environmental microbiology

Environmental Microbiology (2021) **23**(12), 7617–7631 doi:10.1111/1462-2920.15860

Ruminant-associated *Listeria monocytogenes* isolates belong preferentially to dairy-associated hypervirulent clones: a longitudinal study in 19 farms

Carla Palacios-Gorba,[1†] Alexandra Moura,[2,3†] Jesús Gomis,[1] Alexandre Leclercq,[2,3] Ángel Gómez-Martín,[1] Hélène Bracq-Dieye,[2,3] María L. Mocé,[1] Nathalie Tessaud-Rita,[2,3] Estrella Jiménez-Trigos,[1] Guillaume Vales,[2,3] Ángel García-Muñoz,[1] Pierre Thouvenot,[2,3] Empar García-Roselló,[4] Marc Lecuit [2,3,5*] and Juan J. Quereda [1*]

[1]*Departamento Producción y Sanidad Animal, Salud Pública Veterinaria y Ciencia y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Cardenal Herrera-CEU, CEU Universities, Valencia, Spain.*

[2]*Institut Pasteur, National Reference Centre and WHO Collaborating Centre for Listeria, Paris, France.*

[3]*Institut Pasteur, Université de Paris, Inserm U1117, Biology of Infection Unit, Paris, 75015, France.*

[4]*Departamento Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad Cardenal Herrera-CEU, CEU Universities, Valencia, Spain.*

[5]*Necker-Enfants Malades University Hospital, Division of Infectious Diseases and Tropical Medicine, Institut Imagine, APHP, Paris, France.*

Summary

Studies have shown that ruminants constitute reservoirs of *Listeria monocytogenes*, but little is known about the epidemiology and genetic diversity of this pathogen within farms. Here we conducted a large-scale longitudinal study to monitor *Listeria* spp. in 19 dairy farms during three consecutive seasons ($N = 3251$ samples). *L. innocua* was the most prevalent species, followed by *L. monocytogenes*. *Listeria monocytogenes* was detected in 52.6% of farms and more frequently in cattle (4.1%) and sheep (4.5%) than in goat farms (0.2%). Lineage I accounted for 69% of *L. monocytogenes* isolates. Among animal samples, the most prevalent sublineages (SL) and clonal complexes (CC) were SL1/CC1, SL219/CC4, SL26/CC26 and SL87/CC87, whereas SL666/CC666 was most prevalent in environmental samples. Sixty-one different *L. monocytogenes* cgMLST types were found, 28% common to different animals and/or surfaces within the same farm and 21% previously reported elsewhere in the context of food and human surveillance. *Listeria monocytogenes* prevalence was not affected by farm hygiene but by season: higher prevalence was observed during winter in cattle, and during winter and spring in sheep farms. Cows in their second lactation had a higher probability of *L. monocytogenes* faecal shedding. This study highlights dairy farms as a reservoir for hypervirulent *L. monocytogenes*.

Introduction

The genus *Listeria* currently includes 26 recognized species of ubiquitous small rod-shaped Gram-positive bacteria (Quereda *et al*., 2020; Carlin *et al*., 2021). Only two of these species, *L. monocytogenes* and *L. ivanovii*, are considered pathogens (Vázquez-Boland *et al*., 2001). *Listeria monocytogenes* is an important foodborne pathogen that can cause human and animal listeriosis, a severe invasive infection with high hospitalization and fatality rates in humans (20%–30%) (Charlier *et al*., 2017). In immunocompromised individuals and the elderly, listeriosis manifests mostly as septicemia and central nervous system (CNS) infections. In pregnant women, listeriosis can lead to foetal or neonatal complications (Swaminathan and Gerner-Smidt, 2007; Charlier *et al*., 2017).

Domestic ruminants can become infected by *L. monocytogenes* through ingestion of contaminated silage (Vázquez-Boland *et al*., 2001), which can result in rhombencephalitis, septicemia and abortion. Animals may also be asymptomatic carriers and shed the bacterium in their faeces (Skovgaard and Morgen, 1988; Ho *et al*., 2007; Esteban *et al*., 2009; Haley *et al*., 2015;

Received 3 August, 2021; accepted 24 November, 2021. *For correspondence. E-mail marc.lecuit@pasteur.fr. juan.quereda@uchceu.es. †These authors share the first authorship. Carla Palacios-Gorba and Alexandra Moura contributed equally to this work, and the rank was determined according to increasing seniority.

Hurtado *et al*., 2017; Castro *et al*., 2018). In dairy ruminants, *L. monocytogenes* can be transmitted to bulk tank milk (BTM) from faecal or environmental contamination of the udder surface (Husu *et al*., 1990; Sanaa *et al*., 1993; Winter *et al*., 2004; Castro *et al*., 2018; Addis *et al*., 2019), as a consequence of poor hygiene in the milking parlour or as a consequence of intramammary infection (Winter *et al*., 2004; Addis *et al*., 2019). The prevalence of *L. monocytogenes* in BTM of dairy cow farms can range between 1.2% and 16% (Jayarao *et al*., 2006; Vilar *et al*., 2007; Van Kessel *et al*., 2011; Castro *et al*., 2018) and contaminated milk poses several risks for producers and consumers, namely: (i) the development of listeriosis after consumption of raw milk contaminated products; (ii) the development of biofilms in the milking equipment that contributes to persistent contamination of the BTM and (iii) cross-contamination of dairy processing plants, pasteurized dairy products or other food-associated environments (MacDonald *et al*., 2005; Oliver *et al*., 2005; Latorre *et al*., 2010, 2011; Fox *et al*., 2011). Faecal shedding of *L. monocytogenes* also poses a risk for inter-animal transmission in dairy farms and contamination of agricultural environments and raw vegetables at the pre-harvest stages (Schlech *et al*., 1983).

Listeria monocytogenes population is heterogeneous and can be classified into lineages (Wiedmann *et al*., 1997), PCR genoserogroups (Doumith *et al*., 2004), CCs (clones) and sequence types (STs) as defined by multilocus sequence typing (MLST) (Ragon *et al*., 2008), and sub-lineages (SLs) and cgMLST types (CTs), as defined by core genome MLST (cgMLST) (Moura *et al*., 2016). *Listeria monocytogenes* genetic heterogeneity also reflects different pathogenic potential among *L. monocytogenes* isolates, with some clones being more frequently isolated from humans (e.g. CC1, CC2, CC4 and CC6) (Maury *et al*., 2016, 2019) and ruminants (e.g. CC1) (Dreyer *et al*., 2016) clinical cases. Despite increasing evidence that dairy products and ruminants are important reservoirs of *L. monocytogenes* (Nightingale *et al*., 2004; Borucki *et al*., 2005; Ho *et al*., 2007; Esteban *et al*., 2009; Dell'Armelina Rocha *et al*., 2013; Haley *et al*., 2015; Hurtado *et al*., 2017; Castro *et al*., 2018; Maury *et al*., 2019; Hafner *et al*., 2021), little is still known about the genetic diversity, transmission dynamics and persistence of pathogenic *L. monocytogenes* in farm environments.

The objectives of the present study were: (i) to determine the prevalence of *Listeria* spp. in individual dairy ruminants and the farm environment in Spanish farms by a longitudinal study design; (ii) to characterize the genetic diversity and population structure of *L. monocytogenes* in dairy farms using whole-genome sequencing; and (iii) to understand the transmission of *L. monocytogenes* at the farm level and the risk factors [season, production hygiene, lactation number and the days in milk (DIM) of current lactation] that influence it.

Results

Prevalence of Listeria *spp. in dairy farms*

A total of 3251 samples were collected from 19 Spanish dairy farms over three consecutive seasons (Fig. 1; Table S1): 2081 from animals (2080 faeces and one brain sample from a CNS infection case) and 1170 from the surrounding farm environment (195 feed, 390 food and water troughs, 195 beddings, 195 milk filters and 195 milking station floor). Each farm was sampled one time per season except farm 'Sheep B' which was subjected to eight additional samplings from 2019 to 2020 ($n = 400$ extra samples: 144 from farm environment and 256 from animal faeces, see M&M Fig. 1 and Fig. S1). None of the farms reported listeriosis cases, except one farm ('Sheep C'), where a listeriosis outbreak occurred on the last season sampled (Spring 2020).

Listeria spp. was detected in 94.7% (18/19) of farms and in all sampling seasons (Fig. 1). Overall, *Listeria* spp. prevalence was 11.2% (318/2850), and similar in faeces samples (10.2%; 186/1824) and farm environment samples (12.9%; 132/1026) (Fig. 1, Table S2). Prevalence varied significantly between farms from 0% to 43.3% and was overall higher in cattle and sheep farms (Table S2). The most prevalent species were *L. innocua* (64.7%; 275/425) and *L. monocytogenes* (30.6%; 130/425) (Table 1). Co-occurrence of the two *Listeria* species was detected in 0.8% (14/1824) of individual animal faeces and 1.1% (11/1026) environmental samples (Table S3). *Listeria monocytogenes* was detected in 52.6% of farms (10/19), and prevalence in positive farms ranged between 0.7% and 21.3%, frequently higher for cattle farms (Fig. 1B, Table S2). *Listeria monocytogenes* prevalence was similar in faeces samples (3.8%; 70/1824) and farm environment samples (2.5%; 26/1026), although, in some farms, *L. monocytogenes* was most frequently present in faeces samples compared to environmental samples in a specific season (Fig. 1B). *Listeria innocua* was present in 6.7% (123/1824) of faeces samples and in 10.8% (111/1026) of farm environmental samples (Table S3).

Among environmental samples, *L. monocytogenes* was detected in all sampled sites except in the milking station floor where other *Listeria* spp. were present (eight *L. innocua* and one *L. newyorkensis*). *Listeria monocytogenes* occurred on food troughs (5.8%), beddings (3.5%), feed (2.9%), milk filter socks (MFS) (2.3%) and the water troughs (0.6%) (Tables S2 and S3). Of note, no *L. monocytogenes* was detected in feed, food troughs and water troughs from four out of nine of the farms where faecal shedders were detected.

A

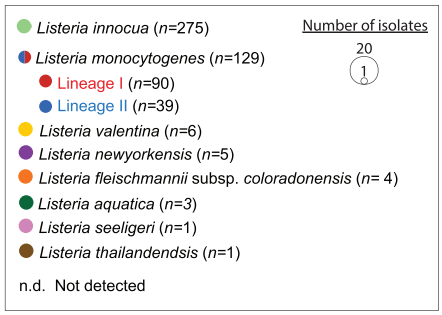
FARMS SAMPLED FROM AUTUMN 2018 TO SPRING 2019

	Autumn 2018		Winter 2018-2019		Spring 2019	
	Animal	Farm environment	Animal	Farm environment	Animal	Farm environment
Cattle A	n.d.	n.d.	n.d.		n.d.	
Cattle B	n.d.	n.d.	n.d.			
Cattle C	n.d.	n.d.				
Cattle D	n.d.	n.d.	n.d.	n.d.		
Cattle E	n.d.	n.d.				
Sheep A	n.d.	n.d.	n.d.	n.d.		
Sheep B	n.d.	n.d.				
Goat A		n.d.	n.d.	n.d.	n.d.	n.d.
Goat B	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

FARMS SAMPLED FROM AUTUMN 2019 TO SPRING 2020

	Autumn 2019		Winter 2019-2020		Spring 2020	
	Animal	Farm environment	Animal	Farm environment	Animal	Farm environment
Cattle F	n.d.	n.d.	n.d.	n.d.	n.d.	
Cattle G					n.d.	
Cattle H					n.d.	
Cattle I						
Cattle J	n.d.		n.d.		n.d.	
Sheep C	n.d.	n.d.				
Sheep D						
Sheep E						
Goat C		n.d.	n.d.	n.d.	n.d.	n.d.
Goat D	n.d.	n.d.			n.d.	n.d.

	Extra sampling dates	Season	Animal	Farm environment
Sheep B	2018-Nov-17	Autumn	n.d.	n.d.
	2019-Feb-27	Winter		
	2019-Mar-13			
	2019-Apr-10	Spring		
	2019-May-02			
	2019-May-20			
	2019-July-15	Summer		
	2019-Sept-16	Autumn		
	2020-Jan-29	Winter		
	2020-Feb-26			n.d.
	2020-June-06	Spring	n.d.	n.d.



B

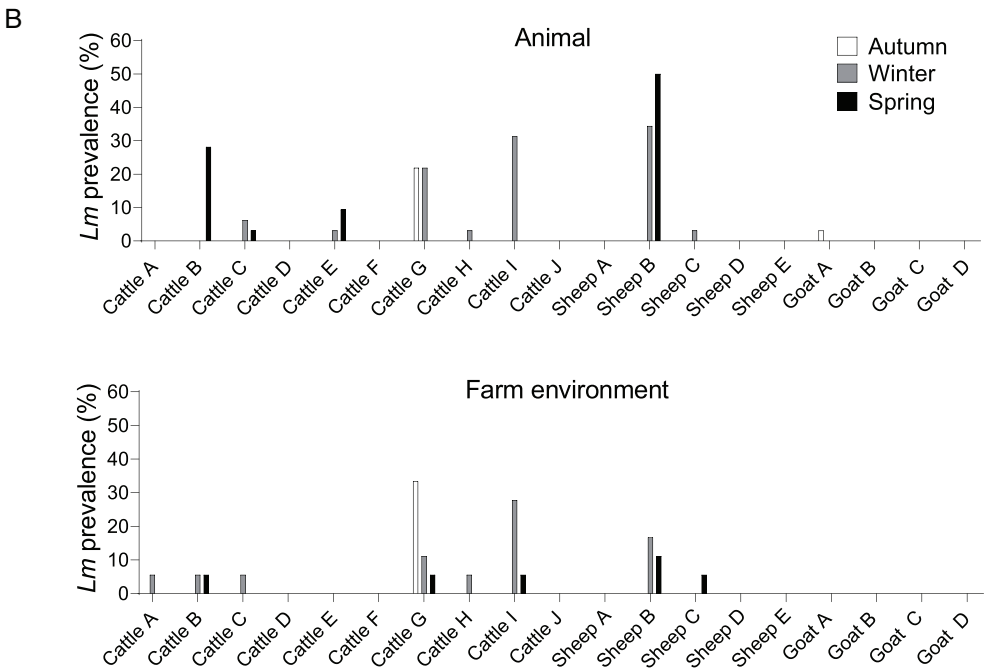


Fig. 1. (A) *Listeria* spp. isolated in this study from dairy cattle, sheep and goat farms during three consecutive seasons. Farm 'Sheep B' was sampled 11 times during seven consecutive seasons from autumn 2018 to spring 2020 (see Material and methods). Circle size is proportional to the number of isolates.
B. Prevalence of *L. monocytogenes* in faeces samples and the farm environment during three consecutive seasons. For consistency among farms, only data from three consecutive seasons (autumn 07-Nov-2018, winter 27-Feb-2019 and spring 10-Apr-2019) were considered for prevalence calculation on farm 'Sheep B'.

Table 1. Number of isolates obtained in this study ($N = 425$).

Listeria species	Animal	Farm env.	Feed	Total
L. monocytogenes	89 (20.9%)	32 (7.5%)	9 (2.1%)	130 (30.6%)
L. innocua	150 (35.3%)	92 (21.6%)	33 (7.8%)	275 (64.7%)
L. valentina	5 (1.2%)	1 (0.2%)		6 (1.4%)
L. newyorkensis		5 (1.2%)		5 (1.2%)
L. fleischmannii subsp. *coloradonensis*	1 (0.2%)	2 (0.5%)	1 (0.2%)	4 (0.9%)
L. aquatica	3 (0.7%)			3 (0.7%)
L. seeligeri		1 (0.2%)		1 (0.2%)
L. thailandensis	1 (0.2%)			1 (0.2%)

Among animals, different seasonal patterns of *L. monocytogenes* shedding were observed (Fig. 1B). While no *L. monocytogenes* was detected in animals from farm 'Cattle B' during autumn and winter, it increased sharply in the spring season (28.1%). Farm 'Cattle G' was characterized by a high *L. monocytogenes* prevalence of faecal shedders during autumn and winter (21.9% in both seasons), but none was detected in spring. In farm 'Cattle I', although no *L. monocytogenes* was detected in autumn, it increased sharply to 31.2% in winter and disappeared in the spring sampling. Finally, while none of the tested sheep shed *L. monocytogenes* during autumn in farm 'Sheep B', the prevalence increased to 34.3% during winter and to 50% during spring (Fig. 1B).

Interestingly, in farm 'Sheep C', where an 8-week listeriosis outbreak occurred in the spring of 2020, no *L. monocytogenes* was isolated from any of the faeces or environmental samples collected. The outbreak caused 89 abortions (from a total of 974 pregnant sheep) and CNS symptoms (inappetence, recumbency, difficulties in swallowing, drooping eyelid, ear and lip, head-tilt and circling) in six animals (1.6% total mortality). On samples taken *post-mortem* from the bedding surfaces, faeces and the brainstem of one diseased sheep, *L. monocytogenes* was isolated from the brainstem and from one bedding sample but only *L. innocua* was detected on the faeces (Table S3).

Population structure and genetic diversity of Listeria *spp. in dairy farms*

Altogether, 425 *Listeria* spp. isolates were obtained from the farm environment (176/425, 41.4%) and animal samples (249/425, 58.6%) and further characterized at the genomic level. Eight different *Listeria* species were identified: *L. monocytogenes* ($n = 130$, 30.6%), *L. innocua* ($n = 275$, 64.7%), *L. valentina* ($n = 6$, 1.4%), *L. newyorkensis* ($n = 5$, 1.2%), *L. fleischmannii* subsp. *coloradonensis* ($n = 4$, 0.9%), *L. aquatica* ($n = 3$; 0.7%), *L. seeligeri* ($n = 1$, 0.2%) and *L. thailandensis* ($n = 1$, 0.2%) (Figs 1 and 2; Table 1).

Listeria monocytogenes belonged to lineages I ($n = 91$; 70%; genoserogroups IVb, $n = 70$ and IIb, $n = 21$) and II ($n = 39$; 30%; genoserogroup IIa) (Figs 2 and 3). Among animal samples, the most prevalent SLs and CCs were SL1/CC1 ($n = 18$, 13.8%), SL219/CC4 ($n = 14$, 10.8%), SL26/CC26 ($n = 10$, 7.7%) and SL87/CC87 ($n = 12$, 9.2%), whereas SL666/CC666 ($n = 12$, 9.2%) was most prevalent in environmental samples (Fig. 2A; Table 2).

Sixty-one different CTs were detected: 48 (79%) unique to this study and 13 (21%) previously reported in BIGSdb-*Listeria*, sharing two to seven allelic differences with existing cgMLST profiles (Table S4). The majority of CTs detected comprised only one isolate (44/61, 72.1%), whereas 17 (27.9%) comprised 2–16 isolates sharing 0–6 allelic differences (Table 2; Fig. 2B).

Up to five and eight different CTs could be isolated from environmental and faecal materials respectively, on a single sampling day (Table S3). Overall, a significantly higher CT diversity was found in animal faecal samples than environmental samples (Shannon indexes 3.4 vs. 2.8; Hutcheson *t*-test, $P = 0.006$). No CTs were common to multiple farms, except for L1-SL219-ST219-CT5814 which was detected in farms 'Sheep B' and 'Cattle B', separated by 73 km and sampled 7 days apart (Table S3). 16.4% (10/61) CTs were common to both environmental and animal samples from the same farm, three of them collected at different time points (Tables 2 and S3). Persistent *L. monocytogenes* strains [i.e. continued presence over time, at a specific location (Stasiewicz *et al*., 2015)] were identified in six dairy farms (Table 2) and in one sheep (Tables S3 and S5).

Listeria pathogenic islands LIPI-3 and LIPI-4 were present in 50% (65/130) and 32.3% (42/130) *L. monocytogenes*, whereas all isolates carried LIPI-1, which is part of the *L. monocytogenes* core genome (Fig. 3). Acquired resistance traits towards antibiotics, disinfectants or other stress conditions, were rare (Table 3, Table S3; Fig. 3). Two isolates (1.5%, CTs L1-SL2-ST2-CT6147 and L1-SL2-ST2-CT6148) harboured genes conferring resistance to macrolides (*ermG*, *mefA* and *msrD* genes) or to benzalkonium chloride (*bcrABC* and Tn*6188*::*ermC*; CT

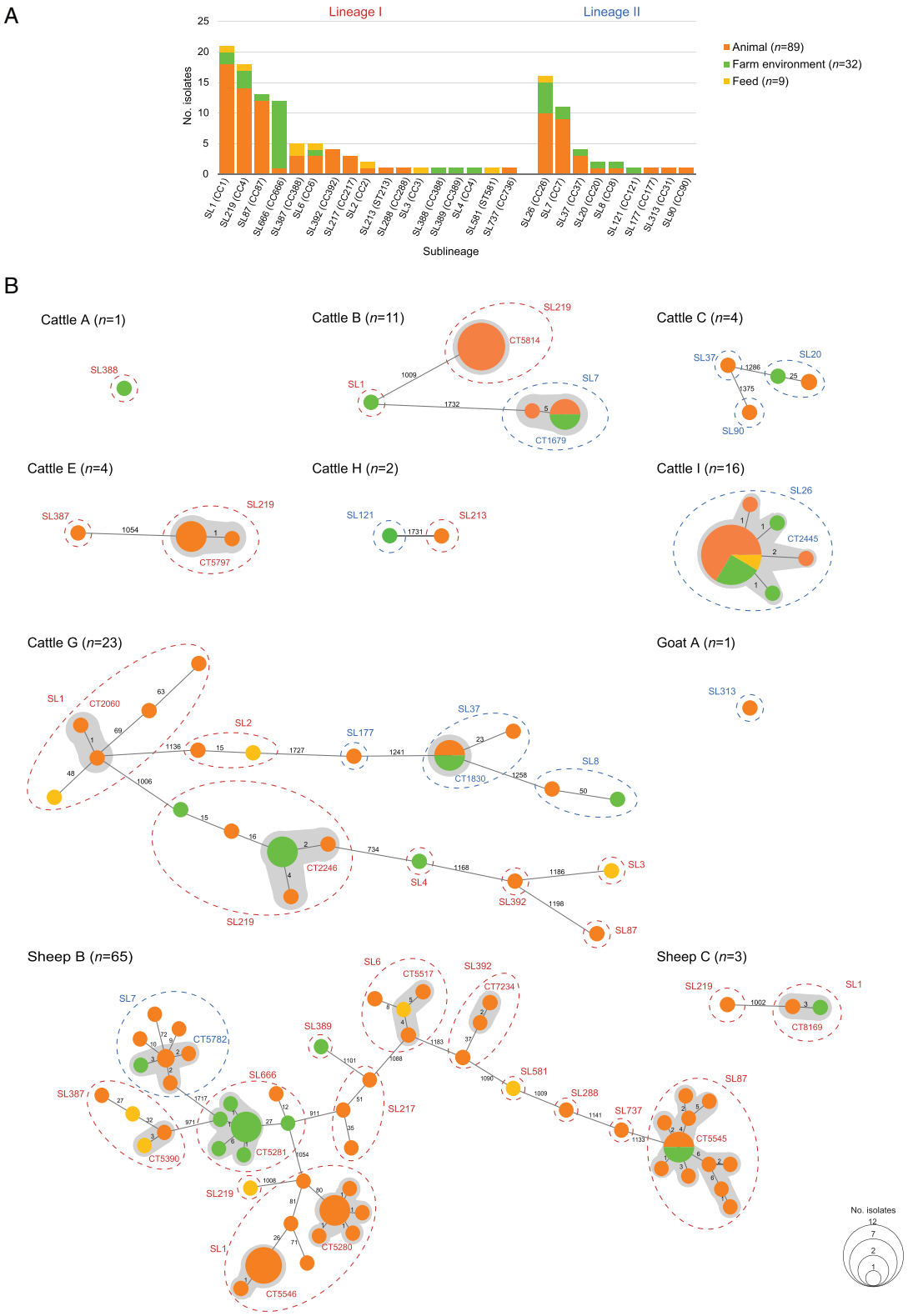


Fig. 2. Diversity of *L. monocytogenes* isolates collected in this study ($n = 130$) based on cgMLST (1748 loci) analyses.
A. Distribution of sublineages (SLs) in animals, feed and farm environmental sources. Corresponding clonal complexes (CCs), defined on the basis of seven-locus MLST, are shown in brackets.
Fig. 2. Legend on next page.

Table 2. *Lm* cgMLST types detected comprising two or more isolates ($n = 17$ types out of 61).

cgMLST type	No. isolates	Farm(s)	Season(s)	Source (s)	Sources description	Traits
Lineage I						
L1-SL87-ST1591-CT5545 (CC87, IIb)	12	Sheep B	Spring 2019 (Apr, May)	A, E	Faeces (11), bedding (1)	LIPI-1, LIPI-4
L1-SL666-ST666-CT5281 (CC666, IVb)	10	Sheep B	Winter 2019 (Feb, Mar), Spring 2019 (Apr, May), Summer 2019 (Jun), Autumn 2019 (Sep)	E	Milk filter (10)	LIPI-1, LIPI-3
L1-SL219-ST219-CT5814 (CC4, IVb)	8	Sheep B, Cattle B	Spring 2019 (May)	A, E	Faeces (7), feed (1)	LIPI-1, LIPI-3, LIPI-4
L1-SL1-ST1-CT5280 (CC1, IVb)	6	Sheep B	Winter 2019 (Feb), Spring 2019 (Apr)	A	Faeces (6)	LIPI-1, LIPI-3
L1-SL1-ST1-CT5546 (CC1, IVb)	4	Sheep B	Spring 2019 (Apr)	A	Faeces (4)	LIPI-1, LIPI-3
L1-SL219-ST219-CT2246 (CC4, IVb)	4	Cattle G	Autumn 2019 (Oct)	A, E	Faeces (2), food trough (2)	LIPI-1, LIPI-3, LIPI-4
L1-SL219-ST219-CT5797 (CC4, IVb)	3	Cattle E	Spring 2019 (Apr)	A	Faeces (3)	LIPI-1, LIPI-3, LIPI-4
L1-SL6-ST6-CT5517 (CC6, IVb)	3	Sheep B	Winter 2019 (Mar)	A, E	Faeces (1), feed (1), bedding (1)	LIPI-1, LIPI-3
L1-SL1-ST1-CT2060 (CC1, IVb)	2	Cattle G	Winter 2020 (Feb)	A	Faeces (2)	LIPI-1, LIPI-3
L1-SL6-ST6-CT5393 (CC6, IVb)	2	Sheep B	Winter 2019 (Feb, Mar)	A	Faeces (2)	LIPI-1, LIPI-3
L1-SL392-ST392-CT7234 (CC392, IIb)	2	Sheep B	Winter 2020 (Jan)	A	Faeces (2)	LIPI-1
L1-SL387-ST388-CT5390 (CC388, IVb)	2	Sheep B	Winter 2019 (Feb)	A, E	Faeces (1), feed (1)	LIPI-1, LIPI-4
L1-SL1-ST1-CT8169 (CC1, IVb)	2	Sheep C	Spring 2020 (May)	A, E	Brain (1), bedding (1)	LIPI-1, LIPI-3
Lineage II						
L2-SL26-ST26-CT2445 (CC26, IIa)	16	Cattle I	Winter 2020 (Feb), Spring 2020 (May)	A, E	Faeces (10), feed (1), food trough (3), bedding (2)	LIPI-1, SSI-1
L2-SL7-ST7-CT5782 (CC7, IIa)	5	Sheep B	Spring 2019 (Apr, May)	A, E	Faeces (4), milk filter (1)	LIPI-1, SSI-1
L2-SL7-ST7-CT1679 (CC7, IIa)	3	Cattle B	Spring 2019 (May)	A, E	Faeces (2), bedding (1)	LIPI-1, SSI-1
L2-SL37-ST37-CT1830 (CC37, IIa)	2	Cattle G	Winter 2020 (Feb)	A, E	Faeces (1), food trough (1)	LIPI-1

Abbreviations: A, animal; E, environment.

L2-SL313-ST325-CT1188 and L2-SL121-ST121-CT909 respectively). SSI-1 (32/130 isolates, 24.6%, tolerance to low pH and high salt), SSI-2 (1/130, 0.7%; tolerance to alkaline and oxidative stress conditions) and LGI-3 (1/130, 0.7%; tolerance to cadmium) genomic regions were also present (Table S3). Acquired resistance genes were also present in *L. innocua* (19/275, 6.9%) and *L. aquatica* (1/3, 33.3%), with *tetM* (resistance to tetracyclines) being the most prevalent resistance trait detected (Table 3 and Table S3).

The most prevalent *L. monocytogenes* CTs detected in this study (L1-SL87-ST1591-CT5545, $n = 12$; L1-SL666-ST666-CT5281, $n = 10$; L1-SL219-ST219-CT5814, $n = 8$; and L2-SL26-ST26-CT2445, $n = 16$) harboured at least one of the following genomic regions: LIPI-3, LIPI-4 or SSI-1 (Table 2; Fig. 3).

Impact of farm type, season and farming practices in the prevalence of L. monocytogenes

L. monocytogenes was detected more frequently in cattle (7/10 positive farms, prevalence 4.1%) than in sheep (2/5 positive farms, prevalence 4.5%) and goat farms (1/4 positive farms, prevalence 0.2%) (Table S2). Although *L.*

B. Minimum spanning tree based on the cgMLST profiles *L. monocytogenes* observed in each farm sampled in this study. Circle sizes are proportional to the number of isolates and are coloured by source type, as in panel A. Dashed lines delimitate SLs and are coloured according to the phylogenetic lineage (red, lineage I; blue, lineage II). Values shown in connecting lines denote the number of allelic differences between profiles. Grey zones delimitate isolates within the same CT [cut-off of seven-allelic differences (Moura *et al*., 2016)] and CTs common to more than one isolate are labelled.

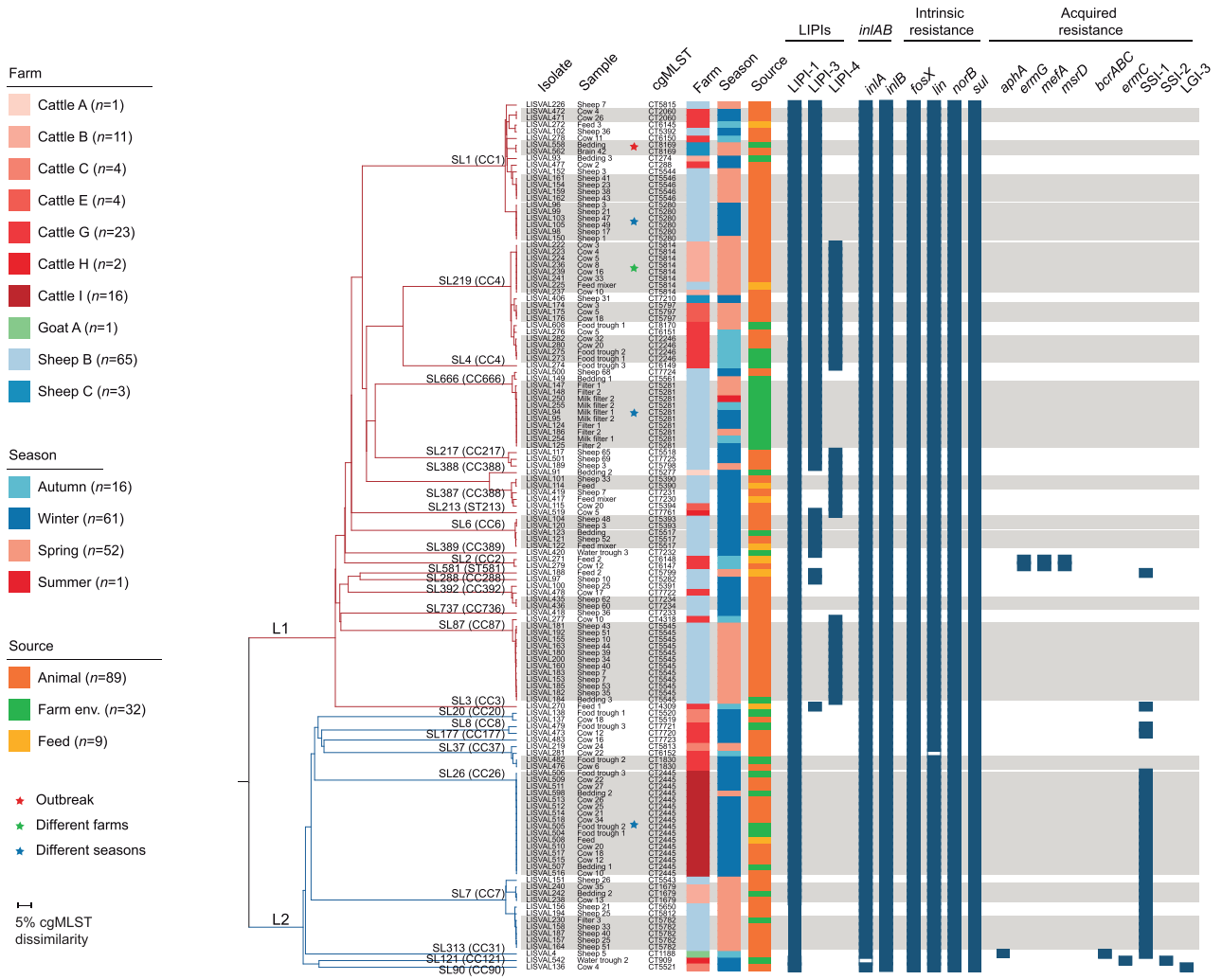


Fig. 3. Genetic diversity of the 130 *L. monocytogenes* isolates sequenced in this study. cgMLST single linkage dendrogram. Branches are coloured by phylogenetic lineage (L1, red; L2, blue) and labelled by SLs. Corresponding clonal complexes (CCs), defined on the basis of seven-locus MLST, are shown in brackets. Isolates' names, type of sample and CTs are shown in tips. Isolates belonging to the same CT are highlighted in horizontal grey boxes. Vertical coloured boxes indicate the sampling farm, season and source, coloured according with the key panel respectively. Colour-filled dark blue boxes indicate the presence of selected genetic traits: *Listeria* pathogenic islands (LIPI-1, LIPI-3 and LIPI-4), internalins (*inlA*, *inlB*), intrinsic antibiotic resistance (*fosX*, *lin*, *norB*, *sul*) and acquired resistance loci towards antibiotics (*aphA*, *ermG*, *mefA*, *msrD*), benzalkonium chloride (*bcrABC*, *ermC*), pH or oxidative stress (SSI-1, SSI-2) and metals (LGI-3). White-filled blue boxes represent genes with truncations leading to premature stop codons.

Table 3. Acquired antibiotic resistance genes detected in this study.

Listeria species	No. isolates	Genes[a]	Phenotypic resistance[b]	Farm(s)	Source(s)
L. monocytogenes	2	*mefA*, *msrD*, *ermG*	ERY, CLI	Cattle G	A, E
	1	*aphA*	CLI	Goat A	A
L. innocua	13	*tetM*	CLI, TET	Cattle A, E, G, J; Sheep A, E	A, E
	4	*dfrD*, *lnuA*, *tetM*	CLI, TET	Sheep E	A, E
	1	*dfrD*, *lnuA*, *tetS*, *tetM*	CLI, TET, STX	Goat D	A
	1	*lnuA*, *tetM*	CLI, TET	Cattle J	E
L. aquatica	1	*dfrK*	ERY, CLI, TET	Sheep A	A

Abbreviations: A, animal; E, environment.
[a]Gene traits of antibiotic resistance towards: folate inhibitors (*dfrD*, *dfrK*); lincosamides (*lnuA*); macrolides (*ermG*, *mefA*, *msrD*); tetracyclines (*tetM*, *tetS*).
[b]Antibiotics: erythromycin (ERY), clindamycin (CLI), tetracycline (TET), trimethoprim/sulfamethoxazole (STX).

monocytogenes was detected more frequently in 'Cattle G' and 'Sheep B' farms, no remarkable differences in management practices were detected compared to other farms of the same animal species where the prevalence of *Listeria* spp. was lower (Table S1). Since only one out of four goat farms was positive for *L. monocytogenes* (Fig. 1C), and the prevalence was extremely low in this farm (0.7%), goat farms were not included in further statistical analyses.

L. monocytogenes presence in consecutive seasons was only detected in farm 'Cattle G' (Fig. 1A). In cattle farms, the overall prevalence was higher in winter than in autumn (χ^2 test, $P < 0.05$) (Figs. 1B and 4A). In sheep farms, the overall prevalence was higher in winter and spring than in autumn (χ^2 test, $P < 0.05$) (Figs 1B and 4B). Interestingly, cows were more likely to shed *L. monocytogenes* on the second lactation than on the first, fourth or higher lactations (χ^2 test, $P < 0.05$) (Fig. 4C), but DIM did not impact the frequency of *L. monocytogenes* faecal shedding (Fig. 4D). In sheep, no significant association was found between the lactation number and frequency of *L. monocytogenes* faecal shedding (Fig. 4E). Differences in production hygiene were observed between both cattle and sheep farms, but there was no significant correlation between hygiene scores and *L. monocytogenes* prevalence (Table S6).

Genotypes isolated from faeces samples did not occur in MFS, indicating that pre-milking teat disinfection used in these farms was effective to prevent *L. monocytogenes* milk contamination, with exception for farm 'Sheep B', in which this procedure was not typically performed (similarly to all small ruminants' dairy farms). Accordingly, L2-SL7-ST7-CT5782 isolated from sheep faeces samples was identified in MFS in the farm 'Sheep B'.

Although all farms reported usage of antibiotics for treatment purposes (Table S1), acquired genetic traits of antibiotic resistance were rare in *L. monocytogenes*. Interestingly, among *L. innocua*, 19 isolates harboured

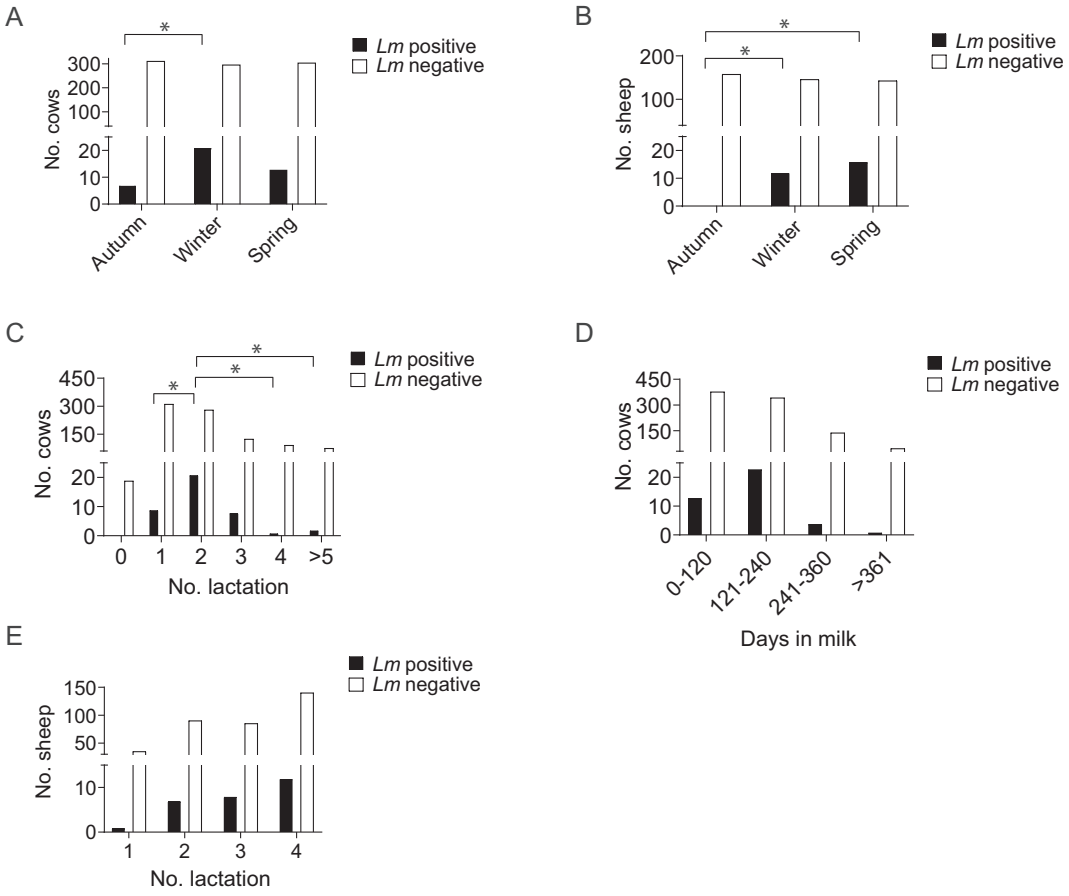


Fig. 4. Impact of seasons, lactation number and DIM in the number of *L. monocytogenes* faecal shedders.
A, B. Number of *L. monocytogenes* faecal shedders per season in cattle and sheep farms respectively.
C, E. Number of *L. monocytogenes* faecal shedders per lactation in cattle and sheep farms respectively.
D. Number of *L. monocytogenes* faecal shedders per DIM range in cattle farms. Statistically significant differences were evaluated by chi-square tests. Stars denote *P*-values <0.05.

tetM genes and showed resistance towards tetracycline (Table 3), 13 (68.4%) of which were isolated from farms using tetracyclines.

Discussion

Understanding *L. monocytogenes* population dynamics and its biodiversity is essential for effective disease surveillance and the development of control strategies. To the best of our knowledge, this is the largest longitudinal study on the prevalence, ecology and genomic characteristics of *L. monocytogenes* in individual dairy ruminants and the farm environment. Other reports have either applied a longitudinal study design with a reduced number of farms (one to three farms) (Ho *et al*., 2007; Haley *et al*., 2015; Castro *et al*., 2018; Chow *et al*., 2021) and/or analysed faeces samples from randomly chosen farm ruminants, which limits the understanding of global and individual faecal shedding patterns over time (Haley *et al*., 2015; Castro *et al*., 2018).

In this study, the prevalence of *L. monocytogenes* detected in dairy farms (3.8% in faeces samples and 2.5% in farm environment samples) was lower than previously reported in dairy farms with no clinical listeriosis cases (faecal sample prevalence of 0%–60% in cattle and 14.2% in sheep) in farms from USA and Europe (Skovgaard and Morgen, 1988; Nightingale *et al*., 2004; Ho *et al*., 2007; Esteban *et al*., 2009; Castro *et al*., 2018; Chow *et al*., 2021). Differences in climate and farm management (e.g. feed used) between different geographical regions may account for the low prevalence of *L. monocytogenes* in our study compared to previous studies performed in northern countries (Yusuf *et al*., 2007; Cavicchioli *et al*., 2019; Ianevski *et al*., 2019).

L. monocytogenes was detected more frequently in cattle farms than in small-ruminant farms, in agreement with previous studies concerning the epidemiology of listeriosis in ruminants (Nightingale *et al*., 2004; Esteban *et al*., 2009; Hurtado *et al*., 2017; Hafner *et al*., 2021). Interestingly, the pathogenic species, *L. ivanovii*, often reported in small ruminants (Ramage *et al*., 1999; Orsi and Wiedmann, 2016; Hafner *et al*., 2021) was not detected in any of our farms, which could be due to its relatively low prevalence (Sauders *et al*., 2012; Orsi and Wiedmann, 2016) or to possible biases of isolation protocols that have typically been optimized for recovery of *L. monocytogenes* (Orsi and Wiedmann, 2016). Our results are in line with other reports using cultivation-based approaches showing that the incidence of *L. innocua* in ruminant faeces is higher (9.7%–22.7%) than that of *L. monocytogenes* (1.8%–9.3%) (Vilar *et al*., 2007; Zhao *et al*., 2021), though it has been shown that *L. innocua* can outgrow *L. monocytogenes* during enrichment protocols masking its detection (Keys *et al*., 2013).

Although consumption of spoiled silage is thought to be the principal source of infection for ruminants (Vázquez-Boland *et al*., 2001), up to a third of animal listeriosis cases lack an evident link between listeriosis and silage feeding (Walland *et al*., 2015). In this study, in 50% of the farms where faecal shedders were detected, no *L. monocytogenes* could be detected in feed, food troughs or water troughs. It has been suggested that wildlife, farm staff or visitors, acquisition of new animals and/or farm equipment could also vehiculate *L. monocytogenes* into farms (Sanaa *et al*., 1993; Cooper and Walker, 1998; Murinda *et al*., 2004; Nightingale *et al*., 2004; Haley *et al*., 2015).

The majority of isolates retrieved here belonged to lineage I (particularly to SL1/CC1, SL219/CC4 and SL87/CC87) which is significantly associated with a clinical origin both in humans and animals (Gray *et al*., 2004; Maury *et al*., 2016; Papić *et al*., 2019). CC1 and CC4 have been shown to be highly associated with dairy products (Maury *et al*., 2019; Painset *et al*., 2019), being more invasive (hypervirulent) and colonizing better the intestinal lumen (Dreyer *et al*., 2016; Maury *et al*., 2019; Papić *et al*., 2019) and a cause of multiple human listeriosis outbreaks (Linnan *et al*., 1988; Costard *et al*., 2017). CC87 has been previously reported as predominant in foodborne and clinical isolates in China and related to two outbreaks in Northern Spain (Pérez-Trallero *et al*., 2014; Wang *et al*., 2018; Zhang *et al*., 2020).

Interestingly 21% of the CTs here detected were not unique to this study and included genotypes previously detected in the context of listeriosis surveillance in Europe and Oceania (Kwong *et al*., 2016; Moura *et al*., 2016, 2017, 2021; Hurley *et al*., 2019; Painset *et al*., 2019) (Table S4). These findings highlight the importance of surveillance programs in farm animals, even in the absence of disease signs, to prevent pathogen transmission to humans through the food chain. This would be also of particular importance in cows on their second lactation and during winter times, when *L. monocytogenes* prevalence was significantly higher. Previous reports also showed that *L. monocytogenes* prevalence in cattle farms was higher during winter (Husu, 1990; Mohammed *et al*., 2009; Castro *et al*., 2018) and that an inadequate transition from the first to the second lactation could impair immune function (Roche *et al*., 2009) and predispose to *L. monocytogenes* colonization. Our findings also highlight the importance of antibiotic stewardship in veterinary medicine, since tetracycline resistance was detected more frequently in *L. innocua* isolates from farms using this antibiotic.

Here, the same genotypes were found in multiple animals and surfaces within the same farms, although the majority of them (72%) were sporadic. Moreover, with exception for one sheep, identical genotypes could not be detected in the same animal along different seasons,

suggesting that faecal shedding period is shorter than the time frame between our sampling dates (14–135 days). Indeed, faecal carriage of *L. monocytogenes* in healthy human adults is also reported to be transient (Grif *et al*., 2003) and previous experimental studies in sheep inoculated orally with a high dose of *L. monocytogenes* (10^{10} colony forming units) have shown that faecal shedding lasted for only 10 days (Zundel and Bernard, 2006). Studies in wild and domestic ruminants suggest that animals can silently carry *L. monocytogenes* in tonsils even without faecal shedding (Zundel and Bernard, 2006; Palacios-Gorba *et al*., 2021), which could explain why *L. monocytogenes* was not detected in the faeces of sheep herd where a listeriosis outbreak occurred.

In summary, our data show that (i) *L. innocua* and *L. monocytogenes* were the most prevalent *Listeria* spp. in both dairy ruminant faeces and farm-associated environments; (ii) single ruminants can harbour *L. monocytogenes* alone or together with *L. innocua* without clinical signs of infection; (iii) *L. monocytogenes* could be isolated from half of the dairy farms sampled; (iv) CC1 and CC4 hypervirulent *L. monocytogenes* clones, which are among the most common *L. monocytogenes* CCs responsible for human infection, represented 30% of the *L. monocytogenes* isolates retrieved in this study and were mainly obtained from host-associated samples (faeces); (v) the overall *L. monocytogenes* prevalence was higher in winter than in autumn in cattle farms and higher in winter and spring than autumn in sheep farms; and (vi) *L. monocytogenes* faecal shedding was intermittent and cows were more likely to shed *L. monocytogenes* on their second lactation.

Our data are consistent with the hypothesis that dairy farms may favour the selection of invasive *L. monocytogenes* clones, which are shed in the faeces more efficiently than hypovirulent clones (Maury *et al*., 2019), and constitute a reservoir for hypervirulent strains that can colonize dairy products. This study improves the understanding of *Listeria* spp. prevalence and ecology in the dairy ruminant environment and may contribute to the development of effective disease surveillance and control strategies to reduce the number of both human and animal listeriosis cases.

Experimental procedures

Farms

The study population consisted of 19 dairy ruminant farms (10 cattle, five sheep and four goats) with different housing systems, management practices and herd sizes located in the provinces (administrative division in Spain) of Valencia, Alicante, Castellón, Murcia and Albacete (mid-east and south-east of Spain) (Table S1; Fig. S1).

No history of clinical listeriosis had been observed in any of the farms before and/or during the sampling period, except for farm 'Sheep C' which suffered a listeriosis outbreak in the last season sampled (spring 2020).

Sample collection

During the sampling period (winter 2018 to spring 2019 and winter 2019 to spring 2020), each farm was visited once per season (autumn, winter and spring), for a total of three visits per farm (Fig. S1). Farm characteristics, sampling dates and *Listeria* spp. isolated are indicated in Tables S1 and S3. Farm 'Sheep B' was sampled 11 times during seven consecutive seasons from autumn 2018 to spring 2020 for *Listeria* genomic diversity analysis due to the discovery of the new species *L. valentina* in the 27-Feb-2019 sampling (Quereda *et al*., 2020). For consistency with data from other farms, only data from three consecutive seasons (autumn 07-Nov-2018, winter 27-Feb-2019 and spring 10-Apr-2019) in this farm were considered for prevalence and statistics calculations.

On each farm visit, 50 samples [32 samples of faeces from individual animals, three samples of feed, three samples of bedding, three MFS, and nine surface swabs (three from milking station floor, three from water troughs, three from food troughs)] were collected during three consecutive seasons (autumn, winter and spring) amounting to 150 samples per farm. The same 32 animals sampled during the first farm visit were monitored in the course of followings evaluations (three seasons total) by veterinarians during the usual handling of the animals, following the guidelines of European Union Directive 2010/63/EU for the protection of animals used for scientific purposes (European Union, 2010). Cows, sheep, or goats that were sold in the intervals between the sampling periods were replaced in the study with a new animal. MFS were selected for sampling since the prevalence of *L. monocytogenes* is twice that in BTM (Castro *et al*., 2018). Each sample was collected into a sterile bag by the use of clean gloves or sampling utensils. Rectal faecal grab samples were collected from randomly selected animals in each farm to have a representation of all lactation numbers. Faecal samples were obtained by rectal grab to avoid cross-contamination among animals. This routine veterinary practice does not require the approval of the Animal Ethics and Experimentation Committee. Bedding samples, food troughs samples, water troughs samples and milking station floor samples were collected from diverse locations on each farm. All samples were collected using disposable gloves by aseptic conditions and stored in clean coolers with ice packs for transit to the laboratory. Samples were processed within 2–12 h of collection (Fig. S1).

Animal cleanliness and production hygiene

A numerical scoring system for assessing animal cleanliness of five body areas (tail head, ventral abdomen, udder, upper rear limb and lower rear limb) was used for the individual animals as previously described (scale of 1–5, where score 1 = very clean, score 5 = heavily soiled) (Reneau *et al*., 2005). Production hygiene was evaluated based on the cleanliness of the premises (milk room, milking station, feed troughs, water troughs and beddings) on farm visits on a scale of 1–3 as previously described (Castro *et al*., 2018). A score of '1' corresponded to a major deficit in production hygiene, '2' to a minor deficit in production hygiene and '3' to no notable deficit in production hygiene.

Listeria *spp. isolation and identification*

Listeria spp. were isolated as previously described (Quereda *et al*., 2020; Palacios-Gorba *et al*., 2021). Briefly, 8 g of rectal faecal samples or bedding samples were diluted 1/10 in Half-Fraser broth (Scharlab, Spain), homogenized and incubated at 30°C for 24 h for enrichment. Swab samples (feed troughs, water troughs and milking station floor) were placed in 10 ml Half Fraser broth, vortexed for 2 min and incubated at 30°C for 24 h for enrichment. Entire MFS socks or 8 g of feed samples were used as sample material for primary enrichment in Half Fraser broth (30°C, 24 h). Samples were homogenized manually for 1 min until the solid matter was completely suspended in the enrichment solution. One hundred microliters of the incubated suspension were transferred to 10 ml Fraser broth (Scharlab) and incubated at 37°C for 24 h. After the second enrichment, 100 μl enriched culture and two 10-fold dilutions were transferred to RAPID'*L.mono* plates (BioRad, USA) and incubated at 37°C for 24 h. Characteristic *Listeria* spp. colonies were blue or white, with or without a yellow halo, round, convex, 1–2 mm [*L. monocytogenes* (PIPLC +/xylose-) forms blue colonies, *L. ivanovii* (PIPLC +/xylose+) forms blue-green colonies with distinct yellow halos, other *Listeria* spp. form white colonies]. When more than one type of colony was present in RAPID'*L. mono* plates, one *L. monocytogenes* colony (no *L. ivanovii* was detected) and one non-pathogenic *Listeria* spp. were picked and further confirmed in selective Oxford agar plates for *Listeria* (Scharlab) (colonies were approximately 2 mm in diameter, grey-green with a black sunken centre and a black halo) and Columbia CNA agar with 5% sheep blood agar plates (colonies were opaque, flat, 1–2 mm). Isolates were preserved in glycerol at −80°C and sent to the World Health Organization Collaborating Centre for *Listeria* (Institut Pasteur, Paris, France) for characterization. Species identification was performed by matrix-assisted laser desorption ionization-time of flight mass spectrometry using the MicroFlex LT system with the last MBT library DB-7854 (Bruker Daltonics, Germany), as previously described (Thouvenot *et al*., 2018) and by whole genome sequencing as previously described (Quereda *et al*., 2020).

Isolates carrying acquired resistance genes ($n = 23$) were subjected to antibiotic susceptibility testing using the disc diffusion method on Mueller Hinton Fastidious Agar (Becton-Dickinson, Germany) and the following antibiotics: ampicillin (10 μg), clindamycin (2 μg), erythromycin (15 μg), gentamycin (10 μg), kanamycin (30 μg), tetracycline (30 μg) and trimethoprim/sulfamethoxazole (1.25/23.75 μg). Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute (CLSI), 2017; EUCAST, 2020).

Genome sequencing and assembly

DNA extraction was carried out with the NucleoSpin Tissue purification kit (Macherey-Nagel, Germany) from 0.9 ml Brain heart infusion (Difco, USA) cultures grown overnight at 35°C. DNA libraries were prepared using the Nextera XT DNA Sample kit (Illumina, USA) and sequenced in a NextSeq 500 platform (Illumina) using 2 × 150 bp runs, according to the manufacturer's protocol. Raw reads were trimmed with fqCleaner v.3.0 (Alexis Criscuolo, Institut Pasteur, Paris) as previously described (Quereda *et al*., 2020; Palacios-Gorba *et al*., 2021), and assembled with SPAdes v.3.12.0 (Prjibelski *et al*., 2020) using automatic k-mer selection and the --only-assembler and --careful options.

Molecular typing and phylogenetic analysis

In silico typing was performed from the assemblies using the genoserogrouping (Doumith *et al*., 2004), MLST [seven loci (Ragon *et al*., 2008)], cgMLST profiles [1748 loci (Moura *et al*., 2016)], resistance and virulence schemes (244 loci) implemented at using BIGSdb-*Listeria* v.1.30 [https://bigsdb.pasteur.fr/listeria; (Jolley and Maiden, 2010; Moura *et al*., 2016)]. Genes were scanned using the BLASTN algorithm, with minimum nucleotide identity and alignment length coverage of 70% and word size of 10, as previously described (Moura *et al*., 2016). MLST profiles were classified into ST and grouped into CCs as previously described (Ragon *et al*., 2008). cgMLST profiles were grouped into CTs and SLs, using the cut-offs of 7 and 150 allelic mismatches respectively, as previously described (Moura *et al*., 2016). Minimum spanning trees and single linkage dendrograms were built from cgMLST profiles using Bionumerics 7.6

software (Applied Maths, Belgium) and annotated with iTol v.4.2 (Letunic and Bork, 2021). Assemblies were also screened for antimicrobial resistance genes and the presence of plasmids using ABRicate v.1.0.1 (https://github.com/tseemann/abricate) and MOB-suite v.2.0.1 (Robertson and Nash, 2018) respectively.

Statistical analysis

For DIM analysis cows were grouped into different categories considering the dairy cattle lactation curve and classified into 'early lactation' (0–120 days), 'mid-lactation' (121–240 days), 'late lactation' (241–360) and 'end of lactation' (>361 DIM). DIM were not analysed in sheep farms since all the ewes in the same farm were synchronized using intravaginal sponges and delivered approximately the same day. Shannon diversity indices and Hutcheson *T*-test (Hutcheson, 1970) were calculated using the web https://www.dataanalytics.org.uk/comparing-diversity/. The rest of the statistical analyses were conducted with IBM SPSS Statistics version 25. The significance level for all statistical tests was $P < 0.05$. Chi-square tests were performed to determine the effect of season, the number of lactation and DIM on the number of *L. monocytogenes* faecal shedders. Spearman's rank-order correlations were done to evaluate the association between the farm hygiene score and *L. monocytogenes* prevalence.

Acknowledgements

We are grateful for the willingness of the farmers to participate in this study. This work was supported by Generalitat Valenciana (Project references GV/2018/A/183, AICO/2021/278), the Spanish Ministry of Science and Innovation (Project reference PID2019-110764RA-I00/AEI/10.13039/501100011033) and Universidad CEU Cardenal Herrera Programa INDI 20/40 (J.J.Q.), the Spanish Ministry of Science and Innovation (Project reference PID2020-119462RA-I00/AEI/10.13039/ 501100011033) (A.G.-M.), Institut Pasteur, Inserm, Santé Publique France, and Fondation Le Roch-Les Mousquetaires (M.L.). J. J. Quereda is supported by a 'Ramón y Cajal' contract of the Spanish Ministry of Science, Innovation, and Universities (RYC-2018-024985-I). C. Palacios-Gorba is supported by a Predoctoral contract from the Universidad Cardenal Herrera-CEU. The funders had no role in study design, data collection, and interpretation, or the decision to submit the work for publication.

Data Availability Statement

Sequences obtained in this study are publicly available at the European Nucleotide Archive (BioProjects PRJEB45781 and PRJEB36008) and BIGSdb-Listeria (bigsdb.pasteur.fr/listeria).

References

Addis, M.F., Cubeddu, T., Pilicchi, Y., Rocca, S., and Piccinini, R. (2019) Chronic intramammary infection by *Listeria monocytogenes* in a clinically healthy goat – a case report. *BMC Vet Res* **15**: 229.

Borucki, M.K., Gay, C.C., Reynolds, J., McElwain, K.L., Kim, S.H., Call, D.R., and Knowles, D.P. (2005) Genetic diversity of *Listeria monocytogenes* strains from a high-prevalence dairy farm. *Appl Environ Microbiol* **71**: 5893–5899.

Carlin, C.R., Liao, J., Weller, D., Guo, X., Orsi, R., and Wiedmann, M. (2021) *Listeria cossartiae* sp. nov., *Listeria immobilis* sp. nov., *Listeria portnoyi* sp. nov. and *Listeria rustica* sp. nov., isolated from agricultural water and natural environments. *Int J Syst Evol Microbiol* **71**: 004795.

Castro, H., Jaakkonen, A., Hakkinen, M., Korkeala, H., and Lindström, M. (2018) Occurrence, persistence, and contamination routes of *Listeria monocytogenes* genotypes on three Finnish dairy cattle farms: a longitudinal study. *Appl Environ Microbiol* **84**: 1–14.

Cavicchioli, R., Ripple, W.J., Timmis, K.N., Azam, F., Bakken, L.R., Baylis, M., *et al*. (2019) Scientists' warning to humanity: microorganisms and climate change. *Nat Rev Microbiol* **17**: 569–586.

Charlier, C., Perrodeau, É., Leclercq, A., Cazenave, B., Pilmis, B., Henry, B., *et al*. (2017) Clinical features and prognostic factors of listeriosis: the MONALISA national prospective cohort study. *Lancet Infect Dis* **17**: 510–519.

Chow, J.T.H., Gall, A.R., Johnson, A.K., and Huynh, T.N. (2021) Characterization of *Listeria monocytogenes* isolates from lactating dairy cows in a Wisconsin farm: antibiotic resistance, mammalian cell infection, and effects on the fecal microbiota. *J Dairy Sci* **104**: 4561–4574.

Clinical and Laboratory Standards Institute (CLSI) (2017) Performance standards for antimicrobial susceptibility testing – approved guideline M100, 27th ed.

Cooper, J., and Walker, R.D. (1998) Listeriosis. *Vet Clin North Am Food Anim Pract* **14**: 113–125.

Costard, S., Espejo, L., Groenendaal, H., and Zagmutt, F.J. (2017) Outbreak-related disease burden associated with consumption of unpasteurized cow's milk and cheese, United States, 2009–2014. *Emerg Infect Dis* **23**: 957–964.

Dell'Armelina Rocha, P.R., Lomonaco, S., Bottero, M.T., Dalmasso, A., Dondo, A., Grattarola, C., *et al*. (2013) Ruminant rhombencephalitis-associated *Listeria monocytogenes* strains constitute a genetically homogeneous group related to human outbreak strains. *Appl Environ Microbiol* **79**: 3059–3066.

Doumith, M., Buchrieser, C., Glaser, P., Jacquet, C., and Martin, P. (2004) Differentiation of the major *Listeria monocytogenes* Serovars by multiplex PCR. *J Clin Microbiol* **42**: 3819–3822.

Dreyer, M., Aguilar-Bultet, L., Rupp, S., Guldimann, C., Stephan, R., Schock, A., *et al*. (2016) *Listeria monocytogenes* sequence type 1 is predominant in ruminant rhombencephalitis. *Sci Rep* **6**: 36419.

Esteban, J.I., Oporto, B., Aduriz, G., Juste, R.A., and Hurtado, A. (2009) Faecal shedding and strain diversity of *Listeria monocytogenes* in healthy ruminants and swine in northern Spain. *BMC Vet Res* **5**: 1–10.

EUCAST. (2020) Disk diffusion method for antimicrobial susceptibility testing, version 9.

European Union. (2010) Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. *Off J Eur Union*: 33–79.

Fox, E., Hunt, K., O'Brien, M., and Jordan, K. (2011) *Listeria monocytogenes* in Irish Farmhouse cheese processing environments. *Int J Food Microbiol* **145**: S39–S45.

Gray, M.J., Zadoks, R.N., Fortes, E.D., Dogan, B., Cai, S., Chen, Y., *et al*. (2004) *Listeria monocytogenes* isolates from foods and humans form distinct but overlapping populations. *Appl Environ Microbiol* **70**: 5833–5841.

Grif, K., Patscheider, G., Dierich, M.P., and Allerberger, F. (2003) Incidence of fecal carriage of *Listeria monocytogenes* in three healthy volunteers: a one-year prospective stool survey. *Eur J Clin Microbiol Infect Dis* **22**: 16–20.

Hafner, L., Pichon, M., Burucoa, C., Nusser, S.H., Moura, A., Garcia-Garcera, M., and Lecuit, M. (2021) Listeria monocytogenes faecal carriage is common and depends on the gut microbiota. *Nat Commun* **12**: 6826.

Haley, B.J., Sonnier, J., Schukken, Y.H., Karns, J.S., and Van Kessel, J.A.S. (2015) Diversity of *Listeria monocytogenes* within a U.S. Dairy Herd, 2004-2010. *Foodborne Pathog Dis* **12**: 844–850.

Ho, A.J., Ivanek, R., Gröhn, Y.T., Nightingale, K.K., and Wiedmann, M. (2007) *Listeria monocytogenes* fecal shedding in dairy cattle shows high levels of day-to-day variation and includes outbreaks and sporadic cases of shedding of specific *L. monocytogenes* subtypes. *Prev Vet Med* **80**: 287–305.

Hurley, D., Luque-Sastre, L., Craig, T.P., Huynh, S., Eshwar, A.K., Nguyen, S.V., *et al*. (2019) Whole-genome sequencing-based characterization of 100 *Listeria monocytogenes* isolates collected from food processing environments over a four-year period. *mSphere* **4**: e00252-19.

Hurtado, A., Ocejo, M., and Oporto, B. (2017) *Salmonella* spp. and *Listeria monocytogenes* shedding in domestic ruminants and characterization of potentially pathogenic strains. *Vet Microbiol* **210**: 71–76.

Husu, J.R. (1990) Epidemiological studies on the occurrence of *Listeria monocytogenes* in the feces of dairy cattle. *J Vet Med Ser B* **37**: 276–282.

Husu, J.R., Seppänen, J.T., Sivelä, S.K., and Rauramaa, A. L. (1990) Contamination of raw Milk by *Listeria monocytogenes* on dairy farms. *J Vet Med Ser B* **37**: 268–275.

Hutcheson, K. (1970) A test for comparing diversities based on the shannon formula. *J Theor Biol* **29**: 151–154.

Ianevski, A., Zusinaite, E., Shtaida, N., Kallio-Kokko, H., Valkonen, M., Kantele, A., *et al*. (2019) Low temperature and low UV indexes correlated with peaks of influenza virus activity in northern Europe during 2010–2018. *Viruses* **11**: 207.

Jayarao, B.M., Donaldson, S.C., Straley, B.A., Sawant, A.A., Hegde, N.V., and Brown, J.L. (2006) A survey of foodborne pathogens in bulk tank milk and raw milk consumption among farm families in Pennsylvania. *J Dairy Sci* **89**: 2451–2458.

Jolley, K.A., and Maiden, M.C.J. (2010) BIGSdb: scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics* **11**: 595.

Keys, A.L., Dailey, R.C., Hitchins, A.D., and Smiley, R.D. (2013) Postenrichment population differentials using buffered *Listeria* enrichment broth: implications of the presence of *Listeria innocua* on *Listeria monocytogenes* in food test samples. *J Food Prot* **76**: 1854–1862.

Kwong, J.C., Mercoulia, K., Tomita, T., Easton, M., Li, H.Y., Bulach, D.M., *et al*. (2016) Prospective whole-genome sequencing enhances national surveillance of listeria monocytogenes. *J Clin Microbiol* **54**: 333–342.

Latorre, A.A., Pradhan, A.K., Van Kessel, J.A.S., Karns, J. S., Boor, K.J., Rice, D.H., *et al*. (2011) Quantitative risk assessment of listeriosis due to consumption of raw milk. *J Food Prot* **74**: 1268–1281.

Latorre, A.A., Van Kessel, J.S., Karns, J.S., Zurakowski, M. J., Pradhan, A.K., Boor, K.J., *et al*. (2010) Biofilm in milking equipment on a dairy farm as a potential source of bulk tank milk contamination with *Listeria monocytogenes*. *J Dairy Sci* **93**: 2792–2802.

Letunic, I., and Bork, P. (2021) Interactive tree of life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res* **49**: W293–W296.

Linnan, M.J., Mascola, L., Lou, X.D., Goulet, V., May, S., Salminen, C., *et al*. (1988) Epidemic listeriosis associated with Mexican-style cheese. *N Engl J Med* **319**: 823–828.

MacDonald, P.D.M., Whitwam, R.E., Boggs, J.D., MacCormack, J.N., Anderson, K.L., Reardon, J.W., *et al*. (2005) Outbreak of listeriosis among Mexican immigrants as a result of consumption of illicitly produced Mexican-style cheese. *Clin Infect Dis* **40**: 677–682.

Maury, M.M., Bracq-Dieye, H., Huang, L., Vales, G., Lavina, M., Thouvenot, P., *et al*. (2019) Hypervirulent *Listeria monocytogenes* clones' adaption to mammalian gut accounts for their association with dairy products. *Nat Commun* **10**: 2488.

Maury, M.M., Tsai, Y.H., Charlier, C., Touchon, M., Chenal-Francisque, V., Leclercq, A., *et al*. (2016) Uncovering *Listeria monocytogenes* hypervirulence by harnessing its biodiversity. *Nat Genet* **48**: 308–313.

Mohammed, H.O., Stipetic, K., McDonough, P.L., Gonzalez, R.N., Nydam, D.V., and Atwill, E.R. (2009) Identification of potential on-farm sources of *Listeria monocytogenes* in herds of dairy cattle. *Am J Vet Res* **70**: 383–388.

Moura, A., Criscuolo, A., Pouseele, H., Maury, M.M., Leclercq, A., Tarr, C., *et al*. (2016) Whole genome-based population biology and epidemiological surveillance of *Listeria monocytogenes*. *Nat Microbiol* **2**: 16185.

Moura, A., Lefrancq, N., Wirth, T., Leclercq, A., Borges, V., Gilpin, B., *et al*. (2021) Emergence and global spread of *Listeria monocytogenes* main clinical clonal complex. *Sci Adv* **7**. https://pubmed.ncbi.nlm.nih.gov/34851675/

Moura, A., Tourdjman, M., Leclercq, A., Hamelin, E., Laurent, E., Fredriksen, N., *et al*. (2017) Real-time whole-genome sequencing for surveillance of *Listeria monocytogenes*, France. *Emerg Infect Dis* **23**: 1462–1470.

Murinda, S.E., Nguyen, L.T., Nam, H.M., Almeida, R.A., Headrick, S.J., and Oliver, S.P. (2004) Detection of sorbitol-negative and sorbitol-positive Shiga toxin-producing *Escherichia coli*, *Listeria monocytogenes*, *Campylobacter jejuni*, and *Salmonella* spp. in dairy farm environmental samples. *Foodborne Pathog Dis* **1**: 97–104.

Nightingale, K.K., Schukken, Y.H., Nightingale, C.R., Fortes, E.D., Ho, A.J., Her, Z., *et al*. (2004) Ecology and transmission of *Listena monocytogenes* infecting ruminants and in the farm environment. *Appl Environ Microbiol* **70**: 4458–4467.

Oliver, S.P., Jayarao, B.M., and Almeida, R.A. (2005) Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. *Foodborne Pathog Dis* **2**: 115–129.

Orsi, R.H., and Wiedmann, M. (2016) Characteristics and distribution of *Listeria* spp., including *Listeria* species newly described since 2009. *Appl Microbiol Biotechnol* **100**: 5273–5287.

Painset, A., Björkman, J.T., Kiil, K., Guillier, L., Mariet, J.-F., Félix, B., *et al*. (2019) LiSEQ – whole-genome sequencing of a cross-sectional survey of *Listeria monocytogenes* in ready-to-eat foods and human clinical cases in Europe. *Microb Genomics* **5**: e000257.

Palacios-Gorba, C., Moura, A., Leclercq, A., Gómez-Martín, Á., Gomis, J., Jiménez-Trigos, E., *et al*. (2021) *Listeria* spp. isolated from tonsils of wild deer and boars: genomic characterization. *Appl Environ Microbiol* **87**: e02651-20.

Papić, B., Pate, M., Félix, B., and Kušar, D. (2019) Genetic diversity of *Listeria monocytogenes* strains in ruminant abortion and rhombencephalitis cases in comparison with the natural environment. *BMC Microbiol* **19**: 299.

Pérez-Trallero, E., Zigorraga, C., Artieda, J., Alkorta, M., and Marimón, J.M. (2014) Two outbreaks of *Listeria monocytogenes* infection, northern Spain. *Emerg Infect Dis* **20**: 2155–2157.

Prjibelski, A., Antipov, D., Meleshko, D., Lapidus, A., and Korobeynikov, A. (2020) Using SPAdes de novo assembler. *Curr Protoc Bioinformatics* **70**: e102.

Quereda, J.J., Leclercq, A., Moura, A., Vales, G., Gómez-Martín, Á., García-Muñoz, Á., *et al*. (2020) *Listeria valentina* sp. nov., isolated from a water trough and the faeces of healthy sheep. *Int J Syst Evol Microbiol* **70**: 5868–5879.

Ragon, M., Wirth, T., Hollandt, F., Lavenir, R., Lecuit, M., Le Monnier, A., and Brisse, S. (2008) A new perspective on *Listeria monocytogenes* evolution. *PLoS Pathog* **4**: e1000146.

Ramage, C.P., Low, J.C., McLauchlin, J., and Donachie, W. (1999) Characterisation of *listeria ivanovii* isolates from the UKusing pulsed-field gel electrophoresis. *FEMS Microbiol Lett* **170**: 349–353.

Reneau, J.K., Seykora, A.J., Heins, B.J., Endres, M.I., Farnsworth, R.J., and Bey, R.F. (2005) Association between hygiene scores and somatic cell scores in dairy cattle. *J Am Vet Med Assoc* **227**: 1297–1301.

Robertson, J., and Nash, J.H.E. (2018) MOB-suite: software tools for clustering, reconstruction and typing of plasmids from draft assemblies. *Microb Genomics* **4**: e000206.

Roche, J.R., Friggens, N.C., Kay, J.K., Fisher, M.W., Stafford, K.J., and Berry, D.P. (2009) Invited review: body condition score and its association with dairy cow productivity, health, and welfare. *J Dairy Sci* **92**: 5769–5801.

Sanaa, M., Poutrel, B., Menard, J.L., and Serieys, F. (1993) Risk factors associated with contamination of raw milk by *Listeria monocytogenes* in dairy farms. *J Dairy Sci* **76**: 2891–2898.

Sauders, B.D., Overdevest, J., Fortes, E., Windham, K., Schukken, Y., Lembo, A., and Wiedmann, M. (2012) Diversity of *Listeria* species in urban and natural environments. *Appl Environ Microbiol* **78**: 4420–4433.

Schlech, W.F., Lavigne, P.M., Bortolussi, R.A., Allen, A.C., Haldane, E.V., Wort, A.J., *et al*. (1983) Epidemic listeriosis — evidence for transmission by food. *N Engl J Med* **308**: 203–206.

Skovgaard, N., and Morgen, C.A. (1988) Detection of *Listeria* spp. in faeces from animals, in feeds, and in raw foods of animal origin. *Int J Food Microbiol* **6**: 229–242.

Stasiewicz, M.J., Oliver, H.F., Wiedmann, M., and den Bakker, H.C. (2015) Whole-genome sequencing allows for improved identification of persistent *Listeria monocytogenes* in food-associated environments. *Appl Environ Microbiol* **81**: 6024–6037.

Swaminathan, B., and Gerner-Smidt, P. (2007) The epidemiology of human listeriosis. *Microbes Infect* **9**: 1236–1243.

Thouvenot, P., Vales, G., Bracq-Dieye, H., Tessaud-Rita, N., Maury, M.M., Moura, A., *et al*. (2018) MALDI-TOF mass spectrometry-based identification of *Listeria* species in surveillance: a prospective study. *J Microbiol Methods* **144**: 29–32.

Van Kessel, J.A.S., Karns, J.S., Lombard, J.E., and Kopral, C.A. (2011) Prevalence of *Salmonella enterica*, *Listeria monocytogenes*, and *Escherichia coli* virulence factors in bulk tank milk and in-line filters from U.S. dairies. *J Food Prot* **74**: 759–768.

Vázquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., *et al*. (2001) *Listeria* pathogenesis and molecular virulence determinants. *Clin Microbiol Rev* **14**: 584–640.

Vilar, M.J., Yus, E., Sanjuán, M.L., Diéguez, F.J., and Rodríguez-Otero, J.L. (2007) Prevalence of and risk factors for *Listeria* species on dairy farms. *J Dairy Sci* **90**: 5083–5088.

Walland, J., Lauper, J., Frey, J., Imhof, R., Stephan, R., Seuberlich, T., and Oevermann, A. (2015) *Listeria monocytogenes* infection in ruminants: is there a link to the environment, food and human health? A review. *Schweiz Arch Tierheilkd* **157**: 319–328.

Wang, H., Luo, L., Zhang, Z., Deng, J., Wang, Y., Miao, Y., *et al*. (2018) Prevalence and molecular characteristics of *Listeria monocytogenes* in cooked products and its comparison with isolates from listeriosis cases. *Front Med* **12**: 104–112.

Wiedmann, M., Bruce, J.L., Keating, C., Johnson, A.E., McDonough, P.L., and Batt, C.A. (1997) Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. *Infect Immun* **65**: 2707–2716.

Winter, P., Schilcher, F., Bagò, Z., Schoder, D., Egerbacher, M., Baumgartner, W., and Wagner, M. (2004) Clinical and histopathological aspects of naturally occurring mastitis caused by *Listeria monocytogenes* in cattle and ewes. *J Vet Med Ser B Infect Dis Vet Public Heal* **51**: 176–179.

Yusuf, S., Piedimonte, G., Auais, A., Demmler, G., Krishnan, S., Van Caeseele, P., *et al*. (2007) The

relationship of meteorological conditions to the epidemic activity of respiratory syncytial virus. *Epidemiol Infect* **135**: 1077–1090.

Zhang, H., Chen, W., Wang, J., Xu, B., Liu, H., Dong, Q., and Zhang, X. (2020) 10-year molecular surveillance of *Listeria monocytogenes* using whole-genome sequencing in Shanghai, China, 2009–2019. *Front Microbiol* **11**: 551020.

Zhao, Q., Hu, P., Li, Q., Zhang, S., Li, H., Chang, J., *et al*. (2021) Prevalence and transmission characteristics of *Listeria* species from ruminants in farm and slaughtering environments in China. *Emerg Microbes Infect* **10**: 356–364.

Zundel, E., and Bernard, S. (2006) *Listeria monocytogenes* translocates throughout the digestive tract in asymptomatic sheep. *J Med Microbiol* **55**: 1717–1723.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Flow chart showing the process of sample collection and laboratory methods.

Table S1. Characteristics and management practices of the investigated dairy farms.

Table S2. Prevalence of non-pathogenic *Listeria*, *L. monocytogenes*, and total *Listeria* spp. in faeces samples and the farm environment from 19 Spanish dairy farms.

Table S3. *Listeria* isolates characterized in this study.

Table S4. cgMLST types previously defined at BIGsdb-*Listeria* detected in this study ($n = 13$).

Table S5. Individual animals where *L. monocytogenes* were isolated repeatedly in different samplings

Table S6. Correlation of hygienic scores and prevalence of *L. monocytogenes* on the investigated dairy farms. Hygienic scores were calculated as described in Material and Methods to assess animal cleanliness and production hygiene.