ORIGINAL ARTICLE

Characterization of *Listeria monocytogenes* isolated from wildlife in central New York

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Abstract

Background: *Listeria monocytogenes* (*Lm*) present in farming soil and food-processing facilities threatens food safety, but little is known about the carriage of *Lm* by wildlife.

Objectives: We estimated the prevalence of faecal *Lm* shedding among wildlife admitted to a veterinary medical teaching hospital in central New York and characterized a subset of the *Lm* isolates.

Methods: Wildlife samples were collected between May 2018 and December 2019. We characterized the *Lm* isolates by assessing the growth at three temperatures approximating the body temperatures of reptiles (25°C), mammals (37°C), and birds (42°C) and identifying genotypic characteristics related to transmission and virulence.

Results: The apparent prevalence of faecal *Lm* shedding was 5.6% [18/324; 95% confidence interval (CI), 3.3%–8.6%]. Among 13 isolates that represented two lineages and 11 clonal complexes, three and five isolates were grouped into the same SNP clusters with human clinical isolates and environmental isolates, respectively. However, specific SNP difference data showed that *Lm* from wildlife was generally not closely related (>22 SNP differences) to *Lm* from human clinical sources and the food-processing environment. While the stress response locus *SSI-2* was absent, *SSI-1* was found in four isolates. Virulence genes *prfA*, *plcA*, *hly*, *mpl*, *actA*, *plcB*, *inlA*, *inlB*, *inlC*, *inlE*, *inlH*, *inlJ*, and *inlK* were present, without any premature stop codons, in all isolates. Virulence loci *Listeria* pathogenicity island 3 (*LIPI-3*) and *LIPI-4*, which have been linked to hypervirulence, and *inlG* were found in four, three, and seven isolates, respectively.

Conclusions: Wildlife represents a potential reservoir for genetically diverse and putatively hypervirulent *Lm* strains. No statistically significant association between growth parameters and hosts was observed. However, compared to lineage I isolates, lineage II isolates showed significantly ($p < 0.05$) faster growth at 25°C and significantly slower growth at 42°C, suggesting that wildlife *Lm* isolates that belong to lineages I and II differ in their ability to grow at 25°C and 42°C.

KEYWORDS
epidemiology, *Listeria*, wildlife, zoonoses

1 | INTRODUCTION

Listeria monocytogenes (*Lm*) is a Gram-positive, rod-shaped, intracellular foodborne pathogen that causes listeriosis. The consequences of listeriosis in people include septicaemia, encephalitis, meningoencephalitis, abortion, and even death (Vazquez-Boland, Dominguez-Bernal, et al., 2001). Among the major foodborne pathogens, *Lm* accounts for an estimated 19% of all deaths due to domestically acquired foodborne illness caused by known pathogens in the United States (Scallan et al., 2011). Based on genetic characterization, *Lm* is divided into four evolutionary lineages (I, II, III, and IV) (Orsi et al., 2010). Most *Lm* isolated from natural environments and farm animals belong to lineages I and II (Sauders et al., 2006). Lineage I is typically over-represented among human listeriosis cases, while lineage II is typically over-represented among environmental and food isolates; however, lineage II isolates also cause a number of sporadic human cases of listeriosis (Gray et al., 2004). Even though lineage II is widespread, most human listeriosis outbreaks are associated with lineage I (Vazquez-Boland, Dominguez-Bernal, et al., 2001). Lineage III is more frequently found in animal cases of listeriosis than in human listeriosis (Jeffers et al., 2001; Roberts et al., 2006). Lineage IV was first reported as a separate lineage in 2008; prior to this, lineage IV isolates were classified as lineage III (Ward et al., 2008). While specific data on lineage IV frequency are thus difficult to ascertain from studies published before 2008, both lineages III and IV appear to be rarely isolated from various sources, specifically food and environmental samples.

Lm virulence depends on several virulence factors, most of which are present in the *Listeria* pathogenicity island 1 (*LIPI-1*). *LIPI-1* contains *prfA*, which encodes the major positive transcriptional regulator of other virulence genes, as well as *hly*, *plcA*, *plcB*, *actA*, and *mpl*, which are involved in the *Lm* intracellular life cycle and help *Lm* escape from the phagosomes inside animal host cells (Radoshevich & Cossart, 2018; Vazquez-Boland, Kuhn, et al., 2001). Other *Lm* virulence factors include more than 25 internalin proteins, which are encoded in a number of distinct loci throughout the *Lm* genome. Some of these internalin proteins have been shown to play an important role in host–cell invasion; among them, internalin A (encoded by *inlA*) is particularly crucial for the virulence of *Lm* (Lecuit et al., 2001). Internalin A is a surface molecule that interacts with the host cell surface protein E-cadherin and is critical for *Lm* invasion into the host intestinal epithelial cells. Sequences of *inlA* with premature stop codons (PMSC) leading to a truncated internalin A protein are associated with compromised invasion capability and hypovirulence (Nightingale et al., 2008, 2005; Orsi et al., 2007). Compared to lineage I, lineage II isolates more commonly show hypovirulence due to premature stop codon mutations in *inlA* as well as other virulence genes (Nightingale et al., 2005; Orsi et al., 2007; Roche et al., 2005). While some *Lm* subtypes are hypovirulent, other *Lm* subtypes have been hypothesized to be hypervirulent based on their increased infectivity and severity of symptoms in infected individuals (Maury et al., 2016). For example, subtypes associated with infection of the human central nervous system and maternal-neonatal infections have been considered hypervirulent (Maury et al., 2016). *Lm* hypervirulence is also associated with presence of *Listeria* pathogenicity island 3 (*LIPI-3*) and *Listeria* pathogenicity island 4 (*LIPI-4*) (Cotter et al., 2008; Maury et al., 2016).

Lm can survive and grow under different stress conditions including low pH, high osmolarity, low temperature, and oxidative stress, making it a difficult organism to control in food-processing settings. In addition, the ability to survive in certain stress conditions allows *Lm* to infect a wide range of animal hosts. The general alternative sigma factor, Sigma B, is responsible for regulating the expression of several genes involved in stress survival, including the genes present in the stress survival islet 1 (*SSI-1*). *SSI-1* and *SSI-2* are islets containing genes involved in survival and growth under high osmolarity and low pH (Ryan et al., 2010) and survival under high pH and oxidative stress (Harter et al., 2017), respectively.

The ability to survive and grow under different conditions likely facilitates the wide distribution and frequent presence of *Lm* across different environments, leading to *Lm* often being characterized as 'ubiquitous.' For example, *Lm* has frequently been found in soil and water in natural and urban environments, and it can contaminate food-processing plants where it may subsequently contaminate food products. Likewise, *Lm* has been isolated from different animals, including reptiles, birds, and mammals. The reported *Lm* prevalence in wildlife varies across multiple studies depending on habitats and hosts. Studies in China and the Mediterranean found relatively low *Lm* prevalence in wildlife, whereas studies in Finland, Japan, and Germany reported high *Lm* prevalence (Cao et al., 2018; Hellstrom et al., 2008; Najdenski et al., 2018; Weis & Seeliger, 1975; Yoshida et al., 2000). In the study reported here, all the animal hosts could be classified into three categories: birds, mammals, and reptiles (plus one amphibian). Birds' body temperature can range from approximately 39.2 to 41.2°C (Marshall, 1961). Mammals' body temperatures vary among species based on the scaling of body temperature with body mass by species-level phylogenetic analysis (Clarke et al., 2010). The body temperature of turtles is stable at around 22°C (Cabanac & Bernieri, 2000).

Whole-genome sequencing (WGS) allows for the identification of virulence genes and stress response genes, as well as for the assessment of genome-wide diversity. Genome data can also be used for *in silico* multilocus sequencing typing (MLST), which has previously been used to develop a clonal framework for *Lm* (Ragon et al., 2008). Hence, in this study, WGS data of *Lm* isolates obtained from wildlife in central New York were used to (i) assess the diversity of the isolates using *in silico* MLST, (ii) identify virulence genes associated with hypervirulence, (iii) identify mutations in virulence genes associated with hypovirulence, and (iv) identify stress survival islets associated with stress survival and growth. In addition, the *Lm* wildlife isolates were phenotypically assessed for their ability to grow under three different temperatures approximating the body temperatures of the original hosts from which the isolates were obtained.

2 | MATERIALS AND METHODS

2.1 | Isolation of *L. monocytogenes* from wildlife

Wildlife patients admitted to the Janet L. Swanson Wildlife Hospital at Cornell University were sampled for this study between May 2018 and

December 2019. Isolates confirmed to be *Lm* by the end of September 2019 ($n = 13$) were characterized by WGS and phenotypic analysis. Voided faecal samples (birds and mammals) and cloacal swab samples (reptiles and amphibians) were obtained from patients at the Wildlife Hospital, with all admitted patients being eligible for sample collection. Samples were collected upon admission or within 24 h of admission, then placed in Cary–Blair medium and held at room temperature until *Listeria* isolation was performed at the Cornell Food Safety Laboratory. This protocol was approved by the Cornell University Institutional Animal Care and Use Committee. A Buffered *Listeria* Enrichment Broth Base (BLEB) enrichment protocol (U.S. FDA, 2018) was used to isolate *Listeria* spp. from the faecal samples. Briefly, for 5 ml of faecal sample, 45 ml of BLEB (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was added (1:10 enrichment). Then, enrichments were incubated for a total of 48 h at 30°C with BLEB Selective Supplement (Thermo Fisher Scientific, Waltham, MA, USA) added after 4 h of incubation. Each enriched sample was streaked onto one Modified Oxford Agar (MOX) plate (Becton, Dickinson and Company; Thermo Fisher Scientific) and one *L. monocytogenes* plating medium (LMPM) plate (R & F Products, Downers Grove, IL), with 50 μl aliquots of the enrichment media on each plate, after 24 and 48 h of incubation (for a total of two MOX and two LMPM plates per enriched sample). MOX and LMPM plates were incubated for 48 h at 30°C (Curtis et al., 1989) and 35°C, respectively. Putative positive colonies were subjected to *sigB* allelic typing (AT) (Liao et al., 2017) to confirm their *Listeria* classification and preserved in glycerol stock at –80°C.

2.2 | Whole-genome sequencing and analyses

Lm isolates were further characterized by WGS, conducted by the New York State Department of Health using the Illumina NextSeq500 system. WGS data were submitted to NCBI where they were assigned to SNP clusters in the Pathogen Detection database if they matched to at least one other isolate with <50 SNP differences (https://www.ncbi.nlm.nih.gov/pathogens). Sequence type, clonal complex, and lineage assignments were obtained *in silico* using the *Lm* MLST scheme reported by Ragon et al. (2008) and available from the Institut Pasteur MLST and Whole Genome MLST Databases (https://bigsdb.pasteur.fr/listeria/). The nucleotide sequences of all available alleles of (i) 15 virulence genes (*prfA, plcA, hly, mpl, actA, plcB, inlA, inlB, inlC, inlE, inlF, inlG, inlH, inlJ,* and *inlK*), (ii) the genes located in *LIPI-3* and *LIPI-4*, and (iii) the genes located in two stress response operons (*SSI-1* and *SSI-2*) were downloaded from Institut Pasteur MLST and Whole Genome MLST Databases (https://bigsdb.pasteur.fr/listeria/). These sequences were used for identification of the corresponding virulence and stress response genes, as well as mutations leading to premature stop codons in the *Lm* isolates using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). For identification of premature stop codons, nucleotide sequences were translated into amino acid sequences and searched for the '*' symbol, representing a stop codon, inside the sequence using MEGA7 (Kumar et al., 2016).

2.3 | Growth curves

Isolates were assessed for their ability to grow in Brain Heart Infusion (BHI) media at 25, 37, and 42°C using the Synergy H1 Microplate Reader instrument. Each of the *Lm* isolates was retrieved from frozen glycerol stock and streaked on a separate BHI plate, which was incubated for 24 h at 37°C. Five colonies were selected from each of the plates and inoculated into five separate tubes with 5 ml BHI broth. The 65 culture tubes (representing the 13 test isolates and five colonies from each test isolate) plus a blank tube with 5 ml of uninoculated BHI broth were incubated overnight at 37°C with 200 RPM agitation. For each overnight culture tube, 500 μl of the broth was transferred to one corresponding well in a 0.8 ml deep 96-well plate. The location of each *Lm* isolate/replicate was randomly assigned within the 96-well plate for each temperature separately using the 'sample' function implemented in R. Serial 10-fold dilutions were made to reach 10^{-4} of the original concentration, which was used to start the growth curve experiments. Bacterial growth at 25°C, 37°C, and 42°C was measured in 96-well plates using the absorbance capabilities at a wavelength of 600 nm, with measurements conducted every 10 min for 18, 26, and 30 h for cells grown at 42, 37, and 25°C, respectively.

2.4 | Statistical analysis

All data analyses were performed in R Statistical Programming Environment (Pinheiro et al., 2018). The threshold of statistical significance for all analyses was $p = 0.05$.

The ability of the *Lm* isolates to grow at 25, 37, and 42°C was assessed using the growthcurver (v 0.3.0) package (Sprouffske & Wagner, 2016). Growth curve data were fitted with a standard form logistic model using the non-linear least-squares Levenberg–Marquardt algorithm. Two growth parameters were extracted from each of the growth curves, including the area under the curve (auc_l), which was obtained by taking the integral of the logistic equation, and the maximum population size (*k*). The area under the curve can summarize a growth curve, as it incorporates the initial population size, growth rate, and maximum population size into a single value and shows a strong correlation with the growth rate (Sprouffske & Wagner, 2016).

For each temperature (25, 37, and 42°C), the auc_l data were fitted with a simple linear regression model, using the stats (v 4.0.2) package (Team, 2020), with isolate as the independent variable; temperature was not included as an independent variable because growth duration differed between temperatures preventing auc_l comparisons across temperatures. Additionally, the auc_l data were fitted with a mixed effects linear model using the lme4 (v 1.1.17) package (Bates et al., 2015), with lineage as the fixed effect and the BHI media plate used for streaking the stock culture as the random effect. A one-way analysis of variance (ANOVA) was performed on the simple linear model and the mixed effects linear model to explore the difference in auc_l at the isolate and the lineage levels, respectively. Upon the identification of a significant difference by ANOVA, a Tukey's HSD test was performed

TABLE 1 *Listeria monocytogenes* isolates used in this study and their respective hosts

Isolate ID	Host	Host type	Date of sample collection (YYYYMMDD)
FSL-R9-8346	Beaver	Mammal	20180607
FSL-R9-8348	Gray squirrel	Mammal	20180525
FSL-R9-8350	Snapping turtle	Reptile	20180529
FSL-R9-8352	European Starling	Bird	20180531
FSL-R9-8380	Red-winged Blackbird	Bird	20180623
FSL-R9-8502	American Robin	Bird	20180718
FSL-R9-8704	Eastern chipmunk	Mammal	20180901
FSL-R9-8818	Scarlet Tanager	Bird	20190514
FSL-R9-8822	Mouse	Mammal	20190517
FSL-R9-9355	Rock Pigeon	Bird	20190606
FSL-R9-9447	Canada Goose	Bird	20190612
FSL-R9-9449	Snapping turtle	Reptile	20190620
FSL-R9-9581	Gray Catbird	Bird	20190924

for the pairwise comparison of auc_l across isolates or lineages using the emmeans (v 1.4.4) package (Lenth et al., 2020).

The data for *k* were fitted with a simple linear model with isolate and temperature as the independent variables, and with a mixed effects linear model with lineage and temperature as fixed effects and BHI media plate as a random effect. The interaction between temperature and lineage was included in both models. The effect of isolate, temperature, and their interaction on *k* was explored by performing a two-way ANOVA followed by a Tukey's HSD test. Similarly, the effect of lineage, temperature, and their interaction on *k* was explored by performing the same statistical tests on the mixed effects linear model.

3 | RESULTS

3.1 | Prevalence of *L. monocytogenes*

Individual samples were collected from 324 wildlife patients between May 2018 and December 2019. The prevalence of faecal *Lm* shedding was 5.6% [18/324; 95% confidence interval (CI), 3.3%–8.6%]. Reptiles had the highest prevalence (12%, 2/17), followed by mammals (8%, 5/64) and birds (4.5%, 11/242); the one amphibian was negative.

3.2 | Genetic diversity of *L. monocytogenes*

Among the 13 wildlife *Lm* isolates that were characterized (confirmed as *Lm* by the end of September 2019; Table 1), six and seven isolates were classified as lineage I and lineage II, respectively, based on their *sigB* AT classification. In addition, the 13 isolates could be classified into 11 sequence types (STs) and clonal complexes (CC), with three isolates representing ST 388 and CC 388 and all other isolates representing one ST and CC each (Table 2). Upon submission of the WGS data to NCBIt's genome databases, five isolates were

placed into four different NCBI Pathogen Detection SNP clusters, and eight did not match (i.e. >50 SNP differences) with any other isolate in the NCBI Pathogen Detection database and hence were not placed in a cluster. Thus, the 13 wildlife isolates are genetically diverse. Five isolates (FSL-R9-8346, FSL-R9-8380, FSL-R9-8822, FSL-R9-9447, and FSL-R9-9449) were placed within four SNP clusters (PDS000024423.3, PDS000003170.45, PDS000045213.3, and PDS000043859.2) that also contained animal clinical isolates, food isolates, and food-environment isolates (Table 2). Detailed information about these five isolates can be found in the NCBI database (https://www.ncbi.nlm.nih.gov/pathogens/isolates/#FSL-R9-8346%20FSL-R9-8380%20FSL-R9-8822%20FSL-R9-9447%20FSL-R9-9449). Three of these five isolates (FSL-R9-8346, FSL-R9-8380, and FSL-R9-9447) were placed in three SNP clusters (PDS000024423, PDS000003170, and PDS000043859) that also included human clinical isolates. FSL-R9-8346, FSL-R9-8380, and FSL-R9-9447 showed 28, 27, and 23 SNP differences (i.e. SNP differences shown on NCBI Pathogen Detection) to the closest human isolates in their SNP clusters, respectively. Two of the three ST 388 isolates (i.e. FSL R9-8822 and FSL R9-9449) showed only one SNP difference (as shown on NCBI Pathogen Detection) and were placed in the same SNP cluster (PDS000045213). These two isolates were obtained from a mouse and a snapping turtle, suggesting a potential common source of these isolates.

3.3 | Stress-response loci, virulence attenuation, and hypervirulence

While the stress response islet *SSI-2* was not present in any isolate, *SSI-1* was found in four lineage II isolates (FSL-R9-8350, FSL-R9-8704, FSL-R9-8818, and FSL-R9-9581). The virulence genes *prfA*, *plcA*, *hly*, *mpl*, *actA*, and *plcB* (representing LIPI-1) as well as *inlA*, *inlB*, *inlC*, *inlE*, *inlH*, *inlJ*, and *inlK* were present in all isolates, and no premature stop codon

TABLE 2 *Listeria monocytogenes* diversity

Isolate ID	*sigB* AT	SNP cluster‡	ST	CC	Lineage
FSL R9-8346	61	PDS000024423.3	389	389	I
FSL R9-8348	57	–	795	795	II
FSL R9-8350	57	–	37	37	II
FSL R9-8352	86	–	391	89	II
FSL R9-8380	61	PDS000003170.45	554	554	I
FSL R9-8502	57	–	365	14	II
FSL R9-8704	57	–	368	368	II
FSL R9-8818	57	–	2469	2469	II
FSL R9-8822	58	PDS000045213.3	388	388	I
FSL R9-9355	58	–	388	388	I
FSL R9-9447	58	PDS000043859.2	1	1	I
FSL R9-9449	58	PDS000045213.3	388	388	I
FSL R9-9581	59	–	2428	1790	II

Note: A '–' indicates that the isolate was not placed into a SNP cluster (i.e. the isolate did not match any other isolate in the NCBI PD database by <50 SNPs). Abbreviations: CC, clonal complex; ST, sequence type; *sigB* AT, *sigB* allelic type; SNP, single nucleotide polymorphism.
‡SNP cluster: single nucleotide polymorphism cluster as assigned by NCBI Pathogen Detection database. A set of isolates different by less than 50 SNP difference in the whole genome is grouped within one SNP cluster.

TABLE 3 Identification of virulence genes[a] and stress genes in *Listeria monocytogenes* isolates

Isolate ID	Lineage	*LIPI-3*	*LIPI-4*	*inlF*	*inlG*	SSI-1	SSI-2
FSL R9-8346	I	+	–	+	–	–	–
FSL R9-8380	I	+	–	+	–	–	–
FSL R9-8822	I	–	+	–	–	–	–
FSL R9-9355	I	–	+	–	–	–	–
FSL R9-9447	I	+	–	+	–	–	–
FSL R9-9449	I	–	+	–	–	–	–
FSL R9-8348	II	–	–	+	+	–	–
FSL R9-8350	II	–	–	+	+	+	–
FSL R9-8352	II	–	–	+	+	–	–
FSL R9-8502	II	–	–	+	+	–	–
FSL R9-8704	II	–	–	+	+	+	–
FSL R9-8818	II	+	–	+	+	+	–
FSL R9-9581	II	–	–	+	+	+	–

Note: '+' and '–' indicate presence and absence of the loci, respectively.
[a]The following virulence genes were identified in all isolates: *prfA, plcA, hly, mpl, actA, plcB, inlA, inlB, inlC, inlE, inlF, inlH, inlJ,* and *inlK*.

was found in any of these genes. The virulence loci *LIPI-3* and *LIPI-4*, as well as *inlF* and *inlG*, were found in four, three, ten, and seven isolates, respectively (Table 3).

3.4 | Growth performance

A significant difference ($p < 0.05$) in auc_l (area under curve) was identified across isolates at 25 and 42°C, but not 37°C (Figure 1). At 42°C, FSL-R9-8352 showed significantly impaired growth performance (based on auc_l values) compared with other isolates, but it had auc_l values at 25 and 37°C that did not significantly differ from other isolates (Figure 1). Compared to lineage I, the auc_l of lineage II isolates was significantly higher ($p < 0.05$) at 25°C, not different at 37°C, and significantly lower ($p < 0.05$) at 42°C (Figure 2).

A significant difference ($p < 0.05$) in maximum population density was identified across isolates at 37°C, but not at 25 and 42°C (Figure 3). The *k*-value also showed no significant difference between
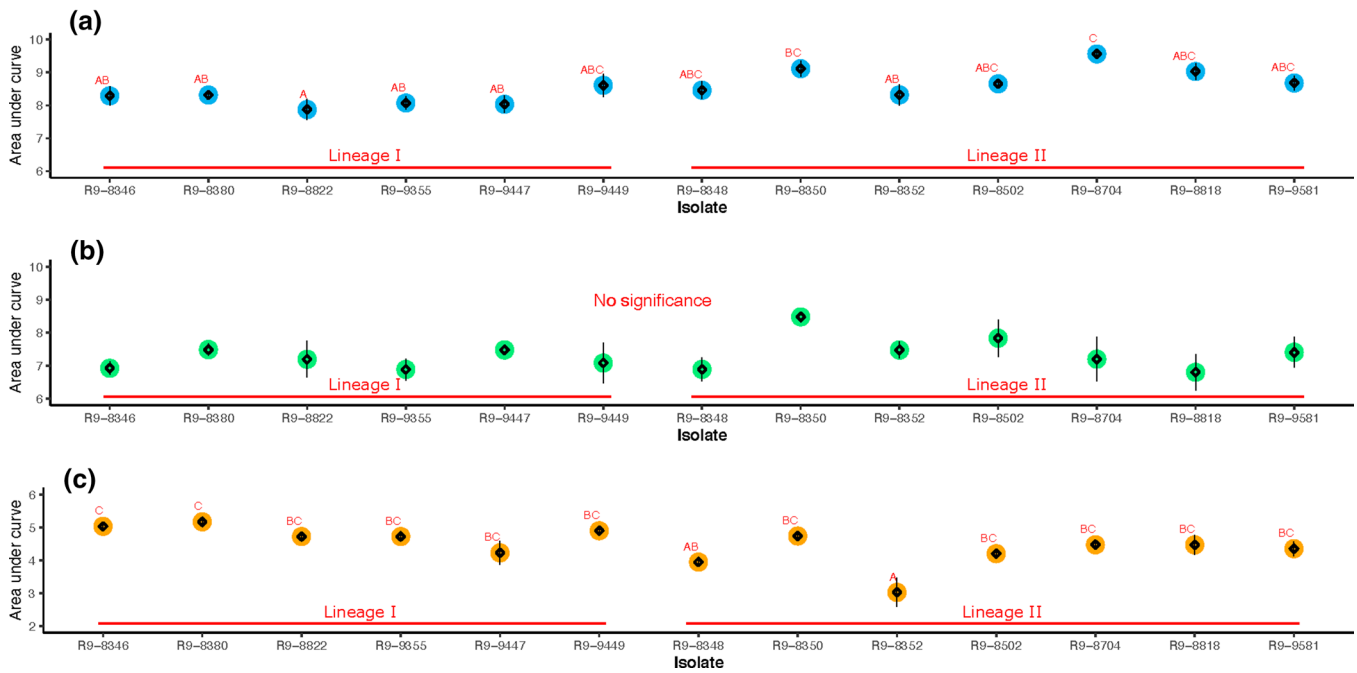
FIGURE 1 Comparison of area under curve of *Listeria monocytogenes* (*Lm*) growth across isolates at (a) 25°C, (b) 37°C, and (c) 42°C. The parameter auc_l represents overall fitness of isolates. The big circle refers to the model estimates, while the diamonds and the error bar present the mean and standard error of the raw data, respectively. Isolates with overlapping letters (on top of data points) are not significantly different from each other. Isolates with distinct letters are significantly different from each other (Tukeyt's $p < 0.05$)
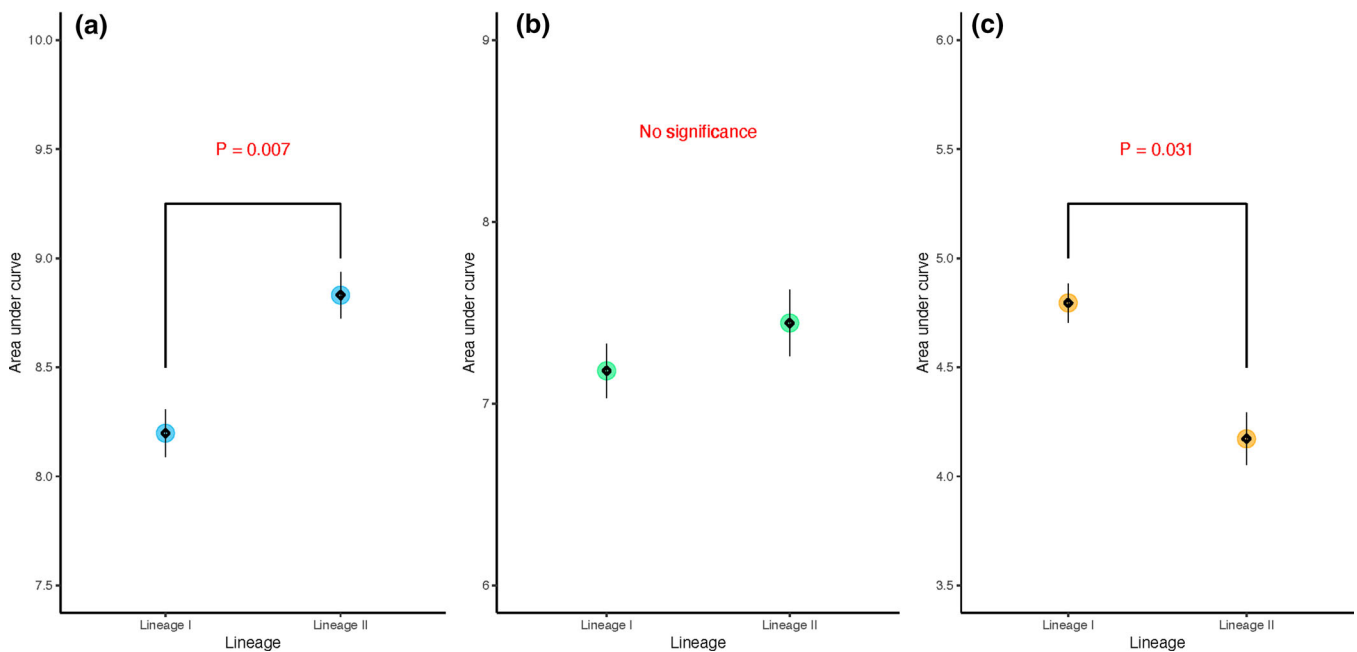

FIGURE 2 Comparison of area under curve of *Listeria monocytogenes* (*Lm*) growth between lineages at (a) 25°C, (b) 37°C, and (c) 42°C. The big circle refers to the model estimates, while the diamonds and the error bar present the mean and standard error of the raw data, respectively
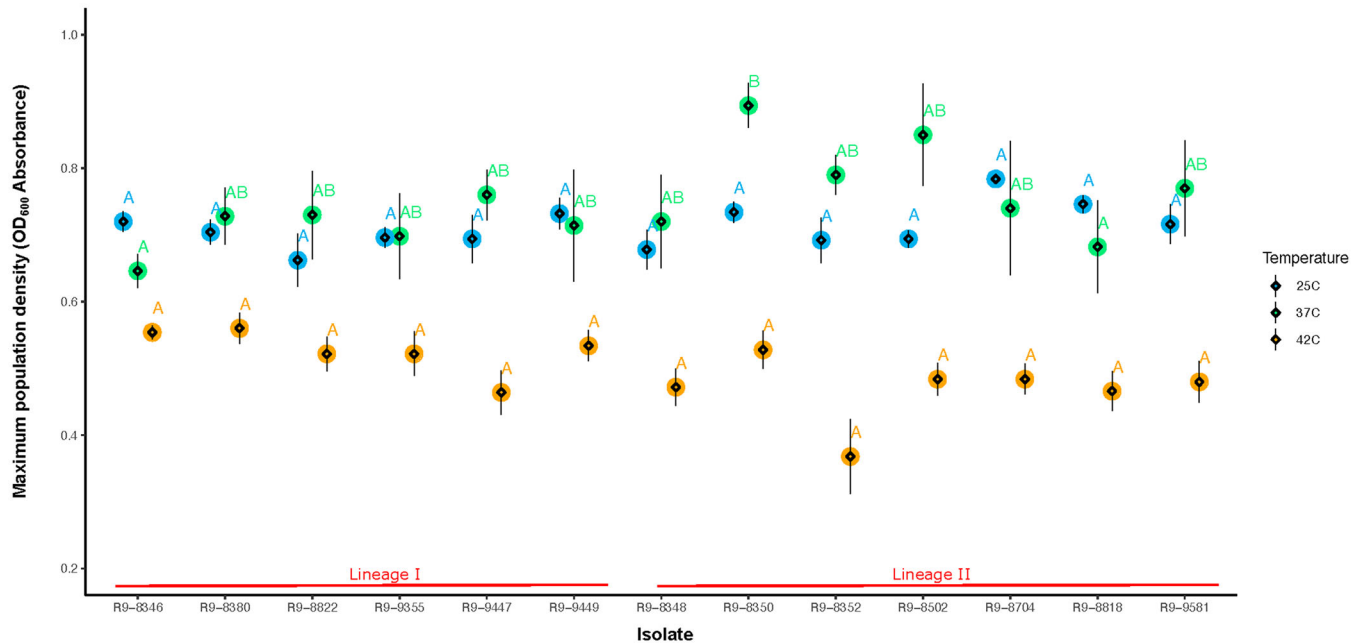
FIGURE 3 Comparison of maximum population density of *Listeria monocytogenes* (*Lm*) growth across isolates at three temperatures. The big circle refers to the model estimates, while the diamonds and the error bar present the mean and standard error of the raw data, respectively. Isolates with overlapping letters (on top of data points) are not significantly different from each other. Isolates with distinct letters are significantly different from each other (Tukey's $p < 0.05$)

lineages at 25°C. Compared to lineage I isolates, lineage II isolates had significantly higher *k*-value at 37°C and a significantly lower *k*-value at 42°C (Figure 4).

4 | DISCUSSION

4.1 | Prevalence of faecal *L. monocytogenes* shedding among wildlife was 6%

Although *Lm* has previously been reported to be more commonly associated with urban environments than with natural environments (Sauders et al., 2006, 2012), wildlife such as black bears and deer have been reported as potential reservoirs of *Lm* (Parsons et al., 2020; Weis & Seeliger, 1975). For example, previous studies reported an *Lm* prevalence of 45% among 231 black bears captured around Asheville, NC (Parsons et al., 2020) and 10% among 102 deer faecal samples collected in Germany (Weis & Seeliger, 1975). Among wildlife in Japan, six *Lm*-positive samples were obtained from a total of 623 mammalian samples (1.0% prevalence), while five *Lm*-positive samples were obtained from 996 avian samples (0.5% prevalence) (Yoshida et al., 2000). In other studies, the *Lm* prevalence in wild birds varied widely. A previous study in China found an *Lm* prevalence of 1.0% among 895 Black-headed Gull faecal samples (Cao et al., 2018), whereas studies in Finland (Hellstrom et al., 2008) and Germany (Weis & Seeliger, 1975) reported an *Lm* prevalence as high as 36.0% and 17.3%, respectively. One study isolated *Lm* from two out of 34 cloacal samples from adult European pond turtles (6% prevalence) (Nowakiewicz et al., 2015), which was lower than the *Lm* prevalence in reptiles in our study.

Although the methods used for isolation of *Lm* from wildlife samples may have differed among the aforementioned studies, the *Lm* prevalence observed here among mammals, reptiles, and birds was within the range of the *Lm* prevalence estimates obtained in these previous studies around the world.

4.2 | *L. monocytogenes* isolates obtained from wildlife faecal samples were genetically diverse but included a substantial number of possibly hypervirulent strains

The 13 *Lm* isolates characterized here by WGS represented considerable diversity, including two lineages and 11 STs. The isolates classified into lineage II, which in a previous study was reported to be more prevalent in natural environments than lineage I (Sauders et al., 2006), included one isolate (FSL-R9-8350) that was classified into ST 37; this ST was previously reported to be predominant in Austrian soil samples (Linke et al., 2014). Classification of the WGS data for the 13 isolates showed that some of the wildlife isolates collected here are closely related to isolates contaminating foods and food-processing environments (26–36 SNP differences), while other isolates were found to group with isolates from human cases (23–28 SNP differences). It is important to note, however, that previous studies have indicated mutation rates of 0.18–0.38 SNPs per year (Harrand et al., 2020; Knudsen et al., 2017; Moura et al., 2016); hence, 23–28 SNP differences indicate that at least a few decades elapsed since the most recent common ancestor between the wildlife isolates and the human and food-associated isolates. In comparison to our findings, a study in Finland
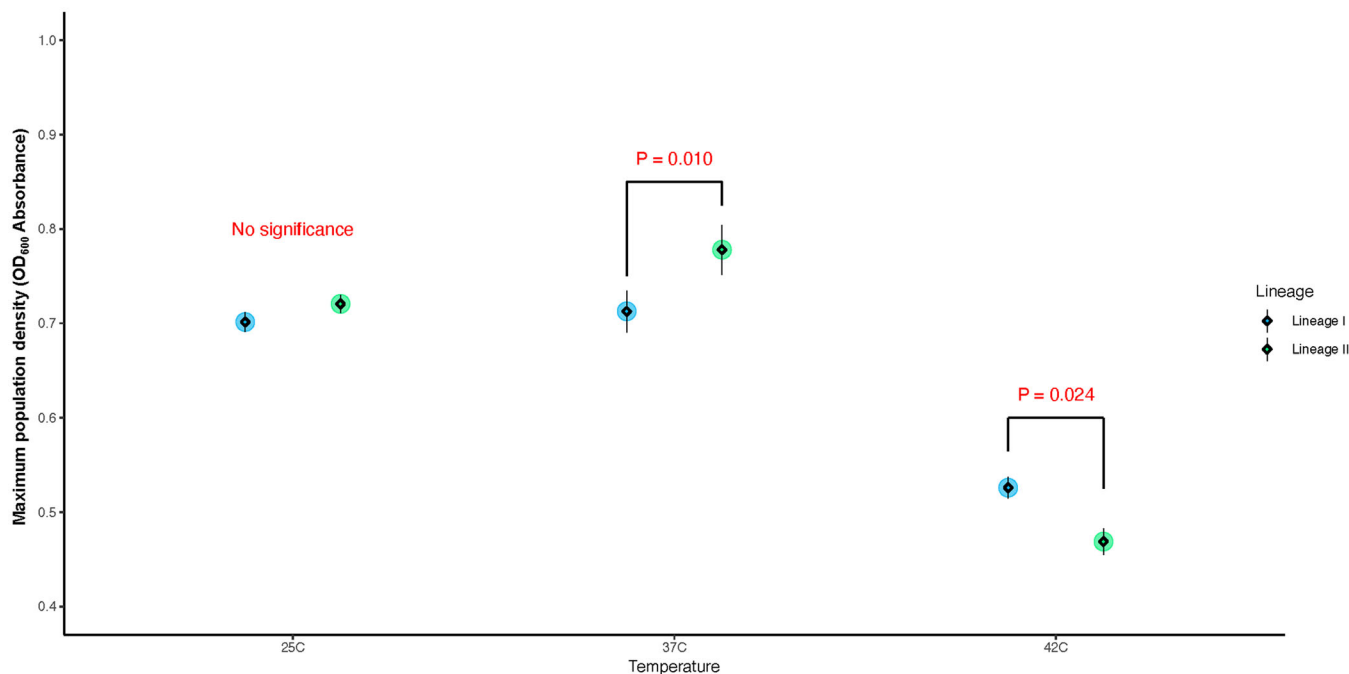
FIGURE 4 Comparison of maximum population density of *Listeria monocytogenes* (*Lm*) growth between lineages at three temperatures. The big circle refers to the model estimates, while the diamonds and the error bar present the mean and standard error of the raw data, respectively

found that 75 out of 212 avian *Lm* isolates had the same pulsotypes as *Lm* isolates from food-processing plants based on pulsed-field gel electrophoresis (PFGE) subtyping (Hellstrom et al., 2008).

WGS data supported that wildlife *Lm* isolates obtained here have the potential to cause human disease, as initially supported by the fact that all 13 isolates contained genes that encoded a full length complement of key virulence factors, including *inlA*, *inlB*, *inlC*, *inlE*, *inlH*, *inlJ*, *plcA*, and *prfA*. While *inlA* and *inlB* gene are critical for cell invasion (Vazquez-Boland, Kuhn, et al., 2001), *hly*, *mpl*, *actA*, and *plcB* are crucial for *Lm* intracellular survival (Vazquez-Boland, Kuhn, et al., 2001). In addition to the virulence genes found in all 13 isolates, *inlG* and *inlF* were found in all seven lineage II isolates, while *inlF* was found in three out of six lineage I and *inlG* was not found in any lineage I isolates. Our results are similar to those obtained by previous studies in which *inlG* was found to be present exclusively in lineage II isolates, while *inlF* was found to be more predominant in lineage II, although also found among lineage I isolates (Jia et al., 2007; Toledo et al., 2018).

WGS-based characterization of the wildlife isolates obtained here also provided evidence that wildlife isolates include clonal groups that may be hypervirulent or at least be likely to cause human disease given exposure to a sufficiently high dose. In addition to 6/13 *Lm* isolates being classified into lineage I, which has been reported to be over-represented among human clinical cases, one of the 13 *Lm* wildlife isolates (FSL-R9-9447) was classified into a hypervirulent CC (i.e. CC1) (Maury et al., 2016); most clinical *Lm* isolates in France belong to seven distinct clones, and CC1 is considered the most diverse and genetically stable (Ragon et al., 2008). In addition, *LIPI-3* and *LIPI-4*, which have been associated with hypervirulence (Maury et al., 2016), were found in four and three wildlife isolates, respectively. Furthermore,

none of the wildlife *Lm* isolates in this study showed premature stop codons (PMSC) in *inlA* or *prfA*; this is important, as previous studies have demonstrated that PMSC mutations in *inlA* and *prfA* in lineage II isolates may cause virulence attenuation (Maury et al., 2017; Nightingale et al., 2008, 2005). Although PMSC mutations in *inlA* are relatively common in lineage II *Lm* isolates, such mutations are rarely observed in linage I isolates (Jacquet et al., 2004; Nightingale et al., 2008; Van Stelten et al., 2010). This may partially explain the lower occurrence of listeriosis outbreaks caused by lineage II isolates as compared to lineage I isolates (Chen et al., 2006).

In addition to evidence that the *Lm* isolated here from wildlife are likely to be fully virulent, including some possibly hypervirulent isolates, we also found that four *Lm* isolates (FSL-R9-8350, FSL-R9-8704, FSL-R9-8818, and FSL-R9-9581) carried *SSI-1* and hence may show enhanced resistance to some stress conditions. The *SSI-1* operon has previously been associated with enhanced capability of survival and growth under suboptimal conditions of low pH and high osmolarity. These conditions can be encountered by *Lm* in foods and in the host gastrointestinal tract (Ryan et al., 2010).

4.3 | No association between original host body temperature and ability to grow at different temperatures was observed, suggesting no genetic adaptation to the original hosts

We tested the growth ability of *Lm* under temperatures (25, 37, and 42°C) that resemble the body temperature of different animal hosts (reptiles, mammals, and birds, respectively). We hypothesized that the

growth parameters of *Lm* isolates would show better fitness under temperatures approximating their hosts' body temperatures. However, we did not find associations between hosts' body temperatures and the growth fitness of isolates, suggesting that these isolates are not pre-adapted to the body temperatures of their original hosts. Although these isolates are not genetically adapted to the hosts' body temperature, they show adaptation to certain growth temperatures (25, 37, and 42°C) depending on lineages. Compared to lineage II, isolates from lineage I demonstrated better growth at 42°C, but reduced growth at 25°C. Interestingly, in a previous study in Switzerland, lineage I isolates (serotype 4b) were more prevalent in water samples in the summer, while isolates from lineage II (serotype 1/2a) were more prevalent in the fall (Schaffter & Parriaux, 2002), which suggests that future studies on the survival of different *Lm* lineages in different environments and under different temperature conditions may be valuable.

5 | CONCLUSIONS

Animals may contaminate pre-harvest environments (i.e. farms) and post-harvest environments (i.e. food-processing facilities) via *Lm* shedding. The *Lm* transmission cycle between animals, environments, humans, sewage, and food-processing plants potentially increases the load of *Lm* in hosts and habitats (Ivanek et al., 2006; Sanaa, et al., 1993; Schlech et al., 1983). Thus, research aimed at understanding the *Lm* transmission pathways between wildlife and humans is needed. This project showed that wildlife may be reservoirs or carriers of potentially hypervirulent *Lm*, but further studies will be needed to assess the virulence of the isolates used in this study. As various wildlife species have been reported as potential sources of zoonotic disease transmission (Lempp et al., 2017), our findings emphasize the value of zoonotic pathogen surveillance among wildlife that are geographically close to human habitats and the food supply chain to support 'One Health.'

CONFLICT OF INTEREST
The authors declare no conflict of interest. The viewpoints expressed in this study do not represent the official positions of the New York State Department of Health.

AUTHOR CONTRIBUTIONS
Formal analysis, investigation, methodology, and writing—original draft: Tong Chen. *Conceptualization, methodology, supervision, writing—original draft, writing—review, and editing*: Renato H. Orsi. *Formal analysis and writing—original draft*: Ruixi Chen. *Data curation, investigation, methodology, and supervision*: Maureen Gunderson. *Data curation and methodology*: Sherry Roof. *Conceptualization, funding acquisition, project administration, resources, supervision, writing—review, and editing*: Martin Wiedmann. *Resources, supervision, writing—review, and editing*: Sara E. Childs-Sanford. *Conceptualization, funding acquisition, project administration, supervision, writing—review, and editing*: Kevin J. Cummings.

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