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Phenotypic and genotypic antimicrobial resistance of *Listeria* spp. in Spain

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ABSTRACT

Listeriosis is a zoonotic disease caused by *Listeria monocytogenes* and *Listeria ivanovii*. The genus *Listeria* currently includes 27 recognized species and is found throughout the environment. The number of systematic studies on antimicrobial resistance *in L. monocytogenes* isolates from domestic farms using antimicrobial substances is limited. Importantly, dairy ruminant farms are reservoir of hypervirulent lineage I *L. monocytogenes* isolates, previously associated with human clinical cases. Considering that the classes of antibiotics used in food-producing domestic animals are frequently the same or closely related to those used in human medicine, studies about the impact of antibiotic use on the acquisition of antibiotic resistance in *Listeria* spp. in domestic animal farms are, therefore, of high importance. Here, susceptibility to 25 antibiotics was determined. Eighty-one animal-related, 35 food and 21 human pathogenic *Listeria* spp. isolates and 114 animal-related non-pathogenic *Listeria* spp. isolates were tested. Whole genome sequencing data was used for molecular characterization. Regarding *L. monocytogenes*, 2 strains from the clinical-associated linage I showed resistance to erythromycin, both related to dairy ruminants. Acquired resistance to one antibiotic was exhibited in 1.5% of *L. monocytogenes* isolates compared with 14% of non-pathogenic *Listeria* spp. isolates. Resistance to tetracycline (7.9%), doxycycline (7.9%), penicillin (4.4%), and ampicillin (4.4%) were the most frequently observed in non-pathogenic *Listeria* spp. While resistance to two or more antibiotics (5.6%) was most common in *Listeria* spp., isolates, resistance to one antibiotic was also observed (1.6%). The present results show that non-pathogenic *Listeria* spp. harbour antimicrobial resistance genes.

1. Introduction

The genus *Listeria* currently includes 27 recognized species of small rod-shaped gram-positive bacteria ubiquitous in nature (Raufu et al., 2022). The species in the genus *Listeria* are divided into two groups, *Listeria sensu stricto* (the clade of interest to public health as it contains *L. monocytogenes* and *L. ivanovii*, as well as other non-pathogenic species, such as *L. innocua*, *L. seeligeri*, *L. marthii*, *L. welshimeri*, *L. cossartiae*, *L. farberi*, and *L. immobilis*) and *Listeria sensu lato* group, which includes the other 18 non-pathogenic *Listeria* spp. (Raufu et al., 2022). *L. monocytogenes* and *L. ivanovii* can cause foodborne infections in humans and other mammals including ruminants, leading to septicemia, meningo-encephalitis, fetal–placental infection and abortion, as opposed to other *Listeria* species which are non-pathogenic (Disson et al., 2021; Quereda et al., 2021). *L. monocytogenes* is the foodborne pathogen associated with the highest case-fatality rate in the western hemisphere

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(e.g., 20–30%) (Charlier et al., 2017; Datta and Burall, 2018; EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), (2021); Kammoun et al., 2022). Natural atypical hemolytic *L. innocua* isolates have been reported. These atypical hemolytic *L. innocua* isolates are virulent albeit to a lesser degree than *L. monocytogenes* (Moura et al., 2019). Rare cases of *L. innocua* septicemia and meningitis infections were previously reported, both in humans and in ruminants (Walker et al., 1994; Perrin et al., 2003; Rocha et al., 2013; Rana et al., 2014). Apart from these sporadic cases of *L. innocua* in humans and animals, non-pathogenic *Listeria* spp. are important since antibiotic resistance genes can be transferred by conjugative plasmids and transposons between species of *Listeria* (Charpentier and Courvalin, 1999). Pathogenic and non-pathogenic *Listeria* spp. are widespread in nature, being found in soil, sewage, water environments, plants, animals, and food (Linke et al., 2014; Orsi and Wiedmann, 2016; Quereda et al., 2021).

Standard therapy for listeriosis is based on ampicillin, amoxicillin or penicillin G combined with gentamicin, although trimethoprim/sulfamethoxazole is generally used in case of intolerance to beta-lactams (Charpentier and Courvalin, 1999; Janakiraman, 2008; Charlier et al., 2017). Aminopenicillins can be substituted by linezolid, cotrimoxazole, rifampicin or fluoroquinolones (Baquero et al., 2020). Erythromycin is used to treat listeriosis in cases of pregnancy and vancomycin is employed in nonmeningeal infections (Jones and MacGowan, 1995; Janakiraman, 2008; Baquero et al., 2020). Several studies have focused on antibiotic resistance of human, environmental and food *L. monocytogenes* isolates, showing susceptibility to common antibiotics used in human and animal infections (Charpentier and Courvalin, 1999; Morvan et al., 2010; Granier et al., 2011; Yan et al., 2019; Baquero et al., 2020; Moura et al., 2023). *Listeria* spp. in nature or food environments are not exposed to the antibiotic concentration commonly used in domestic animal farms, which are a source of hypervirulent *L. monocytogenes* clones (Palacios-Gorba et al., 2021). Importantly, the classes of antibiotics used in food-producing domestic animals are frequently the same or closely related to those used in human medicine (World Health Organisation WHO (2018)). Regarding *Listeria* spp. in domestic animal farms, there is scarce information concerning the impact of antibiotic use in the development of acquired resistance. Unlike *L. monocytogenes*, few studies have focused on *L. ivanovii* and non-pathogenic *Listeria* spp. genotypic and phenotypic antibiotic resistance (MacGowan et al., 1990; Charpentier et al., 1995; Luque-Sastre et al., 2018). Moreover, there is limited information regarding non-pathogenic *Listeria* species harbouring antibiotic resistance genes and their role as future donors for resistance determinants to pathogenic species.

In the present work, pathogenic and non-pathogenic *Listeria* spp. from different ecological niches (dairy ruminants and wild animals, environmental samples in direct contact with animals, human and food isolates) and subjected or not to antibiotic pressure (e.g., isolates obtained from intensive domestic animal farms are under antibiotic pressure) were selected for genotypic and phenotypic antimicrobial resistance determination by using whole genome sequencing, disc diffusion and minimum inhibitory concentration (MIC) methods.

2. Materials and methods

2.1. Bacterial strains

A total of 251 isolates of *Listeria* spp. were selected. Regarding *L. monocytogenes* (n=132), 73 were from previous studies (Palacios-Gorba et al.,2021) and 59 firstly characterized by WGS in the present study (35 isolates from food, 21 clinical isolates from blood culture and cerebrospinal fluid, and 3 animal-related isolates). Regarding the other *Listeria* spp., *L. innocua* (n=89), *L. seeligeri* (n=6), *L. valentina* (n=6), *L. ivanovii* subsp. *ivanovii* (n=5), *L. newyorkensis* (n=5), *L. fleischmannii* subsp. *coloradonensis* (n=4), *L. aquatica* (n=3) and *L. thailandensis* (n=1) were selected (Table S1). One hundred and seven non-pathogenic strains were selected from previous studies (Palacios-Gorba et al.,2021) and 7 firstly characterized by WGS in the present study (Table S1). *L. monocytogenes* strains tested for antimicrobial susceptibility in the present study were selected on the basis of a unique cgMLST type. *L. monocytogenes* and *L. innocua* strains were selected based on clonal type (CT) and sequence type (ST) diversity, respectively. The same *L. monocytogenes* CT (n=17) and *L. innocua* ST (n=20) are present in this study in cases where the same CT or ST originated from a distinct sample source. The isolates were obtained from samples collected in central-eastern Spain between 2019 and 2021 from diverse ecological niches and animal hosts subjected or not subjected to antibiotic pressure (Table 1 and Table S1). In the present study, the classification animal-related isolates included bacterial isolates from animal faeces, udder or tonsils, and environmental samples in a direct contact with animals (e.g., bedding from ruminant farms) (Table S1).

2.2. Bacterial susceptibility testing

All isolates were tested for antimicrobial susceptibility to a panel of 25 antibiotics using disc diffusion method, as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST (2021)): penicillin, ampicillin, ampicillin + sulbactam, amoxicillin + clavulanic acid, cefotaxime, imipenem, meropenem, vancomycin, fosfomycin, ciprofloxacin, levofloxacin, nalidixic acid, rifampicin, trimethoprim-sulfamethoxazole (co-trimoxazole), sulfonamide, doxycycline, tetracycline, tigecycline, kanamycin, gentamicin, fusidic acid, erythromycin, clindamycin, linezolid, and chloramphenicol. Furthermore, all isolates were tested for Minimum Inhibitory Concentration (MIC) to 4 antibiotics of clinical interest: ampicillin [0.016–256 mg/L], meropenem [0.002–32 mg/L], trimethoprim*-sulfamethoxazole (co-trimoxazole) [0.002–32* mg/L] and erythromycin [0.016–256 mg/L], using Liofilchem® MTS™ (MIC Test Strip). Both tests were carried on Mueller Hinton Fastidious Agar (MH-F) plates (BD 257491).

Data were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Comite de L'antibiogramme de la Societe Francaise de Microbiologie CA-SFM ("CA-SFM. 2013," n.d.; EUCAST (2021)). *Staphylococcus aureus* ATCC 25923 and *Streptococcus pneumoniae* ATCC 49619 were used as quality control strains.

2.3. Whole genome sequencing and molecular characterization

As previously described (Moura et al., 2023), genomic DNA was extracted from 0.9 mL Brain heart infusion cultures grown overnight at 35°C using either the DNeasy Blood and Tissue Extraction kit (Qiagen) or the NucleoSpin Tissue purification kit (Macherey-Nagel). Illumina NextSeq 500 was used to sequence the DNA libraries prepared using the Nextera XT DNA Sample kit (Illumina). Using fqCleaner v.21.1 (gitlab.pasteur.fr/GIPhy/fqCleanER/), raw reads were trimmed and assembled using unicycler v.0.4.8 (https://github.com/rrwick/Unicycler).

Molecular characterization of all 251 isolates was done using the *Listeria* sequence typing database available on BIGSdb platform [http://bigsdb.pasteur.fr/listeria; last accessed November 27th 2023]. Genome search of 25 antimicrobial resistance genes was done using BIGSdb (*aacA4*, *aadC*, *aadE*, *aphA*, *cat_CHL*, *dfrD*, *dfrK*, *erm*(B), *erm*(G), *fexA*, *fosX*, *lmo0919*, *lnu*(A), *lnu*(G), *mef*(A), *mph*(B), *mprF*, *msr*(D), *norB*, *penA*, *qnrB*, *str* , *sul*, *tet*(M), *tet*(S)).

Single gene alignments were performed using BLAST software against β-Lactamases and ESBLs families (Ambler class A-D β-lactamases and ESBLs), macrolide–lincosamide–streptogramin B (MLS), tetracyclines and acquired chloramphenicol resistance genes (van Hoek et al., 2011).

Apart from the analyzed 25 antibiotic resistance genes available in BIGSdb, reference sequences for the *mph*(B) gene and *tet*(M)/*tet*(W)/*tet*

Table 1
Number of isolates obtained in this study (N=251).

	Listeria spp.	Dairy ruminants (*n*=105)	Wild animals (*n*=5)	Farm environment (*n*=85)	Human (*n*=21)	Food (*n*=35)	Total (N=251)
Sensu stricto	*L. monocytogenes* (lineage I)	33 (31.4%)		19 (22.3%)	13 (61.9%)	5 (14.3%)	70 (27.9%)
	L. monocytogenes (lineage II)	16 (15.2%)		8 (9.4%)	8 (38.1%)	30 (85.7%)	62 (24.7%)
	L. innocua	41 (39%)		48 (56.5%)			89 (35.4%)
	L. ivanovii subsp. *ivanovii*	5 (4.8%)					5 (2%)
	L. seeligeri		5 (100%)	1 (1.2%)			6 (2.4%)
Sensu lato	*L. valentina*	5 (4.8%)		1 (1.2%)			6 (2.4%)
	L. newyorkensis			5 (5.9%)			5 (2%)
	L. fleischmannii subsp. *coloradonensis*	1 (0.9%)		3 (3.5%)			4 (1.6%)
	L. aquatica	3 (2.8%)					3 (1.2%)
	L. thailandensis	1 (0.9%)					1 (0.4%)
	Total	105 (41.8%)	5 (2%)	85 (33.9%)	21 (8.4%)	35 (13.9%)	251 (100%)

(O)/*tet*(S) were obtained from Uniprot (W7AYF2 and A0A841ZN34, respectively).

2.4. Statistical analysis

Statistical tests were performed with GraphPad Prism V8 software system. Differences in MIC between *L. innocua*, lineage I *L. monocytogenes* and lineage II *L. monocytogenes* were analyzed using the nonparametric Kruskall-Wallis and Mann-Whitney tests. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Antimicrobial susceptibility profiles

Disc diffusion and MIC methods were compared for the reference antibiotics indicated by the European Committee on Antimicrobial Susceptibility Testing (EUCAST (2021)) (ampicillin, erythromycin, meropenem and trimethoprim-sulfamethoxazole). The results obtained by the disc diffusion method and the MIC were equivalent in all isolates and antibiotics tested, classifying the isolates in the same interpretative category (susceptible or resistant) using both methods (Table S2 and Table S3).

All *Listeria* spp. isolates were susceptible to 13 out of the 25 tested antimicrobial compounds: imipenem, meropenem, ampicillin + sulbactam, vancomycin, levofloxacin, rifampicin, trimethoprim-

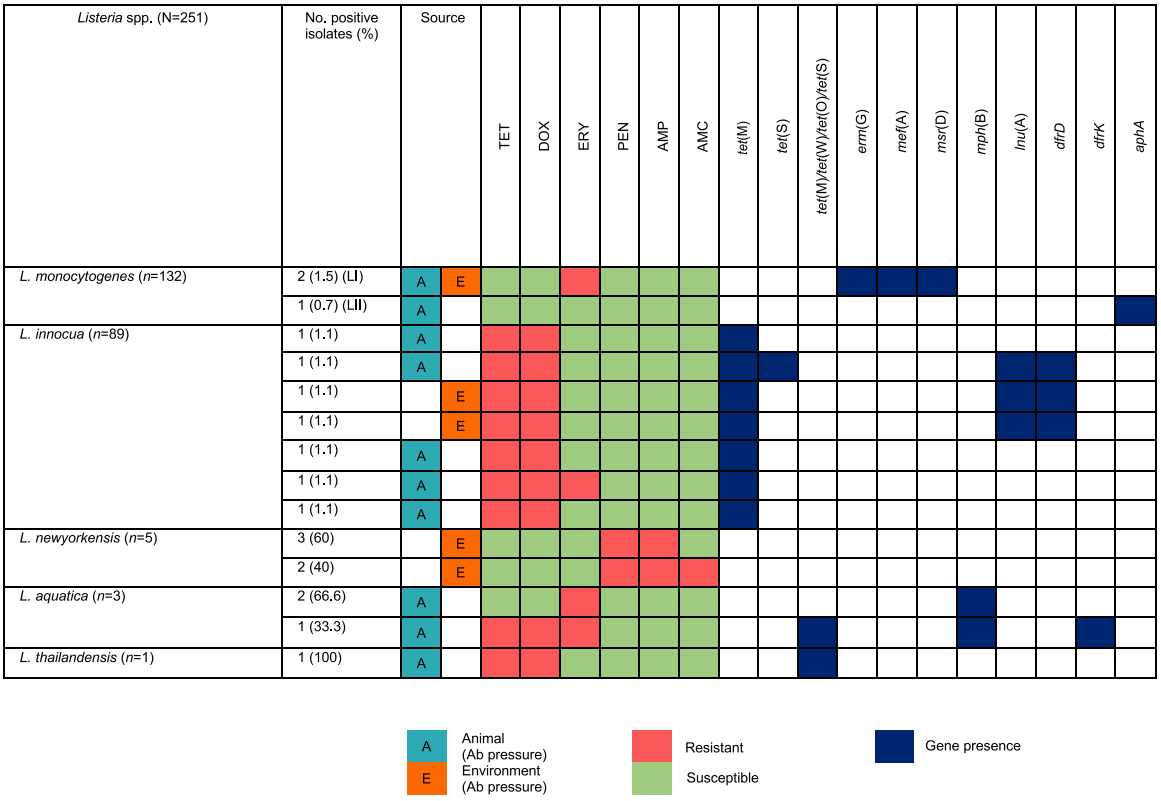

Fig. 1. Antibiotic resistance phenotypes and genotypes of all *Listeria* spp. isolates in this study (N=251). Abbreviations: Ab: antibiotic; LI: lineage I; LII: lineage II. [a]Phenotypic resistance: tetracycline (TET), doxycycline (DOX), erythromycin (ERY), penicillin (PEN), ampicillin (AMP), amoxicillin/clavulanic acid (AMC). [b]Genotypic resistance: tetracycline resistance (*tet*(M), *tet*(S), *tet*(M)/*tet*(W)/*tet*(O)/*tet*(S)); macrolide-lincosamide-streptogramin B class (MLS) resistance (*erm*(G), *mef*(A), *msr*(D), *mph*(B), *lnu*(A)); trimethoprim resistance (*dfrD*, *dfrK*); aminoglycoside resistance (*aphA*).

sulfamethoxazole, sulfonamide, tigecycline, kanamycin, gentamicin, linezolid, and chloramphenicol. All strains were sensitive to ciprofloxacin, except 65 *L. monocytogenes* (49.2%, 65/132), 71 *L. innocua* (79.8%, 71/89), 3 *L. seeligeri* (50%, 3/6), and 1 *L. thailandensis* (100%, 1/1) which were resistant, confirming previous results (Troxler et al., 2000; Leclercq et al., 2019) (Table S3).

All *Listeria* spp. were naturally resistant to nalidixic acid, and naturally resistant to cefotaxime (except 1 *L. seeligeri* (16.7%, 1/6) and 6 strains of *L. valentina* (100%, 6/6) which were borderline resistant to cefotaxime) (Table S3). All *Listeria* spp. were resistant to fosfomycin except 1 *L. monocytogenes* (0.7%, 1/132), 4 *L. ivanovii* subsp. *ivanovii* (80%, 4/5), 1 *L. newyorkensis* (20%, 1/5), 3 *L. seeligeri* (50%, 3/6), and 6 *L. valentina* (100%, 6/6) (Table S3). All strains were resistant to fusidic acid except 9 strains of *L. innocua* (10.1%, 9/89), 1 *L. seeligeri* (16.7%, 1/6), 6 *L. valentina* (100%, 6/6), and 5 *L. ivanovii* subsp. *Ivanovii* (100%, 5/5) (Table S3). All *Listeria* spp. were resistant to clindamycin except 7 lineage II *L. monocytogenes* (5.3%, 7/132), 6 *L. seeligeri* (100%, 6/6), and 1 *L. ivanovii* subsp. *ivanovii* strains (Table S3).

Among the 132 *L. monocytogenes* strains tested, 2 ruminant-related strains (1.5%, 2/132) belonging to the clinical-associated and hypervirulent lineage I were resistant to erythromycin (Fig. 1; Table S2 and Table S3). This contrasts with the higher prevalence of resistance found in non-pathogenic *Listeria* spp., where 16 (14%, 16/114) strains were resistant to at least 1 antibiotic, of which 2 isolates reported multidrug resistance (MDR, defined as resistance to two or more antibiotics of different classes). Importantly, no statistically significant differences in the number of antimicrobial resistances were found in *L. monocytogenes* strains from animal-related isolates (n=76) when compared to *L. monocytogenes* strains isolated from food or human cases (n=56) in the present study (P=0.13). However, the 2 resistant *L. monocytogenes* strains were related to dairy ruminants, subjected to antibiotic pressure. The most frequently observed phenotypic resistance in non-pathogenic *Listeria* spp. were tetracycline (7.9%, 9/114), doxycycline (7.9%, 9/114), penicillin (4.4%, 5/114) and ampicillin (4.4%, 5/114). While resistance to two or more antibiotics (5.6%) was most common in *Listeria* spp., isolates resistant to one antibiotic were also observed (1.6%).

Whole genome sequencing (WGS) identification of antimicrobial resistance genes revealed that in most of the tested *Listeria* spp., 4 intrinsic resistance core genes were detected, *fosX*, *lmo0919*, *norB*, and *sul*, conferring resistance to fosfomycin, lincosamides, quinolones, and sulfonamides, respectively. The *fosX* gene was absent in all *L. aquatica*, *L. fleischmannii* subsp. *coloradonensis*, *L. thailandensis*, *L. valentina*, 4 *L. seeligeri* (66.7%, 4/6) and 1 *L. newyorkensis* (20%, 1/5). The *lmo0919* gene was not present in either of *L. aquatica*, *L. fleischmannii* subsp. *coloradonensis*, *L. thailandensis*, *L. valentina* and *L. newyorkensis* isolates and 1 *L. monocytogenes* isolate (0.75%, 1/132). No antimicrobial resistance genes belonging to lincosamide family were detected in the phenotypically clindamycin-resistant strains that lacked *lmo0919* (van Hoek et al., 2011). All *L. newyorkensis* lacked *norB*. The *sul* gene was present in all isolates, except in all *L. valentina* (100%, 6/6), and *L. newyorkensis* (100%, 5/5).

3.2. Tetracycline antibiotics resistance

None of the pathogenic *Listeria* spp. isolates expressed neither phenotypic nor harbored genotypic resistance to tetracycline. However, 7 *L. innocua* (7.9%, 7/89), 1 *L. aquatica* (33.3%, 1/3), and 1 *L. thailandensis* (100%, 1/1) were phenotypically resistant to tetracycline (Fig. 1; Table S3). The *tet*(M) and *tet*(S) genes, coding for ribosome protection proteins, were detected in the tetracycline-resistant *L. innocua* isolates, where *tet*(M) was detected in all isolates and *tet*(S) was detected in one isolate (Fig. 1). The resistant *L. aquatica* and *L. thailandensis* strains harbored the *tet*(M)/*tet*(W)/*tet*(O)/*tet*(S) gene, encoding family tetracycline resistance ribosomal protection protein.

Seven *L. innocua* (7.9%, 7/89), 1 *L. aquatica* (33.3%, 1/3) and one *L. thailandensis* (100%, 1/1) were found to be phenotypically resistant to doxycycline (Fig. 1; Table S3). Resistance phenotypes correlated with resistance genotypes with either *tet*(M), *tet*(S), or *tet*(M)/*tet*(W)/*tet*(O)/*tet*(S) family tetracycline resistance genes (Fig. 1). In previous studies, tetracycline resistance is the most common resistance phenotype detected in *L. monocytogenes* (0.7% in human clinical isolates, 1–8.7% in food isolates, 7.3% in animal isolates) (Vela et al., 2001; Morvan et al., 2010; Granier et al., 2011; Yan et al., 2019). In the present study involving pathogenic and non-pathogenic *Listeria* spp., tetracycline resistance was the most observed acquired resistance (Fig. 1).

3.3. Macrolide-lincosamide-streptogramin B class (MLS) resistance

Two *L. monocytogenes* (1.5%, 2/132), both belonging to lineage I (Clonal Complex 2 (CC2)), showed phenotypic resistance to erythromycin (MIC>15 mg/L) and harbored *erm*(G), *mef*(A), and *msr*(D) genes (*erm*(G) encoding for rRNA methylases, and *mef*(A) and *msr*(D) encoding for efflux pumps) (Figs. 1 and 2; Table S1, Table S2, and Table S3). The prevalence of erythromycin resistance is similar to that previously reported in *L. monocytogenes* studies (0.06% in human clinical isolates; 1–2.2% in food isolates) (Morvan et al., 2010; Granier et al., 2011; Yan et al., 2019). All *L. aquatica* (n=3) were phenotypically resistant to erythromycin (MIC 1.5–4 mg/L) (Table S2 and Table S3), and harbored the *mph*(B) gene, encoding a macrolide inactivating enzyme (Fig. 1; Table S1). Phenotypic resistance to erythromycin was observed in 1 *L. innocua* (1.1%, 1/89, MIC 1.5 mg/L) (Fig. 1; Table S2 and Table S3). In this non-pathogenic *Listeria*-resistant strain neither macrolide resistant-genes coding for efflux pumps, inactivating enzymes, and ribosome protection proteins previously described (van Hoek et al., 2011) were detected (Fig. 1). Regarding the distribution of MIC values, statistical analysis showed a significant difference in the distribution of MICs between *L. innocua*, lineage I and lineage II *L. monocytogenes* (P=0.0001, Kruskal-Wallis test). A higher erythromycin MICs in *L. innocua* than in lineage I and lineage II *L. monocytogenes* isolates was detected (P<0.01 and P<0.0001, respectively, Mann-Whitney test) (Fig. 2).

3.4. Trimethoprim-sulfamethoxazole resistance

Three *L. innocua* isolates (3.4%, 3/89) harbored the *dfrD* gene encoding a trimethoprim-resistant dihydrofolate reductase but were not phenotypically resistant to trimethoprim- sulfamethoxazole (MIC 0.064 mg/L and 0.047 mg/L) (Fig. 1). Previous reports showed that this resistance is infrequent in *L. monocytogenes*, with rates below 1% in human and food isolates (Morvan et al., 2010; Yan et al., 2019). Regarding the distribution of MIC values, statistical analysis showed a significant difference in the distribution of MICs between *L. innocua*, lineage I and lineage II *L. monocytogenes* (P<0.0001, Kruskal-Wallis test). Higher trimethoprim-sulfamethoxazole MICs in *L. innocua* than in lineage I and lineage II *L. monocytogenes* isolates was detected (P<0.001 and P<0.0001, respectively, Mann-Whitney test) (Fig. 2). Higher trimethoprim-sulfamethoxazole MICs in lineage I than in lineage II *L. monocytogenes* was observed (P<0.01, Mann-Whitney test) (Fig. 2).

3.5. Beta-lactam resistance

Resistance to penicillin was observed in 5 *L. newyorkensis* (100%, 5/5) isolates (Fig. 1; Table S3). Only *L. newyorkensis* isolates (100%, 5/5) were found to be phenotypically resistant to ampicillin (MIC 3–32 mg/L). Altogether, these results confirm previous studies reporting that beta-lactam resistance is infrequent in *L. monocytogenes* (resistance was not detected among 2862 food isolates in China, 4668 human isolates in France) (Morvan et al., 2010; Yan et al., 2019), 60 human and 1040 food and environment isolates collected worldwide (Charpentier et al., 1995), or in 2908 clinical isolates in France (Moura et al., 2023). Two *L. newyorkensis* (40%, 2/5) were phenotypically resistant to amoxicillin + clavulanic acid (Fig. 1). None of the previously reported beta-lactam

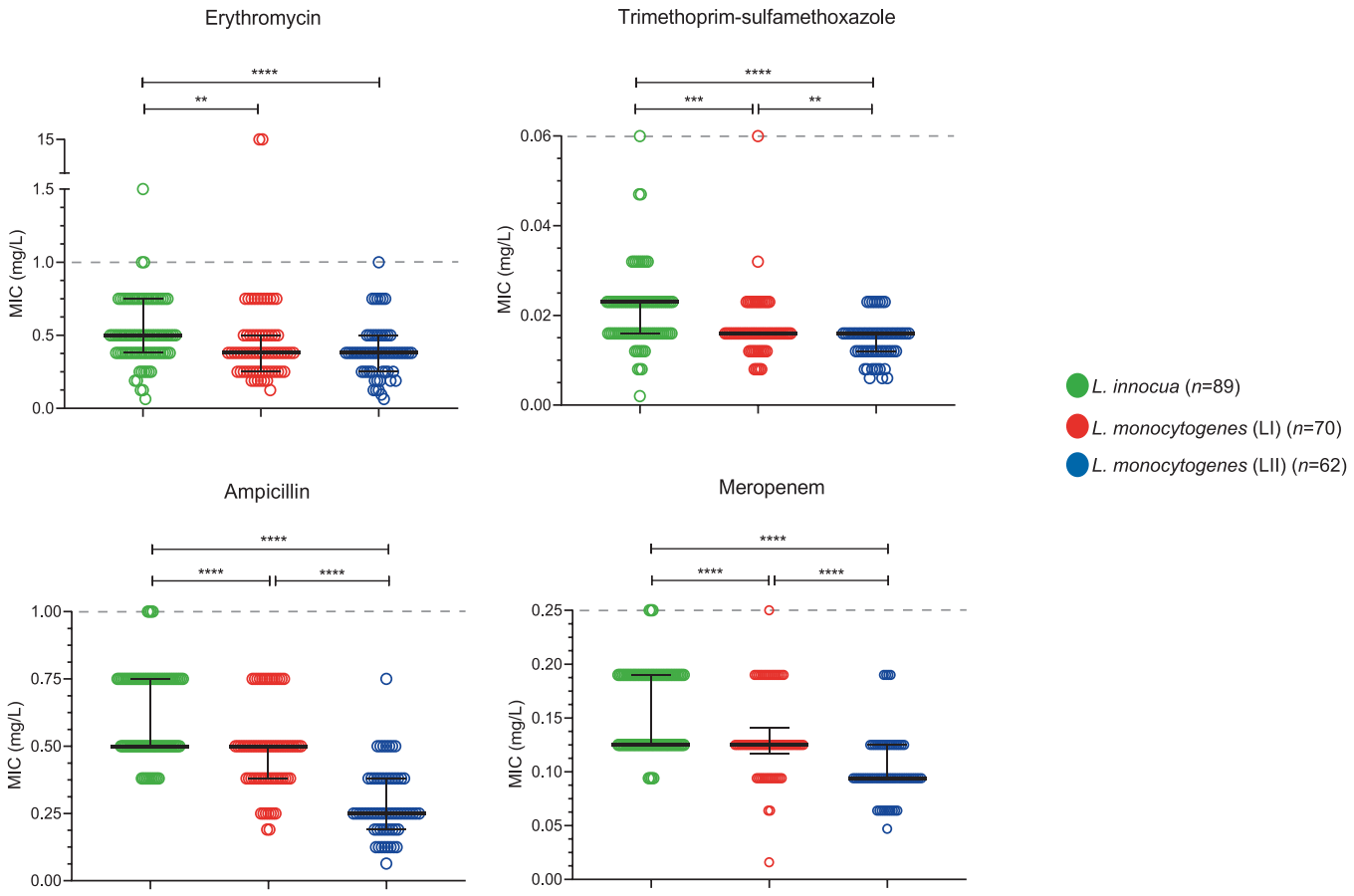

Fig. 2. Minimum Inhibitory Concentration (MIC) of *L. monocytogenes* (n=132) and *L. innocua* (n=89) isolates in this study. Breakpoints (mg/L): Ampicillin S ≤1, R >1; Erythromycin S ≤1, R >1; Meropenem S≤ 0.25, R > 0.25; Trimethoprim-sulfamethoxazole S ≤ 0.064, R > 0.064 (EUCAST). The breakpoints are represented with a discontinuous line in each graph. ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. Medians with interquartile range are shown.

resistance genes were detected in the 251 isolates from the present study (Ambler classes A, B, C and D, β-lactamases and Extended-spectrum beta-lactamases (ESBLs)).

Regarding the distribution of ampicillin and meropenem MIC values, statistical analysis showed a significant difference in the distribution of MICs between *L. innocua*, lineage I and lineage II *L. monocytogenes* (P<0.0001, Kruskal-Wallis test). The results showed a higher ampicillin and meropenem MICs in *L. innocua* than in lineage I *L. monocytogenes* isolates (P<0.0001 Mann-Whitney test) and in *L. innocua* than in lineage II *L. monocytogenes* isolates (P<0.0001, Mann-Whitney test) (Fig. 2). Moreover, higher ampicillin and meropenem MICs in lineage I than in lineage II *L. monocytogenes* was observed (P<0.0001, Mann-Whitney test) (Fig. 2). The reason for these differences in the MIC values of ampicillin/meropenem is unclear and will require further investigation.

3.6. Aminoglycoside resistance

One *L. monocytogenes* isolate (0.7%, 1/132) harbored the *aphA* gene, which codes for aminoglycoside resistance (Fig. 1). However, this isolate did not express phenotypic resistance to either kanamycin or gentamicin.

4. Discussion

The outcome of listeriosis depends on the timely administration of antibiotics. The MONALISA national prospective cohort study (4-year period, 818 patients) showed that administration of anti-*Listeria* beta-lactam, or co-trimoxazole or an aminoglycoside was associated with survival among patients with bacteremia and neurolisteriosis (Charlier et al., 2017). The overuse of antibiotics in farm animals could result in the development of antimicrobial resistance (World Health Organization WHO (2017)). The available literature shows a discrepancy regarding antibiotic resistance rates in *L. monocytogenes* isolates. While comprehensive studies (including up to 2862 food isolates (Yan et al., 2019), 4668 human isolates (Morvan et al., 2010), 2908 clinical isolates (Moura et al., 2023), and 60 human and 1040 food and environment isolates collected worldwide (Charpentier et al., 1995)) report that most *L. monocytogenes* from food, environmental or human clinical cases are susceptible to antibiotics commonly used in clinical settings, other publications report unexpectedly high rates of antimicrobial resistance in *L. monocytogenes*.

The number of systematic studies on antimicrobial resistance in *L. monocytogenes* isolates from domestic farms using antimicrobial substances is limited, and, in addition, the obtained results are contradictory. A previous study that examined the antimicrobial susceptibility of 36 *L. monocytogenes* from sheep with meningoencephalitis (Vela et al., 2001) showed that these strains were susceptible to the antibiotics most commonly used to treat human listeriosis. Another study of 38 *L. monocytogenes* isolates from four dairy farms found that 100% of the isolates were resistant to trimethoprim, 92% to ampicillin, and 45% to tetracycline (Srinivasan et al., 2005). Due to these discrepancies, retesting has been recommended to clarify the current level of antibiotic resistance in *L. monocytogenes* (Baquero et al., 2020). Our results (using WGS and two different laboratory methods, disc diffusion and MIC) show that all the *L. monocytogenes* and *L. ivanovii* subsp. *ivanovii* isolates tested were susceptible to clinically relevant antibiotics. Only erythromycin (n=2) resistance was detected in individual *L. monocytogenes* isolates, in agreement with previous results from comprehensive studies

showing that resistance in *L. monocytogenes* is infrequent (Charpentier et al., 1995; Morvan et al., 2010; Granier et al., 2011; Moura et al., 2023). Both *L. monocytogenes* isolates in which antibiotic resistance were detected, were animal-related and belonged to the hypervirulent and clinical-associated lineage I. Interestingly, Morvan et al. (2010) detected a total of 3 erythromycin-resistant strains out of 4816 (0.06%, 3/4816) clinical human strains from France, whereas Moura et al., (2023) detected only 1 erythromycin-resistant strain out of 2908 (0.03%, 2908) clinical human strains from France (Moura et al., 2023). This low prevalence of erythromycin resistance in human isolates (Morvan et al., 2010) contrasts with the prevalence detected among animal-related isolates in the present study (2.6%, 2/76).

Investigation of the causes of tetracycline and fluoroquinolone resistance emergence in *L. monocytogenes* since 1980 and 1990 discarded clonal spread as a mechanism of transference (Morvan et al., 2010). In non-pathogenic *Listeria* spp. MDR was detected in 2 strains. To the best of our knowledge, there is a lack of genomic data and its correlation with antibiotic phenotypic resistance in non-pathogenic *Listeria* spp. Importantly, the present study shows that non-pathogenic *Listeria* spp. harbour antimicrobial resistance genes. Antibiotic resistance genes can be transferred by conjugation of plasmids and transposons from *Enterococcus-Streptococcus* to *Listeria* and between species of *Listeria* (Charpentier and Courvalin, 1999). Moreover, it has been suggested that the gut (where a high prevalence of *L. innocua* has been detected in domestic ruminants (Palacios-Gorba et al., 2021) is a privileged site for acquisition of conjugative plasmids and transposons from *Enterococcus-Streptococcus* by *Listeria* spp. (Charpentier and Courvalin, 1999). Resistance acquisition by *L. monocytogenes* to these antibiotics would be a therapeutic problem in human and veterinary medicine.

5. Conclusions

In summary, our data show that: i) although *in vitro* susceptibility of *L. monocytogenes* isolated from dairy farms subjected to antibiotic pressure does not differ statistically to the susceptibility of *L. monocytogenes* strains from food or human clinical origins, *L. monocytogenes* resistance strains were only detected in ruminant-related isolates; ii) the infrequent detection of antibiotic resistance in *L. monocytogenes* isolates from Spain are similar to previously described resistance rates in other countries; iii) non-pathogenic *Listeria* spp. harbour antimicrobial resistance genes.

Although acquired resistance in food, clinical, and animal *L. monocytogenes* isolates was infrequent in the present study and did not implicate clinically relevant antibiotics, the prevalence of antimicrobial resistance determinants in non-pathogenic *Listeria* spp. reinforce the need for antimicrobial resistance surveillance in the *Listeria* genus.

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CRediT authorship contribution statement

Yuval Markovich: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Carla Palacios-Gorba:** Writing – review & editing, Formal analysis, Data curation. **Jesús Gomis:** Writing – review & editing, Resources, Investigation. **Ángel Gómez-Martín:** Writing – review & editing, Resources, Investigation. **Susana Ortolá:** Resources, Investigation. **Juan J. Quereda:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of Competing Interest

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

Data Availability

Sequences obtained in this study will be made publicly available at the European Nucleotide Archive (BioProjects PRJEB45781, PRJEB36008 and PRJEB20026) and BIGSdb-*Listeria* (bigsdb.pasteur.fr/listeria) upon publication. The accession numbers are detailed in Table S1.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2024.110086.

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