–09–2023) were used.

All the above procedures were carried out in a Linux environment, using in-house developed bash scripts, except for BIGSdb-Lm platform.

Table 3
Genomic characteristics of isolates of *L. monocytogenes* from dairy farms.

Farm	Isolate	Source type	Lineage	Serogroup	Clonal complex (MLST)	Sequence type (MLST)	Sublineage (cgMLST)	cgMLST type (cgMLST)	Antimicrobials resistance determinants	Stress adaptation and resistance to disinfectants	Pathogenecity Islands	internalins
2	LMV183	Water	I	IVb	CC213	ST213	SL213	CT1846	fosX, lin, mprF, mdrL, norB, sul	*sigB*	LIPI-1, LIPI-3, LIPI-4	inlABCEFHJK
2	LMV196	Feed	I	IIb	CC224	ST224	SL224	CT14280	fosX, lin, mprF, mdrL, norB, sul	sigB, SSI-1	LIPI-1, LIPI-3	inlABCEFHJK
2	LMV197	Feed	I	IVb	CC217	ST217	SL217	CT14279	fosX, lin, mprF, mdrL, norB, sul	*sigB*	LIPI-1, LIPI-3, LIPI-4	inlABCEFHJK
2	LMV198	Feces	I	IVb	CC217	ST217	SL217	CT14279	fosX, lin, mprF, mdrL, norB, sul	*sigB*	LIPI-1, LIPI-3, LIPI-4	inlABCEFHJK
3	LMV194	Water	I	IVbv1	CC554	ST554	SL555	CT501	fosX, lin, mprF, mdrL, norB, sul	*sigB*	LIPI-1, LIPI-3	inlABCEFHJK
3	LMV195	Feed	I	IIb	CC489	ST489	SL489	CT14685	fosX, lin, mprF, mdrL, norB, sul	sigB, SSI-1	LIPI-1, LIPI-3	inlABCEFHJK
6	LMV199	Feces	I	IVbv1	CC183	ST382	SL382	CT14281	fosX, lin, mprF, mdrL, norB, sul	*sigB*	LIPI-1, LIPI-3, LIPI-4	inlABCEFHJK
8	LMV181	Feces	I	IIb	CC392	ST392	SL392	CT14282	fosX, lin, mprF, mdrL, norB, sul	*sigB*	LIPI-1	inlABCEFHJK

Table 4
Antimicrobials resistance detected genes according to platforms used.

Platform	Resistance genes detected
ARGANNOT	*Lin*
CARD	*fosX, lin, mprF, norB*
MEGARES	*fosX, lin, mprF, mdrL, norB*
NCBI	*fosX, lin*
Resfinder	*fosX*
BIGS db	*fosX, mprF, lin, norB, sul*

fosX – lmo1702 gene encoding for Fosfomycin resistance protein FosX.
lin – lmo0919 gene for lincomycin resistance.
mprF – lmo1695 gene encoding MprF membrane protein that modifies the membrane surface conferring resistance against cationic antimicrobial peptides.
norB – lmo2818 gene encoding for NorB, a multidrug efflux pump conferring confers resistance to fluoroquinolones and other structurally unrelated antibiotics like tetracycline.
sul – lmo0224 gene encoding forms of dihydropteroate synthase that confer resistance to sulphonamide.
mdrL – gene encoding for MdrL efflux pump that contributes to the detoxification of antibiotics and heavy metals.

3. Results

3.1. Occurrence of Listeria monocytogenes

Regarding study A, occurrence in raw milk samples, *L. monocytogenes* was not detected in 25 ml in any of the 100 raw milk samples collected from bulk cooling tanks at dairy farms.

Regarding study B, *L. monocytogenes* was detected in 4 out of 8 farms. The positive samples for *L. monocytogenes* were tap water (8.3%) from 2 farms, faeces (12.5%) from 3 farms and feed trough samples (12.0%) from 2 farms, Table 2.

In relation to temporal patterns, most isolates (7 out of 8) were obtained from samples collected during spring months, with only one obtained from samples collected in the autumn/winter period. Table 2 provides a summary of the sample types, those testing positive for *L. monocytogenes*, and the corresponding collection rounds.

3.2. Genomic characteristics of isolates

The results of the genomic characteristics of the eight *L. monocytogenes* isolates are shown in Table 3. Genoserotyping grouped the isolates as IVb (n = 3), IVbv1 (n = 2) and IIb (n = 3). Different serotypes were found in feed samples from farm 2 (IVb and IIb). At farm 3, *L. monocytogenes* isolates from faeces and water samples also showed different serogroups (IIb and IVbv1). MLST identified seven clonal complexes (CC). In farm 2, CC 213 was found in the water sample, CC 224 was found in the feed and CC 217 was found in the faeces and in other feed sample. In farm 3, CC489 was found in the feed and CC 554 in the water. The faecal positive samples found in farm 6 and farm 8 contained CC 183 and CC 392 respectively. None of the CCs found were shared between farms.

3.3. Virulence, stress adaptation, resistance to disinfectants and to antimicrobials genes

Homologues of the virulence factor genes *actA*, *hlyA*, *inlA*, *inlB*, *inlC*, *mpl*, *plcA*, *plcB* and *prfA* were identified in all isolates. All isolates had homologues of the internalins genes *inlC2*, *inlD*, *inlE*, *inlF*, *inlH*, *inlJ* and *inlK*.

LIPI-3 genes were identified in all isolates except LMV181. LIPI-4 homologues genes were detected in the IVb isolates LMV183, LMV197 and LMV198 and in the IVbv1 isolate LMV199.

The stress survival islet–1 (SSI-1) was identified in the IIb isolates LMV195 and LMV196. The *sigB* operon, composed of *bsh* that encodes bile salt hydrolase, and gene *pdgA* that encodes for lysozyme resistance were detected in all isolates. The genes *brcABC* that are related to resistance to benzalkonium chloride were not detected in any isolate but homologues to *mdrL* that encode to an efflux pump were detected in all isolates.

The effectiveness of different platforms in detecting the presence of AMR genes varies, as shown in Table 4. For example, while one platform was able to detect a single gene, other platforms detected up to 5 genes. The existence of the *mprF* and *mdrL* genes, which code for efflux pumps, can be associated with resistance to antimicrobial agents such as macrolides, cefotaxime and other types of antibiotics that are not expressed in the genes. When gathering the results of the different platforms all the isolates have a common resistance genotype to at least six antimicrobial agents.

4. Discussion

L. monocytogenes is naturally present throughout the environment, particularly in soil and on vegetation, and is pathogenic to humans and animals. Listeriosis is one of the deadliest foodborne infections in the EU, particularly in the elderly. Data in animals and feed are generated by non-harmonised surveillance systems as there is no reporting obligation for EU Member States. The available data on the occurrence of *L. monocytogenes* in feed are only collected through clinical investigations in farm animals (EFSA Journal, 2022). *L. monocytogenes* has the potential to multiply at low pH, variable salt concentrations and low temperatures at different stages of food processing (Townsend et al., 2021). In addition, the organism is a public health hazard and readily grows on fresh produce, raw meat, milk, fish and vegetables (Prasad et al., 2023).

Contamination of raw milk can be due to poor hygiene practices, the feed given to the cows, in which the bacterium can multiply, and the water supply. The presence of the pathogen in the environment where the milk is stored may allow the formation of biofilms, a source of contamination reported by Harvey et al. (2007) and Liu et al. (2002).

Previous studies have shown that the incidence of this microorganism in raw milk samples can vary between 2.2% and 16.0% (Castro et al., 2018; Husu, 1990; Vilar et al., 2007; Mohammed et al., 2009; Hag et al., 2021). In the present study, the bacterium was not detected in a total of 100 raw milk samples from the main milk production zone in Portugal. Our occurrence levels may be due to good hygiene practices by farmers, which prevent contamination by faeces or water, and effective disinfection of milking equipment and the udder of the animal during milking. Good hygiene also prevents the formation of biofilms on milking equipment and milk tanks (Matto et al., 2018).

In study B, eight dairy farms were visited to test for *L. monocytogenes*. The aim was to understand the characteristics of the isolates in terms of virulence and adaptation. This research was driven by previous studies in different cheese producers that pointed to milk as a possible source of cheese contamination (Almeida et al., 2013).

The examination of animal's drinking water was part of the analysis due to its potential role as a source of *Listeria* ingestion. In the United States of America (USA) (Mohammed et al., 2009), the prevalence in water trough samples was 66%, in Latvia (Terentjeva et al., 2021) 10% in drinking troughs and in Sudan (Hag et al., 2021) *L. monocytogenes* was detected in 3.3%. The 8.3% prevalence discovered in this study raises significant concern. The water samples were collected from the tap closest to the caption point, indicating that the micro-organism is in the groundwater and will continuously enter the farm, as evidenced by the results from farm 2 and 3. Water can carry various pathogenic bacteria, virus, or parasites capable of causing infectious diseases in cattle. To mitigate the risk of contamination-related infections, it is essential to implement a disinfection protocol. Water plays a crucial role in cleaning farm facilities, such as milking rooms and equipment. However, rinsing surfaces with contaminated water after cleaning could inadvertently

facilitate the establishment of micro-organisms, potentially leading to their transfer from surfaces to milk. Water in neighbouring areas might also be contaminated, posing a risk when used for irrigation as it could spread the micro-organism to the horticultural products being cultivated.

Silage are one of the sources of infections in cattle. Studies assessing the presence of *L. monocytogenes* in silages have reported varying prevalence rates, ranging from0.8% to 38% (Terentjeva et al., 2021; Castro et al., 2018; Husu, 1990; Vilar et al., 2007; Mohammed et al., 2009; Hag et al., 2021; Ho et al., 2007). However, this study did not detect *L. monocytogenes* in any of the silage samples examined. In general, *L. monocytogenes* is detected in poorly fermented silages with a pH above 5.5 (Borucki et al., 2004, 2005). The pH measured in this study ranged between 3.6 and 5, generally, indicating good fermentation. It is known that *Listeria* does not survive at pH below 4 (Bashiry et al., 2022), thus some of the analysed silages can allow the survival of *L. monocytogenes*, or its growth might occur if present in very low levels. Even when good silage-making practices are followed, exposure to oxygen, insects, birds, and potential mold development upon opening can alter its composition. This alteration may lead to alkalization, providing favourable conditions for the growth of microorganisms, which, if present, could be ingested by cattle. In this study silages were "in use" to feed animals and produced with the use of inoculants as starters. These inoculants accelerate pH reduction and increase lactic acid levels. If the strains of inoculants also produce bacteriocins, this could enhance the control of Listeria in both silages and the cattle rumen.

The current study found that 12% of feed samples collected from mangers tested positive. As reported in other studies it can be another source of *L. monocytogenes* ingestion. In Latvia, 29% of samples of mixed feed, including silage and other feed, taken from the feed trough were positive (Terentjeva et al., 2021), and in the USA, the study that found a high number of contaminated water samples described an incidence of 65% in samples taken from the feed trough (Mohammed et al., 2009). In a Spanish study (Varsaki et al., 2022), on farms, 80% of mixed feed samples were positive, the authors also found the microorganism in dry fodder (24%), fresh grass (46%), corn-based fodder (27%), concentrate for dairy cows (29%), showing that feed is a major concern and the risk of listeriosis outbreaks is increased with this high prevalence of *Listeria* compared to our study. Another Spanish study (Palacios-Gorba et al., 2021) showed similar results to our study with 11% of positive samples. Interestingly, is that samples that tested positive were taken on the same round as water samples that also tested positive. However, contaminating strains were different. Mixed feed can include a variety of components such as: silage, fresh grass if available, hay, protein concentrate, etc. some of them more susceptible to carry *L. monocytogenes* than others. The resultant product exhibits different pH values, water activity values, and humidity levels that can create favourable conditions for *Listeria* development. Moreover, the cow itself, as well as insects and other animals like birds or rodents, can serve as vehicles for *Listeria*, potentially contaminating the feed at the manger. Hygiene practices play a crucial role in reducing microbial loads. Implementing measures to enhance cleaning and disinfection of troughs and mangers, as well as disinfecting water sources, could decrease Listeria ingestion by animals and mitigate the spread of the microorganism in the environment.

In this study, 12.5% of the faecal samples tested positive for *L. monocytogenes* Other studies have reported prevalence rates ranging from 2.6% to 43% (Terentjeva et al., 2021; Castro et al., 2018; Townsend et al., 2021; Harvey et al., 2007; Liu et al., 2002; Tsaloumi et al., 2021). The presence of this microorganism in faeces is not unexpected, as it can occur in healthy animals or asymptomatic carriers (Vilar et al., 2007; Mohammed et al., 2009). *L. monocytogenes* frequently inhabits the gut of healthy, asymptomatic ruminants, posing a significant challenge to ensuring food safety. While measures are in place to prevent visibly ill animals from entering milk and meat production chains, the sporadic faecal shedding by asymptomatic carriers can go undetected in both animals and humans (Schoder et al., 2022). Faecal shedding poses a risk for contamination of agricultural environments and animal feed (e.g., through fertilization of fields used to harvest plants for silage preparation), thereby contributing to maintenance of infection cycles in farm ruminants (Ho et al., 2007). This practice is prevalent in the region, where dairy farmers typically prepare surrounding fields for grass planting, followed by maize cultivation for maize silage to feed animals. Ho and his colleagues (Ho et al., 2007) studied day-to-day variability of *L. monocytogenes* faecal shedding in a dairy farm. They concluded that faecal shedding varied considerable over time and was associated with the ingestion of contaminated of silage. Shedding of L. monocytogenes can elevate the concentration of the microorganism in the barn environment, increasing the risk of infection among other animals. In one farm, the same strain was found in both feed and faeces, while in other farms where faecal samples tested positive, establishing a direct link was not feasible, similar to sporadic cases of human listeriosis that often cannot be linked to the consumption of a specific food product. Further extensive data sampling is necessary to identify sources of contamination and infection routes.

In a study conducted to evaluate the seasonality of *Listeria* spp. in dairy farms in Turkey (Atil et al., 2011), it was found that the incidence was higher in spring with 8.7% and lower in summer with 2.9%, which agrees with the results of this study. Controversially, the Spanish colleagues (Palacios-Gorba et al., 2021) concluded that the prevalence was higher in winter than in autumn. In Finland (Lyautey et al., 2007), in a study dealing with the seasonality of this microorganism in cattle faeces, it was observed that the occurrence was higher in the months when the animals were in the barn (October-April) than in the grazing months (May-September). Our results are consistent with those of other studies, which have indicated that healthy cattle may excrete *L. monocytogenes* in their faeces, particularly during colder months (Kevenk and Koluman, 2022). Previous research has suggested that the increased incidence of *Listeria* shedding in faeces among healthy ruminants when housed indoors could be attributed to suboptimal hygiene practices and compromised immune function (Watanabe et al., 2022). Additionally, the type of feed provided to animals during this period could significantly impact the rate of faecal shedding within a herd. Therefore, farm management practices and the nutritional quality of feed provided during indoor periods are crucial factors influencing the immune health of animals, which in turn affects their susceptibility to clinical listeriosis or their status as asymptomatic carriers (Nightingale et al., 2005).

Regarding the characterizations of strains, in general, serogroup IVb is associated with a high number of human listeriosis cases and outbreaks, while IIa and IIb are more common in food and environmental samples (Ho et al., 2007). Chambel et al (Chambel et al., 2007). conducted a study to determine the most common serogroups in the environment of sheep and cow's milk cheese dairies in Portugal. It was found that 69.1% of the isolates were serotype IVb and 24% were serotype IIb. Varsaki et al (Varsaki et al., 2022). collected 424 samples from 14 dairy farms in Spain and most isolates (89%) were classified as serogroup IVb. In the present study, carried out on samples from dairy farm environments, we obtained 37.5%. Although serogroup IVbv1 has never been associated with outbreaks or cases of human listeriosis in the past (Leclercq et al., 2011), it is now considered an emerging risk as it has recently been associated with 4 independent listeriosis events within 2 years. In addition, this serogroup is associated with clinical cases in healthy children (Burall et al., 2017). With regard to serogroup diversity within the same dairy farm, in farm 2, samples were taken from the manger at different times and different serogroups (IIb and IVb) were found. In farm 3, different samples (faeces and water) collected on the same day also showed isolates of different serogroups (IVb and IVbv1), again demonstrating contamination by different strains.

It has been reported that some CCs are classified as hypervirulent and are likely to be more effective at colonising the intestinal tract, which is associated with wider dissemination of the pathogen in the organisms (Maury et al., 2016). The reported prevalence of hypervirulent clones in animals, farm environments and foods highlight the importance of

understanding the ecology and transmission of *L. monocytogenes* in farms (Terentjeva et al., 2021). A search of the Institut Pasteur MLST *Listeria* database (https://bigsdb.pasteur. fr/listeria/) showed that CC183 was previously identified in food in Italy; CC213 was found in clinical cases, cows and potatoes in Mexico; CC217 was found in different matrices such as potatoes (Mexico), meat products, seafood (USA and Spain), urban water, farm feedlot and soil (USA), snail (Greece), sheep (Spain), bovine listeriosis case (USA) and human (USA, Canada and Spain); CC224, described as one of the hypervirulent clones (Fritsch et al., 2018), was implicated in an outbreak in Luxembourg and in human cases in France and in Portugal, it was also found in the farm environment (USA) and in potatoes and avocados (Mexico and Peru); CC392 was found in sheep and cow (Spain), in green beans (Mexico), avocados (Chile) and human (Chile and Canada); CC489 was described in compost (Austria) and in meat-and-bone meal (Russia) and CC554 was found in soil in the USA. The present study contributes to the understanding of the ecology of *Listeria* and shows that CCs already found in food and human clinical cases are also present in the farm environment, with water and feed being the main potential sources of contamination. It also indicates that CC1, which is commonly found in bovines and is the predominant clinical complex in many countries (Moura et al., 2021), has not been detected, despite its prior identification in cheese and clinical isolates in Portugal (Almeida et al., 2013).

A virulence gene cluster essential for intracellular parasitism (*actA*, *hly*, *inlA*, *inlB*, *inlC*, *mpl*, *plcA*, *plcB*) was detected in all isolates. According to Maury et al. (2017) increased virulence in lineage I isolates can be associated with the presence of additional pathogenic islands (LIPI) (Fritsch et al., 2018). In hypervirulent CC4 the mentioned authors associated gene cluster LIPI-4 as a factor specifically implicated in the selective tropism of *L. monocytogenes* for the CNS and foetal-placental unit, in the present study this gene cluster was found in CC183, CC213 and in CC217. Although participating farms in this study do not have constancy of any case of bovine listeriosis.

Analysis of antibiotic, metal and disinfectant resistance genes revealed the presence of intrinsic antibiotic resistance to fosfomycin, quinolones, sulfamethoxazole, oxacillin and cephalosporins, as previously reported by Kurpas et al. (2020) and Hanes and Huang (2022), and the absence of resistance genes for metals and disinfectants. The more common classes of antibiotics used in veterinary medicine are quinolones, aminopenicillins, cephalosporins, tetracyclines, sulphonamides alone or in combination with potentiators, macrolides and glycopeptides (Caneschi et al., 2023). The presence of resistance genes to one or more classes of antibiotics that have been negligently used in livestock for decades, such as tetracyclines and β-lactams, would be expected. However, these results may be a consequence of the European Union's 'Farm to Fork Strategy', which aims to reduce the use of antimicrobials in livestock and aquaculture by 50% by 2030 (World Organisation for Animal Health, 2023). This target is in line with the responsible and prudent use of antibiotics and with good agricultural practices, including the separation of animals receiving treatment from those not receiving treatment in accordance with the Terrestrial Animal Health Code of the World Organisation for Animal Health (Chmielowska et al., 2021).

These isolates from the primary sector could enter the premises of the food industry. Although they lack genes encoding resistance to disinfectants, horizontal gene transfer may be responsible for their survival and persistence in the food production environment. For example, resistance to BC has been reported to range from 7% to 46%, cadmium resistance from 20% to 66% and arsenic resistance from 6% to 21% in food isolates (Schoder et al., 2023). Persistence of strains in the food production environment has been associated with the presence of stress survival islands. We found two isolates carrying SSI-1 genes, both from feed samples and from different farms. This contamination occurred in the farm environment or from the industry where they were produced.

5. Conclusions

This is the first study in Portugal to investigate the presence of *L. monocytogenes* in dairy farms and to identify the main clonal complexes.

The study found no *L. monocytogenes* in raw cow's milk, but 50% of dairy farms tested positive, mostly in spring. The bacterium was present in farm environmental samples such as faeces, feed and water, indicating a risk of listeriosis for animals at high contamination levels.

The study shows that *Listeria* strains found in food and clinical cases are also present in the farm environment, with water and feed being potential sources of contamination. Improved cleaning and disinfection of troughs and water could reduce animal ingestion and environmental spread.

Some clonal complexes involved in human listeriosis cases were identified, emphasising the need to monitor the genetic structure of the micro-organism in potential reservoir animals.

Despite the results of the study, the consumption of untreated raw milk carries a high risk due to other pathogenic micro-organisms. Prevention of disease in ruminants and transmission to humans requires robust surveillance and control measures. Identification of *L. monocytogenes* in natural environments and outdoor production provides information on potential sources and pathways of contamination, suggesting a need for control beyond safety programmes.

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CRediT authorship contribution statement

Bárbara Nunes: Writing – original draft, Writing – review & editing, Data curation, Investigation. **Ana Rita Barata:** Writing – original draft, Investigation, Writing – review & editing, Data curation. **Ricardo Oliveira:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Hugo Guedes:** Writing – review & editing, Investigation, Formal analysis, Conceptualization. **Carina Almeida:** Writing – review & editing, Investigation, Formal analysis. **Gabriela Jorge da Silva:** Writing – review & editing. **Teresa Nogueira:** Writing – review & editing, Investigation, Formal analysis. **Maria José Saavedra:** Writing – review & editing, Supervision, Investigation. **Gonçalo Nieto Almeida:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Conceptualization, Investigation, Methodology.

Declaration of Competing Interest

No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication.

Data Availability

Data will be made available on request.

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