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Reservoirs of *Listeria* Species in Three Environmental Ecosystems

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Soil and water are suggested to represent pivotal niches for the transmission of *Listeria monocytogenes* to plant material, animals, and the food chain. In the present study, 467 soil and 68 water samples were collected in 12 distinct geological and ecological sites in Austria from 2007 to 2009. *Listeria* was present in 30% and 26% of the investigated soil and water samples, respectively. Generally, the most dominant species in soil and water samples were *Listeria seeligeri*, *L. innocua*, and *L. ivanovii*. The human- and animal-pathogenic *L. monocytogenes* was isolated exclusively from 6% soil samples in regions A (mountainous region) and B (meadow). Distinct ecological preferences were observed for *L. seeligeri* and *L. ivanovii*, which were more often isolated from wildlife reserve region C (Lake Neusiedl) and from sites in proximity to wild and domestic ruminants (region A). The higher *L. monocytogenes* detection and antibiotic resistance rates in regions A and B could be explained by the proximity to agricultural land and urban environment. *L. monocytogenes* multilocus sequence typing corroborated this evidence since sequence type 37 (ST37), ST91, ST101, and ST517 were repeatedly isolated from regions A and B over several months. A higher *L. monocytogenes* detection and strain variability was observed during flooding of the river Schwarza (region A) and Danube (region B) in September 2007, indicating dispersion via watercourses.

The genus *Listeria* comprises the species *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri*, and *L. grayi*, highly adapted to soil, water, and vegetation (1, 2). Recently, nine novel species and a subspecies, most of them isolated from natural environments, were introduced: *L. rocourtiae*, *L. marthii*, *L. weihenstephanensis*, *L. fleischmannii* sp. nov., *L. fleischmannii* subsp. *coloradonensis* subsp. nov., *L. floridensis* sp. nov, *L. aquatica* sp. nov., *L. cornellensis* sp. nov., *L. grandensis* sp. nov., and *L. riparia* sp. nov. (3, 4, 5, 6, 7, 8). Some *Listeria* species (*L. monocytogenes*, *L. ivanovii*, and *L. seeligeri*) harbor a gene cluster, *Listeria* pathogenicity island 1 (LIPI-1), that plays a cardinal role in *Listeria* virulence (9, 10). *L. monocytogenes* is pathogenic to both humans and animals, and *L. ivanovii* is pathogenic to animals, particularly ruminants. *L. ivanovii* possesses the separate *Listeria* pathogenicity island 2 (LIPI-2), which encodes phosphocholinesterases for efficient utilization of phospholipids in ruminant erythrocytes. This may explain the susceptibility of ruminants to *L. ivanovii* infection (11).

L. monocytogenes is transmitted to the consumer mainly via contaminated ready-to-eat foods (12, 13). The presence and potential persistence of *Listeria* spp. in food processing facilities are often caused by environmental recontamination at the farm or plant level (14, 15, 16). In order to unravel the transfer of *L. monocytogenes* between niches, molecular subtyping is essential both in outbreak clarification and in the management of contamination events in food business operations (17, 18). However, source tracking of *L. monocytogenes* often remains challenging due to its claimed ubiquity and adaptation to harsh environmental conditions (19, 20, 21). Since the Welshimer and Donker-Voet (22) and Weis and Seeliger (2) publications, most authors have hypothesized that the primary habitat of *Listeria* spp. is soil and decaying vegetation (23). In more recent decades, only a few studies have dealt with the occurrence of *Listeria* in uncultivated natural environments (24, 25).

Important factors that have been speculated to influence the occurrence of *L. monocytogenes* in soil are soil microbiota, fauna, soil composition, temperature, pH, moisture, and strain motility (2, 26, 27, 28). However, if further insight is to be gained into the unique ecological behavior of *L. monocytogenes*, it is necessary to map globally occurring genotypes and strains from environmental habitats (29, 30).

To add more insight to this issue, the objective of this study was to analyze the occurrence of *L. monocytogenes* in soil samples from areas with different soil compositions and, as a novel approach, to compare samples at different altitudes. The latter comparison was based on the hypothesis that *L. monocytogenes* as a cold-adapted organism might have a greater chance of surviving in soil that is exposed to frost conditions for almost half the year. A second objective was to characterize *L. monocytogenes* isolates by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) to estimate globally widely distributed strains. Soil can act as a reservoir for antibiotics produced by other soil microbiota (such as *Streptomyces* and *Nocardia*). Resistance to these compounds could potentially contribute to the survival of *L. monocytogenes* in this niche (31). Therefore, as a third field of inquiry, we

Received 25 March 2014 **Accepted** 25 June 2014
Published ahead of print 7 July 2014
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doi:10.1128/AEM.01018-14

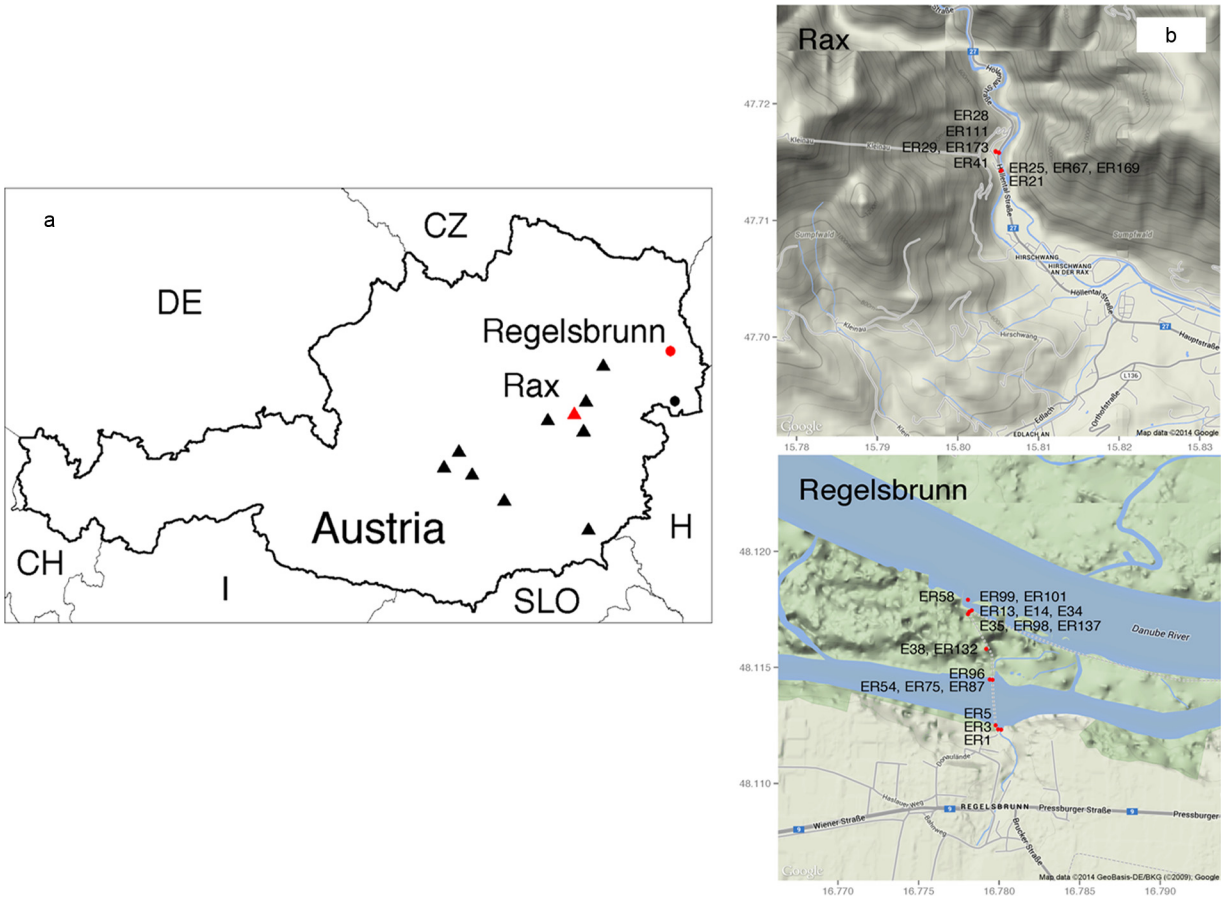

FIG 1 Sampling locations for *Listeria* detection in Austrian soil and water samples visualized in Google Maps. (a) Sampling areas A (Rax) and B (Regelsbrunn) are marked with a red triangle and red circle, respectively. Sampling areas C (Lake Neusiedl) or D to L (Eastern Alps) are labeled with black circles or black triangles, respectively. (b) *L. monocytogenes*-positive sampling locations in regions A and B are marked with red circles. *L. monocytogenes* isolation codes are abbreviated with E or ER. Further *L. monocytogenes* isolate characteristics are listed in Table 4.

investigated the antibiotic resistance of *L. monocytogenes* strains isolated during the survey.

MATERIALS AND METHODS

Sampling and description of sampling areas. In this study, 467 soil and 68 water samples were collected from 12 areas in Austria between 2007 and 2009. Samples were collected from various distinct soil types (humus, sand, and clay). The sampling areas comprised 10 sites located in the eastern Alps (of different soil compositions, here identified as regions A and D through L) at different altitudes (0 to 500 m, 500 to 1,000 m, and ≥1,500 m), two flat-land sampling areas in the east of the country located in the Donauauen National Park adjacent to the River Danube (a humus-rich and wet region [region B]), and an area close to Lake Neusiedl (a sandy and dry region [region C]) straddling the Austrian-Hungarian border (Fig. 1).

Within the selected areas A to C, samples were collected on six separate occasions from discrete sampling points, including different altitudes in region A, during 2007 and 2008. The areas D to L were included to gain more insight into the presence of *Listeria* spp. at higher altitudes in 2009. Soil samples weighing between 50 to 100 g were taken from the surface down to a depth of 5 cm. They were transferred into stomacher bags (Seward Inc., West Sussex, United Kingdom) using sterile shovels. Approximately 500-ml volumes of water were taken aseptically in polypropylene bottles (Nalgene; Thermo Fisher Scientific, Waltham, MA, USA) from rivers and ponds located in areas A to G and I to K. All samples were transported to the laboratory in standardized cooling boxes at 4°C and investigated immediately on arrival. The dominant soil character, the pH value, and the moisture content were recorded.

Isolation of *Listeria* spp. Pathogenic and apathogenic *Listeria* spp. were isolated after selective enrichment in buffered *Listeria* enrichment broth (BLEB; Merck KgA, Darmstadt, Germany) and two selective agar media: Oxoid chromogenic Listeria agar (OCLA; Oxoid Ltd., Hampshire, United Kingdom) and Palcam agar (Biokar Diagnostics). Specifically, 25-g portions of each soil sample were added to 225 ml BLEB and homogenized for 180 s in a Stomacher 400 (Seward Inc.). The 500-ml water samples were filtrated through three sterile analytical filters (Nalgene; Thermo Fisher Scientific) with pore sizes of 1 mm to 0.45 μm. The filters were enriched in 100 ml BLEB. The BLEB enrichments were incubated for 48 h at 30°C. Subsequently, 100-μl aliquots of BLEB enrichment were streaked onto OCLA and Palcam agars and incubated for 24 to 48 h at 37°C.

Identification and confirmation of *Listeria* spp. Up to three *Listeria* colonies were streaked onto Rapid'*L.mono* agar (bioMérieux, Marcy l'Etoile, France) for purification and species differentiation. For further confirmation based on the PCR technique, *Listeria* colonies were dispersed in 100 μl of 0.1 M Tris-HCl buffer (Sigma-Aldrich, St. Louis, MO, USA). Additionally, the whole agar surface was swabbed and dispersed in 1 ml of 0.1 M Tris-HCl buffer (Sigma-Aldrich). DNA isolation was performed applying Chelex 100 resin (Bio-Rad, Hercules, CA, USA) according to the method of Walsh et al. (32). Isolates were confirmed by PCR

TABLE 1 Differentiation of *Listeria* species isolated from soil samples, according to region and altitude

Region	Altitude (m)	No. of single-species samples						Mixed-species samples	
		L. monocytogenes	*L. ivanovii*	*L. innocua*	*L. seeligeri*	Unspecified *Listeria*	*L. welshimeri*	Total no.	Species[a] identified (no. of samples)
A	0–500	9	13	7	6	0	0	5	M + IN (1), M + IV (1), SE + IV (3)
B	0–500	18	0	17	23	8	3	13	M + IN (8), M + SE (2), M + NS (1), M + IN + SE + WE (1), IN + WE (1)
C	0–500	0	0	4	29	2	3	4	SE + WE (3), IN + SE (1)
A	500–1,000	1	3	2	12	1	2	3	SE + WE (2), SE + IV (1)
A	≥1,500	0	1	0	0	0	0	0	0
D to L	≥1,500	0	0	0	3	0	0	0	0

[a] Abbreviations: M, *L. monocytogenes*; IN, *L. innocua*; IV, *L. ivanovii*; SE, *L. seeligeri*; NS, unspecified *Listeria*; WE, *L. welshimeri*.

detection of the *hly* gene, encoding the virulence factor listeriolysin O, and the highly conserved 23S rRNA genes of *Listeria* spp. (33). Subsequently, *Listeria* species were differentiated by multiplex PCR targeting the invasion-associated protein (*iap*) gene (34). Biochemical identification of *Listeria* spp. was performed by applying the API Listeria system (bioMérieux).

AMR testing. The antimicrobial resistance (AMR) of *L. monocytogenes* isolated from soil samples was tested by applying the commercially available Micronaut-S Listeria MIC microtiter plate assay (Merlin; Sifin diagnostics GmbH, Berlin, Germany). A panel of 15 antimicrobials at the concentrations indicated in parentheses were included in the assay: amoxicillin-clavulanic acid (AMC; 0.125/2 to 16/2 μg/ml), ampicillin (AMP; 0.125 to 16 μg/ml), cefotaxime (CTX; 8 to 64 μg/ml), ceftriaxone (CRO; 1 to 64 μg/ml), ciprofloxacin (CIP; 0.24 to 4 μg/ml), clarithromycin (CLR; 0.25 to 8 μg/ml), erythromycin (ERY; 0.25 to 8 μg/ml), gentamicin (GEN; 0.5 to 16 μg/ml), imipenem (IMP; 0.06525 to 4 μg/ml), linezolid (LIZ; 0.5 to 16 μg/ml), penicillin G (0.0625 to 8 μg/ml), rifampin (RAM; 0.125 to 8 μg/ml), tetracycline (TET; 1 to 16 μg/ml), trimethoprim-sulfamethoxazole (T/S; 0.25/4.75 to 2/38 μg/ml), and vancomycin (VAN; 1 to 32 μg/ml). *L. monocytogenes* isolates were grown on Mueller-Hinton agar (Oxoid) for 24 h at 37°C incubation. The overnight cultures were suspended in sterile saline solution (0.85% NaCl) to achieve a turbidity of a McFarland standard of 0.5 and then diluted 1:100 before use. The breakpoints for MICs were determined according to the actual Eucast (http://www.eucast.org/clinical_breakpoints/; accessed 3 March 2014) and Clinical and Laboratory Standards Institute (CLSI) standards for 2010 (35).

Subtyping and epidemiological analysis. Confirmed *Listeria* isolates were incubated overnight in brain heart infusion (BHI; Merck KgA) at 37°C. Subsequently, isolates were cryoconserved in 15% glycerol (Merck KgA) at −80°C in the *Listeria* collection of the Institute of Milk Hygiene, Milk Technology and Food Science (Vetmeduni, Vienna, Austria).

L. monocytogenes PCR serogroups were characterized by applying a multiplex PCR targeting the genes *lmo0737*, *lmo1118*, *ORF2819*, *ORF2110*, and *Listeria*-specific *prs*, published by Doumith et al. (36) and amended by Leclercq et al. (37), for PCR IVb-VI.

The PFGE analysis of *L. monocytogenes* isolated in this study followed the PulseNet protocol (http://www.cdc.gov/pulsenet/pathogens/) with the minor modifications that samples were digested, applying 50 U AscI and ApaI for 4 h at 37°C and 25°C incubation temperatures, respectively. Restricted samples were separated in a 1% (wt/vol) SeaKem gold agarose gel in 0.5× Tris-borate EDTA (TBE) buffer at 6 V/cm on a Chef DR III system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A linear ramping factor with pulse times from 4.0 to 40.0 s at 14°C and an included angle of 120° was applied for 22.5 h. The gels were stained with ethidium bromide (Sigma-Aldrich), digitally photographed with Gel Doc 2000 (Bio-Rad Laboratories, Inc.), and normalized as TIFF images (BioNumerics 6.6 software; Applied Maths NV, Sint-Martens-Latem, Belgium) applying the PFGE global standard *Salmonella enterica* serovar Braenderup H9812.

PCR-restriction fragment length polymorphism (RFLP) for detection of point mutations in the 733-bp fragment of the *inlA* gene followed the protocol published by Rousseaux et al. (38). Thereby, 1 μl of amplified DNA was digested with 10 U AluI (1 h at 37°C) and separated on a 2% (wt/vol) agarose gel containing 3.5 μl SYBR Safe DNA gel stain (Invitrogen, Eugene, OR, USA).

The presence or absence of *L. monocytogenes* stress survival islet 1 (SSI-1) was screened by PCR, targeting the intergenic region between *lmo0443* and *lmo0449*, according to Ryan et al. (39). The polymorphism in the *actA* gene, resulting in a 268-bp or 385-bp product, was determined by a PCR protocol published by Jaradat et al. (40).

MLST based on the seven housekeeping loci *abcZ* (ABC transporter), *bglA* (beta glucosidase), *cat* (catalase), *dapE* (succinyl diaminopimelate desuccinylase), *dat* (D-amino acid aminotransferase), *ldh* (L-lactate dehydrogenase), and *lhkA* (histidine kinase) was performed according to the method of Ragon et al. (41). An allele number was assigned for each housekeeping gene, and sequence types (STs) were determined and compared using the Institut Pasteur *Listeria monocytogenes* MLST database (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Lmono.html).

An allelic profile-based comparison, applying a minimum spanning tree (MST) and the Institut Pasteur online tool, was performed to define the relationships among strains at the microevolutionary level. Clonal complexes (CCs) were defined as groups of STs differing by only one housekeeping gene from another member of the group (41).

Statistical analysis and map design. *t* tests and chi square (χ^2) tests were performed in IBM SPSS (version 19.0; SPSS Inc., Chicago, IL, USA) to determine the statistical significance ($P < 0.05$) of the difference of the distributions in prevalence between various sample categories and parameters (*Listeria* isolation, pH value, moisture content, region, and season).

Sampling locations were georeferenced and inserted into maps applying the ggmap package (42), an open-source tool for spatial visualization with Google Maps within the freely available statistical computing environment R (43).

RESULTS

Occurrence of *Listeria* spp. in Austrian environmental samples. During the investigation of 467 soil samples, 30% ($n = 140$) were determined to be positive for *Listeria* spp., of which 28 samples (6%) were *L. monocytogenes* positive. Twenty-five soil samples contained mixed *Listeria* species, most frequently *L. monocytogenes* and *L. innocua* (Table 1). The distribution of confirmed *Listeria* in soil samples according to species and region is depicted in Fig. 2. *Listeria* was isolated from 26.5% of water samples. The predominant species in soil and water samples were *L. seeligeri* (regions A, B, C, and I), *L. innocua* (regions A, B, and C), and *L. ivanovii* (regions A and C). While the human- and animal-pathogenic *L. monocytogenes* was exclusively isolated from soil samples

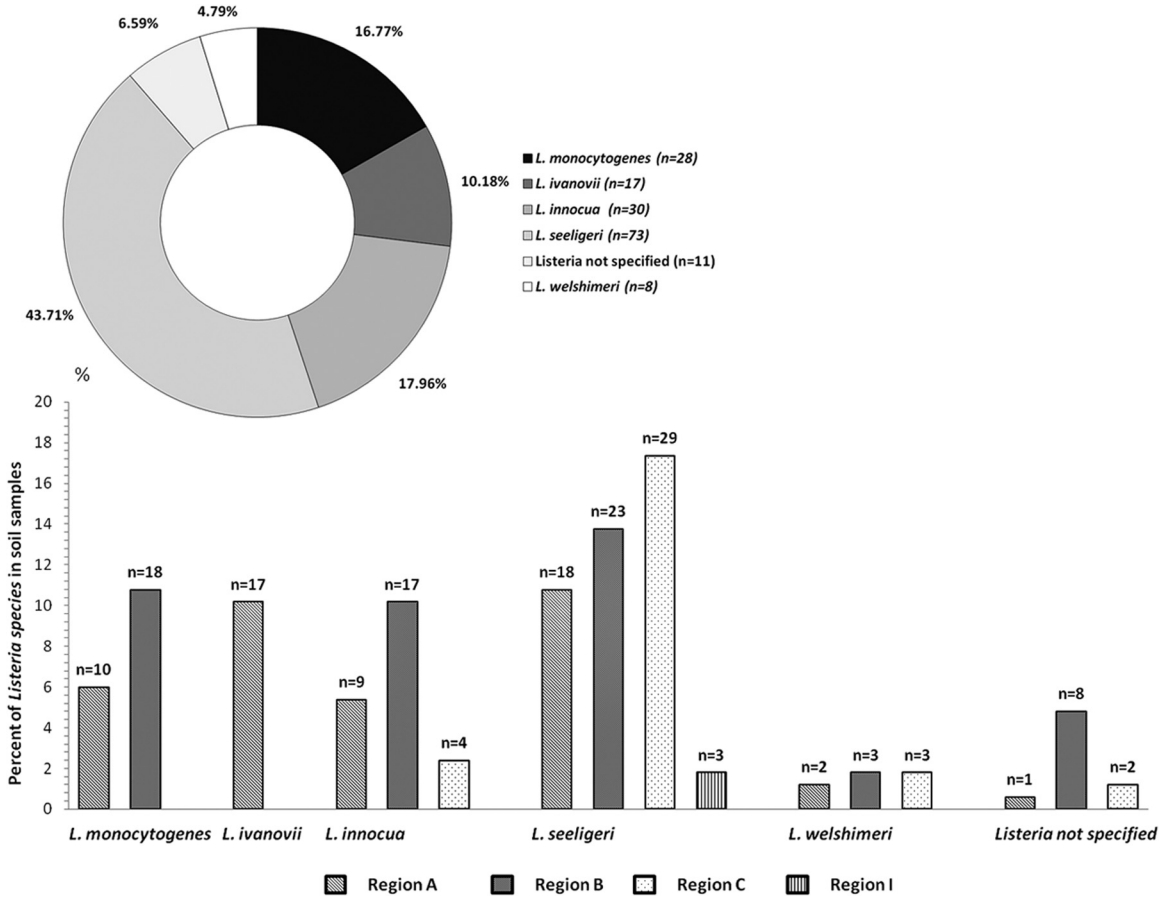

FIG 2 Distribution and percentage of *Listeria* spp., according to species and region.

in regions A and B near the Schwarza and Danube rivers (Fig. 1), the animal pathogen *L. ivanovii* was isolated mainly in region A (mountain) and in water samples in regions B and C (see Table 5). The characteristics of *Listeria*-positive and -negative soil samples according to region, altitude, and dominant soil characteristics are shown in Tables 2 and 3, respectively. The influence of moisture, pH, and soil type on the isolation of *Listeria* spp. from soil samples revealed a significant difference ($P \leq 0.001$). *Listeria* spp. were more frequently isolated from soil samples with low moisture content (22.96%; range, 2% to 80%), neutral pH (average mean, 7.44; range, 3.43 to 9.90), and soil types consisting of a mixture of sand and humus. A possible seasonal effect was observed, whereby the lowest *Listeria* isolation rates (3.33%; $P \leq 0.001$) occurred in July.

Listeria spp. were most frequently isolated from water samples with an average mean pH of 7.94 (range, 7.2 to 8.87). *Listeria* occurrence in soil samples was highest, with 25.27% ($n = 118$), at altitudes between 0 to 500 m, followed by 3.85% ($n = 19$) at altitudes of 500 to 1,000 m. *Listeria* spp. (*L. seeligeri* and *L. ivanovii*) were isolated at low counts at altitudes of >1,500 m (0.86%; $n = 4$).

Higher isolation rates of *L. monocytogenes* and *L. innocua* in regions A and B matched the rising water level and flooding of the Danube and Schwarza rivers in September 2007 (http://ehyd.gv.at/; for the Danube water level, the measuring point was at Wildungsmauer [HZBRNR 207373]; for the Schwarza water level, the measuring point was at Gloggnitz [Adlerbrücke; HZBNR 208710]). *L. seeligeri* was most frequently isolated from soil and water samples in region C (Lake Neusiedl), a waterfowl nature reserve.

Epidemiological investigation. Twenty-seven *L. monocyto-*

TABLE 2 Characteristics of *Listeria*-negative soil samples according to region, altitude, and dominant soil characteristics

Region	Altitude (m)	No. of samples	No. (%) of samples negative for *Listeria*	Avg % moisture content (range)	Avg pH (range)	Dominant soil characteristic(s)
A	0–500	60	30 (50.00)	20.30 (5.00–43.00)	7.56 (6.88–8.29)	Humus; sand/clay
B	0–500	101	47 (46.53)	26.26 (11.00–48.00)	7.47 (6.47–8.65)	Humus/clay; sand
C	0–500	92	58 (63.04)	12.00 (1.00–49.00)	7.74 (6.27–9.90)	Humus/sand; sand/clay
A	500–1,000	62	44 (70.97)	27.97 (5.00–46.00)	7.42 (5.79–7.97)	Humus
A	≥1,500	62	61 (98.39)	48.35 (16.00–75. 00)	6.73 (4.22–7.85)	Humus
D to L	≥1,500	90	87 (96.67)	45.06 (11.20–80.00)	5.26 (3.43–7.71)	Humus; sand/humus

TABLE 3 Characteristics of *Listeria*-positive soil samples according to region, altitude, and dominant soil characteristics

Region(s)	Altitude (m)	No. of samples	No. (%) of samples positive for *Listeria*	Avg % moisture content (range)	Avg pH (range)	Dominant soil characteristic(s)
A	0–500	60	30 (50.00)	18.67 (3.00–37.00)	7.53 (6.63–8.15)	Sand/humus; sand
B	0–500	101	54 (53.47)	28.45 (16.00–51.00)	7.5 (6.71–8.36)	Sand/humus; humus/clay
C	0–500	92	34 (36.96)	16.40 (2.00–31.00)	7.53 (5.55–8.82)	Sand/humus; humus
A	500–1,000	62	18 (29.03)	22.53 (6.00–46.00)	7.24 (5.05–7.97)	Humus
A	≥1,500	62	1 (1.61)	56.00	6.30	Humus
D to L	≥1,500	90	3 (3.33)	33.00 (13.00–45.00)	5.84 (4.92–7.45)	Humus; sand

genes isolates were used for further subtyping (Table 4). The multiplex serogroup PCR assay demonstrated that most of the *L. monocytogenes* isolates (66.67%) clustered in serogroups 1/2a and 3a (genetic lineage II), followed by 33.33% assigned to genetic lineage I (serogroups 4b, 4e, 4d, 1/2b, and 3b). All *L. monocytogenes* isolates were typeable with both macrorestriction enzyme analyses applying AscI and ApaI. The discriminatory power of PFGE typing was higher than that of MLST typing, resulting in 19 AscI and ApaI profiles (SOM1 to SOM19) and 16 multilocus sequence types (STs), respectively (Table 4). Interestingly, MLST analysis revealed that *L. monocytogenes* ST37 was predominant in Austrian soil samples and was repeatedly isolated from regions A and B in June and October 2007. Furthermore, ST91 was isolated from regions A and B in March and August 2008, and ST517 was prevalent in region B soil samples in September and December 2007. The highest diversity of *L. monocytogenes* genotypes was observed during the flooding of the River Danube in September 2007 (ST2, ST4, ST6, ST21, ST59, and ST517). Minimum spanning tree analysis of *L. monocytogenes* lineages I and II isolated in this study, in comparison with the Institut Pasteur strain collection based on identical allelic *abcz* types, is depicted in Fig. 3A and B.

Interestingly, comparison of the isolates recovered in this study with MLST isolates stored on the Institut Pasteur MLST database showed that the majority of genetic lineage I and II isolates were comparable to isolates recovered from compost samples commercially available in Austria in 2009 (ST1, ST4, ST6, ST7, ST20, ST26, ST37, ST59, ST91, and ST517). ST59 soil isolates were comparable to *L. monocytogenes* isolated from deer in Austria. Isolates representing ST1, ST2, and ST91 were also isolated from food in Austria. ST89, ST101, ST120, and ST177 were not present in the Institut Pasteur MLST database among Austrian isolates.

Truncation in the 733-bp fragment of the *inlA* fragment of *L. monocytogenes* soil isolates was found among ST26 (RFLP type 4), ST20, ST37, and ST89 (all RFLP type 1). RFLP type 2 was found solely among genetic lineage I isolates (Table 4). The stress survival islet (SSI-1) inserted into intergenic region lmo0443 to lmo0449 in *L. monocytogenes* was present in ST517 (lineage I; PCR serogroups 4b, 4d, and 4e) and in ST7, ST26, and ST120 (lineage II). Additionally, a polymorphism in the *actA* gene producing a fragment of 268 bp instead of the expected 385 bp was observed for *L. monocytogenes* ST6, ST21, ST59, ST89, ST101, and ST517. A larger amount of lineage II strains harbored at least one of the previously described targets suspected to induce environmental and host adaptation. ST517 and ST89 (environment-associated

TABLE 4 Characteristics of *L. monocytogenes* isolates detected in soil samples between 2007 and 2008

Isolate code(s)	Region(s) and date(s) (mo, yr) of isolation	ST[a]	PCR serogroups	AscI[b]	ApaI[c]	*inlA* type[d]	SSI-1[e]	*actA*[f] (268 bp)
ER 3, ER 5	B (9, 2007)	2	4b, 4d, 4e	SOM1, SOM2	SOM1, SOM2	2	−	−
ER 101	B (9, 2007)	4	4b, 4d, 4e	SOM3	SOM3	2	−	−
E 14	A (6, 2007)	1	4b, 4d, 4e	SOM4	SOM4	2	−	−
ER 99	B (9, 2007)	6	4b, 4d, 4e	SOM5	SOM5	2	−	+
ER 87, ER 96	B (9, 2007); B (12, 2007)	517	4b, 4d, 4e	SOM8	SOM8	2	+	+
ER 1, ER 13	B (9, 2007)	59	1/2b, 3b	SOM6, SOM7	SOM6, SOM7	2	−	+
E 34, E 35, E 38, ER 41, ER 58, ER 111	B (6, 2007); A, B (10, 2007); A (11, 2007)	37	1/2a, 3a	SOM9	SOM9	1	−	−
ER 29	A (9, 2007)	120	1/2a, 3a	SOM10	SOM10	3	+	−
ER 137	B (3, 2008)	7	1/2a, 3a	SOM11	SOM11	5	+	−
ER 75	B (11, 2007)	26	1/2a, 3a	SOM12	SOM12	4	+	−
ER 173	A (8, 2008)	177	1/2a, 3a	SOM13	SOM13	5	−	−
ER 54	B (10, 2007)	89	1/2a, 3a	SOM14	SOM14	1	−	+
ER 98	B (9, 2007)	21	1/2a, 3a	SOM15	SOM15	3	−	+
ER 132, ER 169	B (3, 2008); A (8, 2008)	91	1/2a, 3a	SOM16, SOM17	SOM16, SOM17	3	−	−
ER 21, ER 25, ER 67	A (9, 2007); A (11, 2007)	101	1/2a, 3a	SOM18	SOM18	6	−	+
ER 28	A (9, 2007)	20	1/2a, 3a	SOM19	SOM19	1	−	−

[a] ST, MLST sequence type.
[b] AscI, PFGE type after restriction digest with the enzyme AscI.
[c] ApaI, PFGE type after restriction digestion with the enzyme ApaI.
[d] *inlA*, PCR restriction fragment length polymorphism (RFLP) type for the detection of point mutations in the 733-bp fragment of the *inlA* gene; RFLP types 1 and 4 with truncation in *inlA* gene.
[e] Presence or absence of SSI-1 (stress survival islet 1), in *L. monocytogenes* intergenic region lmo0443–lmo0449.
[f] Polymorphism in the *actA* gene, producing a fragment of 268 bp instead of the expected 385 bp.

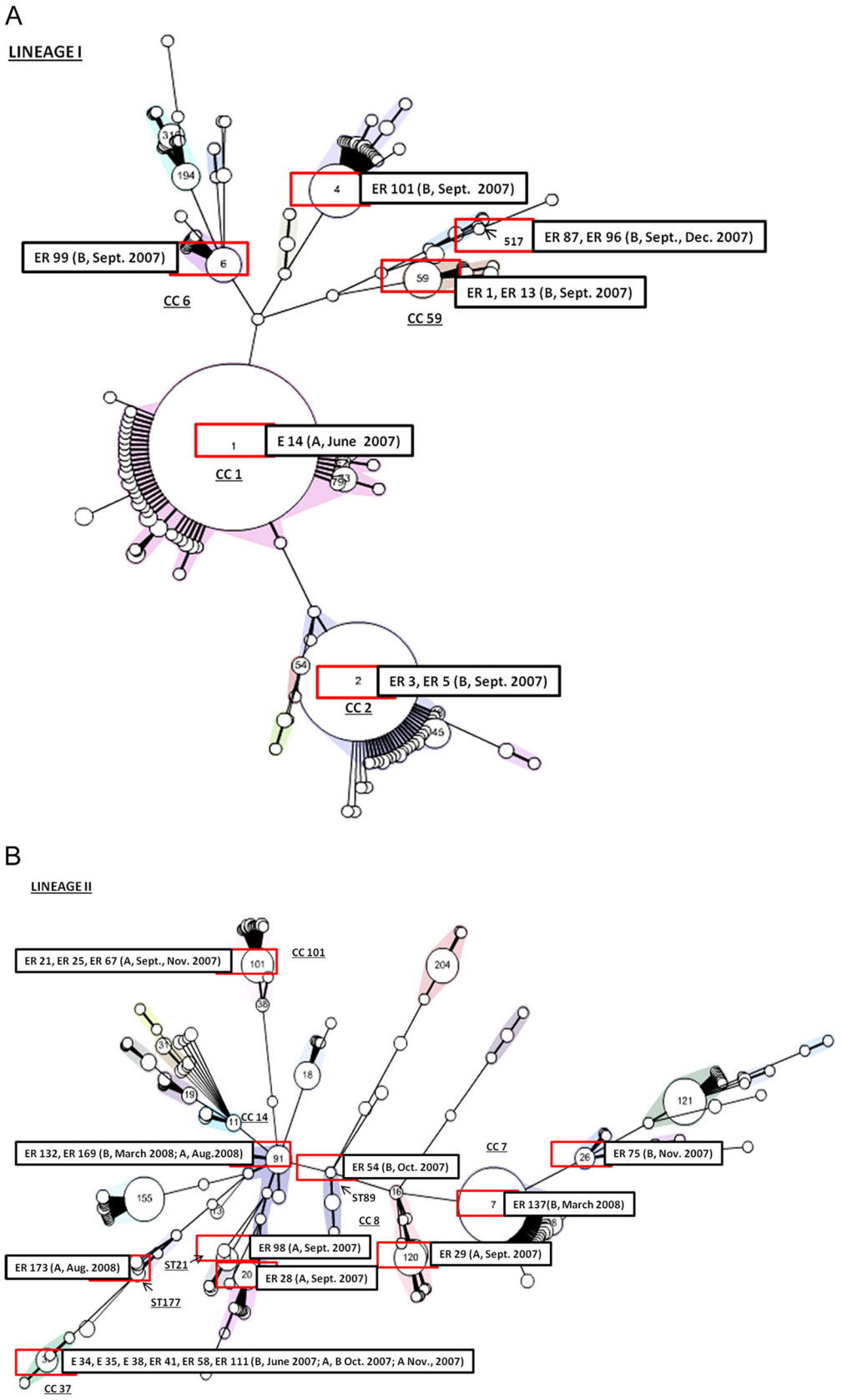

FIG 3 Multilocus sequence typing of *Listeria monocytogenes* isolated from Austrian soil samples, showing genetic lineage I (A) or II (B). The sequence types were clustered according to the *abcz* housekeeping gene using a minimum spanning tree (MST) tool available from the Institut Pasteur MLST database (http://www.pasteur.fr/recherche/genopole/PF8/mlst/). Numbers within circles denote the corresponding ST. *L. monocytogenes* strains were grouped into clonal complexes (CC; underlined; randomly colored), defined as groups of profiles differing by no more than one gene from at least one other profile of the group. Strains with no CC designation correspond to genotypes that are not closely related to any other genotype (singletons) (41, 57). *L. monocytogenes* soil isolates from Austria, including region and date of isolation, are included in each MST.

TABLE 5 Characteristics of *L. ivanovii* isolates detected in soil and water samples between 2007 and 2008

Isolate code(s)	Sample type	Region and date (mo, yr) of isolation	Altitude (m)	Sampling site(s)	API code[a]	AscI[b]	ApaI[c]
ER 24	Soil	A (9, 2007)	0–500	A4	3350	SIV1	IV1
ER 36, ER 41, ER 42, ER43	Soil	A (10, 2007)	0–500	A4, A14, A15	3350	SIV1	IV1
ER 66, ER 110, ER 69, ER 70	Soil	A (11, 2007)	0–500	A5, A11, A28	3350	SIV1	IV1
ER 180, ER 185, ER 186	Soil	A (8, 2008)	500–1,000	A26, A30	3350	SIV1	IV1
ER 30	Soil	A (9, 2007)	0–500	A14	3370	SIV2	IV2
ER 35, ER 37, ER 38	Soil	A (10, 2007)	0–500; ≥1,500	A1, A5	3370	SIV2	IV2
ER 40	Soil	A (10, 2007)	0–500	A8	3370	SIV2	IV2
ER 26	Soil	A (9, 2007)	0–500	A7	3330	SIV3	IV3
ER 122	Water	C (10, 2007)	0–500	C	3330	WIV4	IV4
ER 123	Water	B (11, 2007)	0–500	B	3330	WIV3	IV3
ER 171, 172	Soil	A (8, 2008)	0–500	A11	3350	SIV5	IV5

[a] API code, biochemical profile for species identification: 3330 and 3370, ribose positive; 3350, ribose negative; 3330, glucose-1-phosphate negative.
[b] AscI, PFGE type after restriction digest with the enzyme AscI.
[c] ApaI, PFGE type after restriction digest with the enzyme ApaI.

isolates according to the Institut Pasteur MLST database) and ST26 (most often isolated from wild animals) harbored two of the three investigated genetic modifications (Table 4).

AMR testing of 26 *L. monocytogenes* isolates showed broad susceptibility to the majority of tested antibiotics. Nevertheless, the highest antibiotic resistance rates observed among the panel of *L. monocytogenes* strains (88.46% and 65.38%) were against cefotaxime (CRO; MIC, >2 μg/ml) and erythromycin (ERY; MIC, >1 μg/ml), respectively. Further, 35%, 12%, and 6% of *L. monocytogenes* test strains were resistant to ceftriaxone (CTX; MIC, >2 μg/ml), ciprofloxacin (CIP; MIC, >1 μg/ml), and linezolid (LIZ; MIC, >4 μg/ml). *L. monocytogenes* isolates from region A (assigned to ST37 and ST91) were found to be multiresistant to four antibiotics (CRO, ERY, CTX, and CIP). *L. monocytogenes* ST1, ST2, and ST6 isolates were resistant to third-generation cephalosporins CRO and CTX, and the macrolide ERY. *L. monocytogenes* ST7 was resistant to CRO, ERY, and the fluoroquinolone CIP. ST20 was resistant to CRO, ERY, and LIZ.

Surprisingly, aside from *L. monocytogenes*, we found 22 *L. ivanovii* isolates in our study (Table 5). The analysis resulted in five PFGE profiles each when digested with both restriction enzymes (AscI and ApaI). Similar to what occurred with *L. monocytogenes*, the PFGE *L. ivanovii* profiles SIV1 (54.55% of isolates) and SIV2 (22.73% of isolates) were exclusively isolated from region A during September 2007 and August 2008. The PFGE profile SIV3 was isolated from soil in region A (September 2007) and from water in region B (November 2007) (Table 5).

DISCUSSION

Analysis of a total of 467 soil and 68 water samples from 12 sampling areas in Austria, in national parks or mountain summits, collected between the years 2007 and 2009 permitted further insight into the distribution of saprophytic *Listeria* species. Sampling contrasting geological and ecological areas such as mountainous regions in the eastern Alps, a steppe landscape, and a mixed forest and meadow area has shown that the pathogenic *Listeria* prevalence was higher in lower altitudes.

The overall *Listeria* prevalence in this study was high in uncultivated Austrian soil (30.0%) compared with those in reports from other authors, who describe prevalences ranging between 17.7% and 28% when testing samples originating from sample sites in different continents and in different climates (2, 25, 26, 44). While nonpathogenic *Listeria* species were recoverable from sampling areas A to C and I, *L. monocytogenes* was present in only 6% of soil samples from regions A and B, with a peak of positive findings for the month of September 2007.

Other researchers detected higher *L. monocytogenes* isolation rates (7 to 17%) in the natural environment (25, 26, 45). Many factors were assumed to contribute to a biased *Listeria* detection. These include fewer freezing and thawing cycles before sampling, proximity to water, higher water storage capacities of soil, a high percentage of clay in the soil texture, the absence of endogenous soil microbiota, and the presence of protozoa and nematodes as vectors (23, 26, 43). A recent study indicated, controversially, shorter survival capacities of *L. monocytogenes* in soil models with high clay content (45). *Listeria* persistence in soil was also assumed to be facilitated by strain motility and low temperatures (<8°C) (31). Therefore, outcomes of the different studies might be difficult to compare. Statistical evaluation of our results demonstrated that soil texture (sand/humus), average mean moisture (22.96%), and pH (7.44) have had a distinct influence on *Listeria* isolation in soil. Nevertheless, the impact of these factors should not be overrated since *Listeria* isolation was possible from almost all soil types and along the range of pH values (4.92 to 8.82) and moisture contents (3 to 56%) described. Welshimer and Donker-Voet (22) described a seasonality effect with high *Listeria* isolation rates during spring (86%), explained by higher humidity and remnants of decaying vegetation. In the current study, *Listeria* detection was possible during all seasons. Interestingly, *Listeria* isolation seemed to decrease to nearly zero at higher altitudes, independently from the location tested (regions A and D to L). This falsified our initial hypothesis that cold adaptation might result in a higher isolation frequency in such remote areas. The higher *Listeria* detection rates at lowland sampling points, located at altitudes of 0 to 500 m, could be explained by the proximity to farm/agricultural land and urban environments. Other authors have supposed an impact of wastewater effluents, watercourses, fertilized fields, wildlife feeding grounds and grazing ruminants on pastures on the prevalence of *L. monocytogenes* (29, 46, 47). This suspicion is corroborated by the MLST types found in our study, since *L. monocytogenes* ST517, ST37, ST91, and ST101 could be repeatedly isolated from regions A and B over several months. *L. monocytogenes* ST37 isolates are known to be related to urban, farm, and natural environments.

ST517 is an MLST type that was isolated solely from Austrian compost samples and has not been confirmed elsewhere. ST101 and ST91 are globally distributed strains, the majority of which were isolated from animals and cheese products.

An interesting observation was drawn from the sampling during and after the severe weather period in September 2007, in which flooding of the Danube (sampling area B) and Schwarza (sampling area A) rivers occurred. *Listeria* contamination rates of soil and groundwater were increased, and the highest *L. monocytogenes* strain variability was observed in this period (ST2, ST4, ST6, ST21, ST59, and ST517). Globally distributed *L. monocytogenes* lineage I strains ST1, ST2, ST4, and ST59 are highly correlated with human sources and food, whereas ST21 was most frequently isolated from rodents and birds. ST59 soil isolates were comparable to *L. monocytogenes* isolated from deer in Austria. Surprisingly, *L. monocytogenes* genetic lineages I and II isolated in this study were comparable to isolates from commercially available compost samples in Austria, which were investigated in 2009 and indicated a high prevalence of these genotypes between the years 2007 and 2009 in soil (ST1, ST4, ST6, ST7, ST20, ST26, ST37, ST59, ST91, and ST517). ST89, ST101, ST120, and ST177 were not present in the Institut Pasteur MLST database among the MLST types that were reported from Austrian isolates.

The pattern of recovery of the other pathogenic *Listeria* species in soil, *L. ivanovii*, mimicked to some extent the isolation pattern for *L. monocytogenes*. Using PFGE, the 22 *L. ivanovii* isolates could be differentiated into five subtypes, whereas isolates of PFGE types SIV1 and SIV2 were recurrently isolated between 2007 and 2008 (Table 5) in region A. This observation could also indicate the impact of the flooding season, since abundance of *L. ivanovii* was speculated to occur due to fecal shedding by wildlife or extensively farmed domestic ruminants (2, 44, 48, 49). Similarly, the high abundance of *L. seeligeri* (88.33%) in the Lake Neusiedl nature reserve (region C), which is an arid, sandy endorheic environment comprising small, stagnant lakes and decaying water plants, may be associated with the enormous amount of resident wildlife, mainly waterfowl.

For more insight into the nature of the *L. monocytogenes* isolates identified in this study, we analyzed some genetic traits that were associated either with virulence attenuation (*inlA*) or with adapation (*actA*, SSI-1). Special features of *L. monocytogenes* suspected to induce environmental and mammalian host adaptation were found in 66.67% of all MLST types. A truncation in the 733-bp fragment of the *inlA* gene was solely found among lineage II strains of ST20, ST26, ST37, and ST89. According to the MLST database, these MLST types are most often isolated from the environment and animals. These findings are consistent with those of Orsi et al., 2011 (50), who described a positive selection for 1/2a and 1/2c strains with premature stop codons (PMSCs) important for environmental survival. The *L. monocytogenes* stress survival islet 1 (SSI-1), which encodes proteins allowing *L. monocytogenes* cells to adapt to hostile conditions, such as acid stress, was present in ST517 (lineage I) and in ST7, ST26, and ST120 (lineage II) (39). ST7 and ST120 are globally distributed strains, the majority of which have been isolated from animals (rodents and wild and domestic ruminants), human clinical cases, food, and feed. ST26 is overrepresented among isolates from animals. The *actA* gene polymorphism (producing a 268-bp instead of a 385-bp fragment), which is correlated with enhanced virulence properties and higher biofilm formation in food processing plants, was observed in ST6, ST21, ST59, ST89, ST101, and ST517 isolates (51, 52). Isolates of ST26, ST89, and ST517 (environmental and animal-associated strains) were frequently isolated in our study and harbor two of the three investigated features, suggesting a selective advantage in natural habitats and animal hosts. Even in Austrian soil samples, *L. monocytogenes* sequence types that were comparable to the worldwide most prevalent clonal complexes (CCs) among clinical, animal, environmental, and food isolates were detected (19, 53).

Generally, *L. monocytogenes* isolated from food, food processing environments, and clinical sources have been considered to be susceptible to a broad range of antibiotics. Resistance to clindamycin, linezolid, ciprofloxacin, ampicillin, trimethoprim-sulfamethoxazole, erythromycin, vancomycin, and tetracycline is usually confined to a small number of strains (54, 55).

As soil can act as a reservoir for antibiotics produced by other soil microbiota (such as *Streptomyces* and *Nocardia*), resistance to these compounds could potentially contribute to the survival of *Listeria* spp. in this niche (31, 56). The current study showed that 88.46% of isolates were resistant to third-generation cephalosporins (cefotaxime) and 65.38% to the macrolide antibiotic erythromycin. ST1, ST2, ST6, ST7, ST20, ST37, and ST91, with a high prevalence worldwide (41, 57) and also isolated during our study, were found to be multiresistant to cephalosporins (cefotaxime and ceftriaxone), the macrolide erythromycin, the fluoroquinolone ciprofloxacin, and the oxazolidinone linezolid. A high AMR to the cephalosporin cefoxitin (98%) and moderate resistance to ciprofloxacin (7%) among *L. monocytogenes* isolates from food and the environment have also been reported from Canada (27). Interestingly, a certain level of resistance to antibiotics has also been shown for isolates recovered from wildlife (58). These authors demonstrated resistant strains from carcasses of bisons hunted in North Dakota, USA. Our observations of a high degree of antibiotic resistance to an array of synthetic antibiotics, unexpected in natural samples, suggest that even isolates of *L. monocytogenes* recovered from uncultivated soil might to some extent originate from niches strongly associated with anthropogenic and livestock influences.

Data from this study support the hypothesis that *Listeria* species are widely distributed in nature. Soil is a crucial niche for the persistence of globally distributed *L. monocytogenes* isolates, which can harbor AMR. High numbers of *L. monocytogenes* can be dispersed via watercourses and larger rivers with a proximity to the urban environment and wastewater effluents. A higher environmental contamination was found to be associated with periods of prolonged rainfall and flooding. Distinct ecological preferences were observed for *L. seeligeri* and *L. ivanovii*, which were more often isolated from waterfowl wildlife reserves and from sites in proximity to grazing wild and domestic ruminants. The number of *Listeria* species was nearly zero at higher altitudes, most likely due to the lower human and animal population densities.

ACKNOWLEDGMENTS

The work was supported by the European Union-funded Integrated Project Biotracer (contract 036272) under the 6th RTD Framework.

We acknowledge the Institut Pasteur for providing the MLST database of *L. monocytogenes* in the Genotyping of Pathogens and Public Health Platform, with special thanks to Sylvain Brisse and Jana Haase. Additionally, we thank Petra Apfalter for providing the sample information for *L.*

monocytogenes MLST types isolated from compost. We thank Cameron McCulloch for assistance with the manuscript.

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