

Prevalence and Biological Characteristics of *Listeria* Species Isolated from Livestock and Poultry Meat in Gansu Province, China

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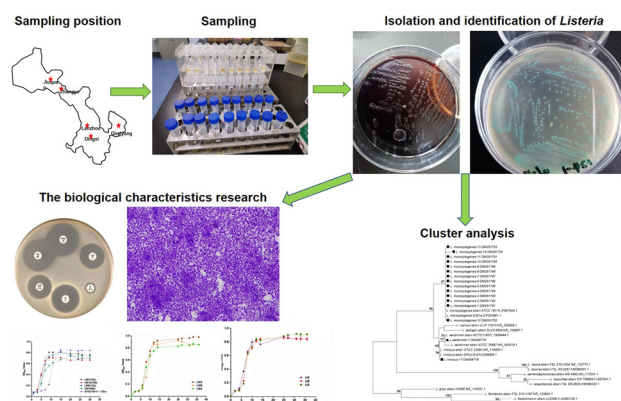
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Abstract

Listeria monocytogenes is a widespread foodborne pathogen contaminating foods during their production or processing stages. Fresh meat is susceptible to such contamination if it is not properly preserved. Our study was conducted to reveal the level of contamination and prevalence of *Listeria* spp. present in livestock and poultry meat from Gansu province. A total of 1,387 samples were collected from five cities in Gansu Province according to standard sampling procedures, of which 174 samples (12.5%) were positive for *Listeria* species. Among them, 14 isolates of *L. monocytogenes* (1.0%), 150 isolates of *Listeria innocua* (10.8%), and ten isolates of *Listeria welshimeri* (0.7%) were identified by conventional bacteriological and molecular identification methods. All isolates were subjected to serological assays, antimicrobial susceptibility tests, growth curve assays, determination of biofilm-forming capacity, and cluster analysis of the 16S rRNA gene sequences. Four predominant serotypes of *L. monocytogenes* were identified, including 1/2a (35.7%, 5/14), 1/2b (14.3%, 2/14), 1/2c (42.9%, 6/14), and 4b (7.1%, 1/14). All *L. monocytogenes* isolates were resistant to tetracycline and cefoxitin. Most *L. innocua* isolates (63.6%, 14/22) and *L. welshimeri* (40%, 4/10) were resistant to tetracycline. The high biofilm-forming



ability was observed among 1/2c and 1/2a serotype isolates. The cluster analysis of the 16S rRNA gene sequences revealed a close genetic relationship between the three *Listeria* species. This study fills the gap in the knowledge of livestock and poultry meat that carry *Listeria* in slaughterhouses and markets in Gansu Province.

Key words: *Listeria monocytogenes*, *Listeria innocua*, *Listeria welshimeri*, livestock and poultry meat, slaughtering, cluster analysis

Introduction

Listeria is sporeless, facultative, and Gram-positive bacteria, widely distributed in the environment and ubiquitously found in decaying vegetation, sewage, and soil (Jadhav et al. 2012). According to the genome sequence, pathogenicity traits, and catabolism characteristics, the genus *Listeria* can be divided into thirteen species, of which *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, and *L. grayi* are most preva-

lent (Schmid et al. 2005). It should be highlighted that *L. monocytogenes* is a zoonotic pathogen that has been ranked among the four foodborne pathogens deemed the greatest threat to public health by the World Health Organization (WHO) in the early 1980s (Chlebicz and Slizewska 2018). Moreover, diseases caused by *L. monocytogenes* mainly occur in immunocompromised individuals and manifest as encephalitis, meningoen- cephalitis, and septicemia. The consequences of the pregnant woman and newborn infection are extremely severe,

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including abortion, premature birth, and pneumonia (Doumith et al. 2004; Indrawattana et al. 2011).

Recently, different studies have reported several food contamination events caused by *L. monocytogenes* worldwide. For example, the prevalence of *L. monocytogenes* in raw milk and milk products in the Samson Province of Turkey was 3.7% (Terzi Gulel et al. 2020). The prevalence of *L. monocytogenes* in sliced cheese and ham in the retail market in southern Brazil was 9.4% (Maia et al. 2019), but in the poultry production chain in Italy was higher and equal to 26.7% (Iannetti et al. 2020). The prevalence of *L. monocytogenes* in slaughterhouses and supermarkets in three neighboring provinces (Chongqing, Shanxi, and Yunnan) of China was 5.5%, and the bacterium was found in 4.0% of foods from Beijing (Fang et al. 2018). Astonishing was that *L. monocytogenes* contamination rates in food from the Sichuan province of China reached 46.9% (Fang et al. 2018; Li et al. 2018a). *L. innocua*, a non-pathogenic bacterium, is widely distributed in meat and meat products. *L. innocua* often accompanies *L. monocytogenes* and is regarded as the indicator bacteria of the latter (den Bakker et al. 2010; Yildirim et al. 2016). *L. welshimeri* is also a non-pathogenic microorganism and does not have virulence genes, including *aadA*, *strA*, *strB*, and *penA* (Davis and Jackson 2009). Although *L. welshimeri* lacks virulence genes for intracellular survival, similar to *L. innocua*, it is more closely related to *L. monocytogenes* than other *Listeria* species (Hain et al. 2007). Davis and Jackson (2009) found that *L. welshimeri* had the broadest resistance range compared to *L. monocytogenes* and *L. innocua*. Therefore, the potential for *L. welshimeri* exists to serve as a reservoir of transferable resistance genes (Davis and Jackson 2009).

Foodborne listeriosis outbreaks have been linked to various meat products (Matle et al. 2020). The first laboratory-confirmed invasive case of listeriosis associated with meat products was in 1988 after consuming contaminated turkey franks (Schwartz et al. 1989). In 1987–2018, worldwide listeriosis outbreaks or sporadic cases comprised of 2,087 cases related to meat products, including processed, vacuum-packaged meat products (Jensen et al. 2016; Chen et al. 2017), and polonies (Smith et al. 2019). These cases were mostly reported in the United Kingdom, Australia, France, the United States, Denmark, and South Africa. Poor hygienic practices and lack of effective monitoring during meat processing are conducive to the growth and survival of *L. monocytogenes*.

Currently, there is no report on the contamination by *Listeria* species in livestock and poultry meat in the Gansu Province, northwest China. In this study, we investigated the prevalence of *Listeria* spp. in livestock and poultry meat from the slaughterhouses and markets of the Gansu province. We conducted a prelimi-

nary study of the biological characteristics of isolated bacteria; further studies are warranted to develop effective prevention and control measures.

Experimental

Materials and Methods

Sample. From July 2020 to October 2020, 1,387 samples were randomly collected from raw pork, chicken, beef, and mutton from slaughterhouses and markets in five cities of the Gansu province (Fig. 1). Forty-one swabs were mainly derived from chopping boards, knives, and instruments from the market environment. The sampling information on 45 *Listeria* spp. isolates was shown in Table SI. The sampling followed the national criteria of meat and meat products-sampling GB/T 9695.19-2008 (2008) with moderate modifications. The meat and the environment surfaces were smeared using cotton swabs soaked in sterile saline solution for sampling, and the collected cotton swabs were put into a tube with 5 ml sterilized phosphate buffer solution (PBS). Samples were placed in the sampling box with the freezer bags and stored at 4°C after delivery to the laboratory.

Isolation and identification of *Listeria* species. *Listeria* strains were isolated according to the microbiology of food testing for *L. monocytogenes* GB 4789.30-2016 (2016) method of China with moderate modifications. The collected cotton swabs were placed into 225 ml Luria-Bertani liquid medium (LB₁) (Solarbio,

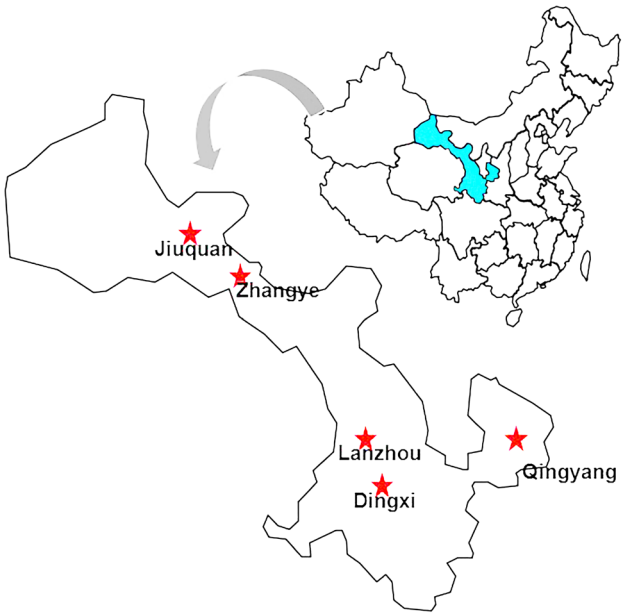


Fig. 1. Distribution diagram of sampling points in Gansu province, China. The geographic locations of samples from slaughterhouses and markets. The five-pointed stars of red represent the different sampling sites.

China) and incubated at 30°C for 36 h. Then, 0.1 ml of enrichment broth was transferred into 10 ml LB₂ for the second-step enrichment at 30°C for 48 h. The isolates were inoculated on PALCAM agar (Huankai Microbial, China) and cultured at 37°C for 36 h. Afterward, 2–3 colonies revealing the characteristic small, round, and grayish-green appearance on PALCAM agar were picked and subcultured on the selected medium for *Listeria* at 37°C for 48 h. Two or three smooth blue-green colonies were then randomly selected for purification and cultured on the solid medium of Brain Heart Infusion (BHI) (Solarbio, China) at 37°C for 8 h. Then, the bacteria were stained according to the Gram procedure. After the above operation, *Listeria* spp. were preliminarily identified. The PCR method that targets specific genes of different *Listeria* species was applied for further verification (Tao et al. 2017). Bacterial DNA was extracted from 3 ml of the above bacterial cultures using the TIANamp Bacterial DNA kit (Tiangen, China) and used as the template to identify the *Listeria* genus-specific *prs* gene by PCR. The positive isolates for the *prs* gene fragments were further identified using the primers for the *L. monocytogenes*-specific *lmo2234* gene. Then, the negative isolates were identified using the primer for the *L. innocua*-specific *lin0464* gene. After amplification, the purified PCR products were sent to the GENEWIZ Technology Co., Ltd., (China) for Sanger sequencing. The sequencing results were analyzed by BLAST in the NCBI database. The PCR primers used in this paper refer to the study of Luo et al. (2017). All the primers used in this study are listed in Table I.

***L. monocytogenes* serogroups.** The bacterial wall-breaking treatment and multiplex PCR method were used to differentiate the major serogroups of *L. monocytogenes*. The multiplex PCR primers (Doumith et al. 2004) were shown in Table I. PCR products were detected by agarose gel electrophoresis, and serogroups of the isolates were determined by analyzing the electrophoresis bands (Doumith et al. 2004). The 16S rRNA sequencing results of the different serogroup isolates were analyzed by BLAST (Basic Local Alignment Search Tool) determine the isolates’ clusters further.

Antimicrobial susceptibility test. The Kirby-Bauer disk diffusion test was used to evaluate the susceptibility of *Listeria* species to ten antibiotics, including penicillin, ofloxacin, cefoxitin, sulfamethoxazole, tetracycline, gentamicin, streptomycin, erythromycin, acetylspiramycin, and fosfomycin. Finally, the diameters of the antimicrobial zone of each antimicrobial susceptibility disc were measured. Results were judged according to Reis et al. (2011).

Measurement of the growth curve. Four *L. monocytogenes* isolates of different serotypes, the reference strain ATCC® 19111™, three *L. innocua* isolates, and three *L. welshimeri* isolates were selected for the growth curve determination. *Listeria* isolates were aseptically inoculated on BHI agar plates and incubated overnight at 37°C. The single colonies were inoculated in 20 ml of BHI liquid culture at 37°C 180×g. Three parallel tests were set for each sample, and one BHI medium was selected as blank control. The growth curve of the isolates was drawn by measuring the OD₆₀₀ value of the bacterial suspension at different times. The multi-mode

Table I
Primer information.

Gene target	Primer sequence (5'-3')	Product size (bp)	Application
<i>prs-F</i>	GCTGAAGAGATTGCGAAAGAAG	370	<i>Listeria</i> species-specific primers
<i>prs-R</i>	CAAAGAAACCTTGGATTTCGCG		
<i>Lmo2234-F</i>	TGTCCAGTTCATTTTAACT	420	<i>Listeria monocytogenes</i> specific primers
<i>Lmo2234-R</i>	TTGTTGTTCTGCTGTACGA		
<i>Lin0464-F</i>	CGCATTTATCGCCAAAACCTC	749	<i>Listeria innocua</i> specific primers
<i>Lin0464-R</i>	TCGTGACATAGACGCGATTG		
<i>lmo0737-F</i>	AGGGCTTCAAGGACTTACCC	691	For the identification of <i>Listeria monocytogenes</i> serogroup
<i>lmo0737-R</i>	ACGATTTCTGCTTGCCATTC		
<i>lmo1118-F</i>	AGGGGTCTTAAATCCTGGAA	909	For the identification of <i>Listeria monocytogenes</i> serogroup
<i>lmo1118-R</i>	CGGCTTGTTCGGCATACTTA		
<i>ORF2819-F</i>	AGCAAAATGCCAAAACCTCGT	471	For the identification of <i>Listeria monocytogenes</i> serogroup
<i>ORF2819-R</i>	CATCACTAAAGCCTCCCATTTG		
<i>ORF2110-F</i>	AGTGGACAATTGATTGGTGAA	597	For the identification of <i>Listeria monocytogenes</i> serogroup
<i>ORF2110-R</i>	CATCCATCCCTTACTTTGGAC		
<i>16S rRNA-27F</i>	AGAGTTTGATCCTGGCTCAG	1,500	Used for cluster analysis
<i>16S rRNA-1492R</i>	GGTTACCTTGTTACGACTT		

microplate reader with model number SpectraMax i3x (Molecular Devices Corporation, USA) was utilized for these studies.

Biofilm formation assay. The method of Hu et al. (2010) for the capacity of bacterial biofilm formation was modified. All *Listeria* isolates were aseptically inoculated on BHI agar plates and incubated overnight at 37°C. The colonies were selected and cultured in 5 ml of BHI liquid medium at 37°C 180 × g for 14 h. After overnight incubation, the bacterial broth was transferred to BHI at a ratio of 1:100 and incubated until the OD₅₆₂ value reached 0.2. The bacterial solution was diluted again at a ratio of 1:100; then the bacteria diluent was transferred 200 µl of each well into a 96-well plate and incubated at 37°C for 46 h. After incubation, the suspension was discarded, and each well was washed with 200 µl sterilized saline three times to remove the planktonic bacteria. Then, the bacteria were fixed with 200 µl methanol for 30 min. After drying, 200 µl of 1% ammonium oxalate crystal violet solution was added for 30 min. Finally, 200 µl of 95% ethanol solution was added and vibrated for 20. The OD₅₆₂ values were measured with a multi-mode microplate reader. Three replicates for each bacterial isolate were measured.

Cluster analysis. The cluster analysis of the *Listeria* genus based on 16S rRNA (Soni and Dubey 2014) was performed using MEGA 5.05 cluster analysis software (Tamura al. 2011). Thirty-three sequences were selected to analyze the *Listeria* spp. cluster relationship, including 14 sequences of *L. monocytogenes*, one sequence of *L. innocua*, one sequence of *L. welshimeri* from this study, and 17 sequences of other *Listeria* species from

the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) database. The 16S rRNA sequencing data used in the experiment have been submitted to the NCBI database, and the accession numbers of all sequencing data have been collated in Table SII. Gaps were treated as missing data, with equal weightings for transitions and transversions.

Statistical analysis. SPSS 22 software (SPSS, Inc., USA) was used for statistical analyses. All experimental data were calculated by the one-way analysis of variance. Results were considered statistically significant at the *p*-value < 0.05 level. *p*-Value < 0.05 was regarded as significantly different, while *p*-value < 0.01 was regarded as highly significantly different. Error bars represent the standard deviation.

Results

Prevalence of *L. monocytogenes*, *L. innocua* and *L. welshimeri* in livestock and poultry meat. The prevalence of *Listeria* species in different samples and different regions of the province has been summarized in Table II. Specific genes of the *Listeria* species were confirmed by agarose gel electrophoresis, including *prs* (370 bp), *lmo2234* (420 bp), and *lin0464* (749 bp). In total, 12.6% (174/1,387) of samples were positive for *Listeria* spp. Among these, *L. innocua* was the most prevalent species, accounting for 86.2% (150/174) of the isolates, whereas *L. monocytogenes* accounted for 8.1% (14/174), and *L. welshimeri* for 5.7% (10/174). Among the meat samples of different categories, the

Table II
Statistic of data on the prevalence of *Listeria* spp. according to regions and sample categories.

	Number of samples	<i>Listeria monocytogenes</i> positive samples (%)	<i>Listeria innocua</i> positive samples (%)	<i>Listeria welshimeri</i> positive samples (%)	<i>Listeria</i> spp. positive samples (%)
Different regions					
Lanzhou City	298	7 (2.4 ^a)	30 (10.1 ^b)	2 (0.7 ^b)	39 (13.1 ^b)
Qingyang City	275	6 (2.2 ^b)	95 (34.6 ^a)	4 (1.5 ^a)	105 (38.2 ^a)
Jiuquan City	129	1 (0.8 ^c)	2 (1.6 ^d)	0 (0 ^c)	3 (2.3 ^d)
Dingxi City	400	0 (0 ^d)	6 (1.5 ^d)	0 (0 ^c)	6 (1.5 ^e)
Zhangye City	285	0 (0 ^d)	17 (6.0 ^c)	4 (1.4 ^a)	21 (7.4 ^c)
Total	1,387	14 (1.0)	150 (10.8)	10 (0.7)	174 (12.6)
Different categories of samples					
Pork	784	8 (1.0 ^b)	81 (10.3 ^d)	7 (0.9 ^a)	96 (12.3 ^c)
Beef and mutton	298	3 (1.0 ^b)	35 (11.7 ^b)	2 (0.7 ^b)	40 (13.4 ^a)
Chicken	264	3 (1.14 ^a)	29 (11.0 ^c)	1 (0.4 ^a)	33 (12.5 ^b)
Environment samples	41	0 (0 ^b)	5 (12.2 ^a)	0 (0 ^d)	5 (12.2 ^c)
Total	1,387	14 (1.0)	150 (10.8)	10 (0.7)	174 (12.6)

^{a-c} – Different superscript lowercase letters indicate that the two sets of data in this column are highly significantly different (*p*-value<0.01), and the same superscript lowercase letters indicate not significantly different values (*p*-value>0.05). With the increase of a to d, the differences between groups are also gradually expanding.

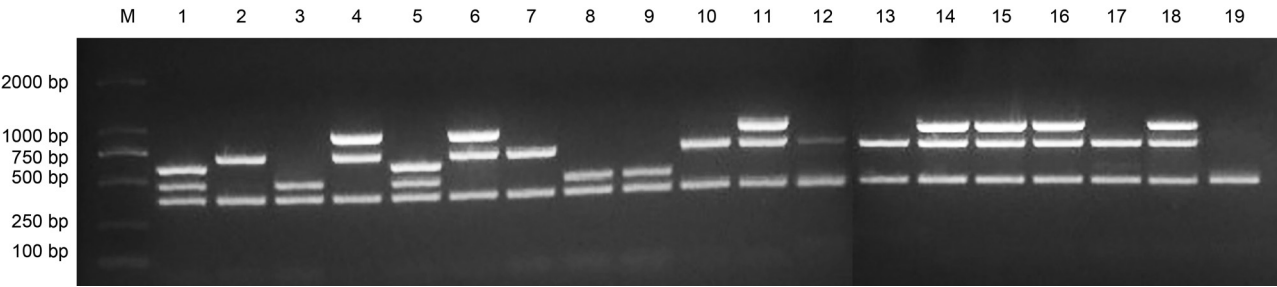


Fig. 2. Serogroups by multiplex-PCR of *L. monocytogenes* isolates.
M – DL 2000 DNA Maker, 1 – ATCC® 19115™ (serogroup 4b), 2 – ATCC® 19111™ (serogroup 1/2a), 3 – NCTC10890 (serogroup 1/2b), 4 – ATCC® 19112™ (serogroup 1/2c), 5–18 – the strains isolated in this study, 19 – *L. innocua* (negative control)

prevalence rate of *Listeria* spp. ranged from 12.2–13.4%. The prevalence rate of *Listeria* spp. in beef and mutton (13.4%) was highly significantly different than in pork (12.3%), chicken (12.5%), and environment samples (12.2%). However, the difference in the *Listeria* spp. prevalence in pork (12.3%) and environment samples (12.2%) was not statistically significant (p -value >0.05). The prevalence rate of *L. monocytogenes* in chicken (1.14%) was highly significantly different than in the other meat sample (1.0%), including pork, beef, and mutton (p -value <0.01). The prevalence rate of *L. welshimeri* in pork (0.9%) was highly significantly different when compared with that in beef (0.7%) or chicken (0.4%) (p -value <0.01). In addition, the prevalence rate of *L. innocua* in environment samples (12.2%) was highly significantly different than in pork (10.3%), beef (11.7%), and chicken (11.0%) (p -value <0.01). Furthermore, the incidence rate of *Listeria* spp. varied from 1.5% to 38.2% among different cities. The occurrence of *Listeria* spp. in Qingyang city was significantly different (p -value <0.01) compared to that in other cities. The incidence of *L. monocytogenes* was highly signifi-

cantly different (p -value <0.01) in the cities of Lanzhou (2.4%), Qingyang (2.2%), and Jiuquan (1.0%).

***L. monocytogenes* serogroups.** The serogroups of *L. monocytogenes* isolates were identified by multiplex PCR (Fig. 2). *L. monocytogenes* isolates were divided into 1/2c (42.9%), 1/2a (35.7%), 1/2b (14.3%), and 4b (7.1%) serogroups. The 1/2c serogroup included two serotypes, 1/2c and 3c. The 1/2a serogroup included 1/2a and 3a serotypes. The 1/2b serogroup was composed of the 3b and 7 serotypes. The 4b serogroup primarily consisted of 4b and 4e serotypes. Combined with the results of multiple PCR and 16s rRNA sequencing analyzed by BLAST, the clusters of the isolates could be identified.

Antimicrobial susceptibility. The antimicrobial susceptibility tests were performed to examine the resistance of isolates (Table III). The results showed that all *L. monocytogenes* isolates were resistant to tetracycline and cefoxitin and sensitive to ofloxacin. Moreover, 92.9% of *L. monocytogenes* isolates indicated a high resistance to penicillin, acetylspiramycin, and erythromycin, and 78.6% were resistant to sulfamethoxazole. The resistance rate of 50.0% of *L. monocytogenes*

Table III
Result of drug susceptibility of *Listeria* spp. isolates.

Antibiotics	<i>Listeria monocytogenes</i>				<i>Listeria innocua</i>				<i>Listeria welshimeri</i>			
	Resistant isolates	Intermediate isolates	Sensitive isolates	Resistance rates (%)	Resistant isolates	Intermediate isolates	Sensitive isolates	Resistance rates (%)	Resistant isolates	Intermediate isolates	Sensitive isolates	Resistance rates (%)
Penicillin	13	0	1	92.9	2	0	20	9.1	3	0	7	30.0
Ofloxacin	1	2	11	7.1	1	13	8	4.6	2	0	8	20.0
Cefoxitin	14	0	0	100	0	0	22	0	2	0	8	20.0
Sulfamethoxazole	11	0	3	78.6	10	1	11	45.5	4	0	6	40.0
Tetracycline	14	0	0	100	14	0	8	63.6	4	0	6	40.0
Gentamicin	3	0	11	21.4	0	0	22	0	1	0	9	10.0
Streptomycin	2	1	11	14.3	5	0	17	23.7	1	0	9	10.0
Erythromycin	13	1	0	92.9	4	4	14	18.2	3	1	6	30.0
Acetylspiramycin	13	0	1	92.9	5	0	17	22.7	3	0	7	30.0
Fosfomycin	7	7	0	50.0	18	0	4	81.8	4	2	4	40.0

isolates to fosfomycin was also observed. *L. innocua* isolates were sensitive to penicillin and gentamicin, whereas they were resistant to fosfomycin (81.8%) and tetracycline (63.6%). The resistance rate for fosfomycin, tetracycline, and sulfamethoxazole in *L. welshimeri* isolates was noteworthy, with a primary resistance rate of 40.0%. In addition, only 10% of *L. welshimeri* isolates were resistant to gentamicin and streptomycin. Overall, the rate of antibiotic resistance of *L. monocytogenes* isolates was higher compared to *L. welshimeri* and *L. innocua* isolates.

Measurement of the growth curve. Four *L. monocytogenes* isolates of different serogroups entered the logarithmic growth phase after 3 h of culture. Serotypes 1/2a and 4b reached the plateau after 20 h, while serotypes 1/2b and 1/2c reached the plateau after 7 h. They apparently showed a faster growth rate compared to serotypes 1/2a and 4b. The *L. monocytogenes* reference strain ATCC® 19111™ (1/2a) entered the logarithmic phase after 8 h incubation and entered the plateau phase after 15 h. The growth rate of the reference strain was significantly slower than that of the four isolates (Fig. 3a). *L. innocua* entered the logarithmic phase between 4 and 10 h and the growth rate began to slow

down after 10 to 31 h of culture, and entered the growth plateau phase after 31 h (Fig. 3b). *L. welshimeri* entered the logarithmic phase after 6 h of culture, which lasted until 12 h, entered the slow growth phase from 12 h to 14 h, and then the plateau phase after 14 h (Fig. 3c).

Biofilm formation assay. The results showed that all *Listeria* isolates of could form a biofilm (Fig. 4). Among *L. monocytogenes* isolates, LM7 formed the highest biofilm while LM14 – was the lowest. The average OD₅₆₂ values for serotypes 1/2c, 1/2a, 4b, and 1/2b were 0.134±0.016, 0.151±0.037, 0.1302±0.017, 0.104±0.011, respectively. Thus, significant differences (*p*-value <0.05) between different serotypes of *L. monocytogenes* were observed. Analysis of the biofilm formation ability of *L. innocua* isolates showed that LI22 exhibited the highest biofilm-forming ability, whereas LI19 showed the weakest biofilm formation. The average OD₅₆₂ value for *L. innocua* was 0.09198±0.01281. A highly significant difference (*p*-value <0.01) was observed between LI22 and other *L. innocua* isolates. For the *L. welshimeri* isolates, LW8 was the strongest biofilm-forming isolate, and LW5 was the weakest. The average OD₅₆₂ value of *L. welshimeri* was 0.09963±0.01257. The significant differences

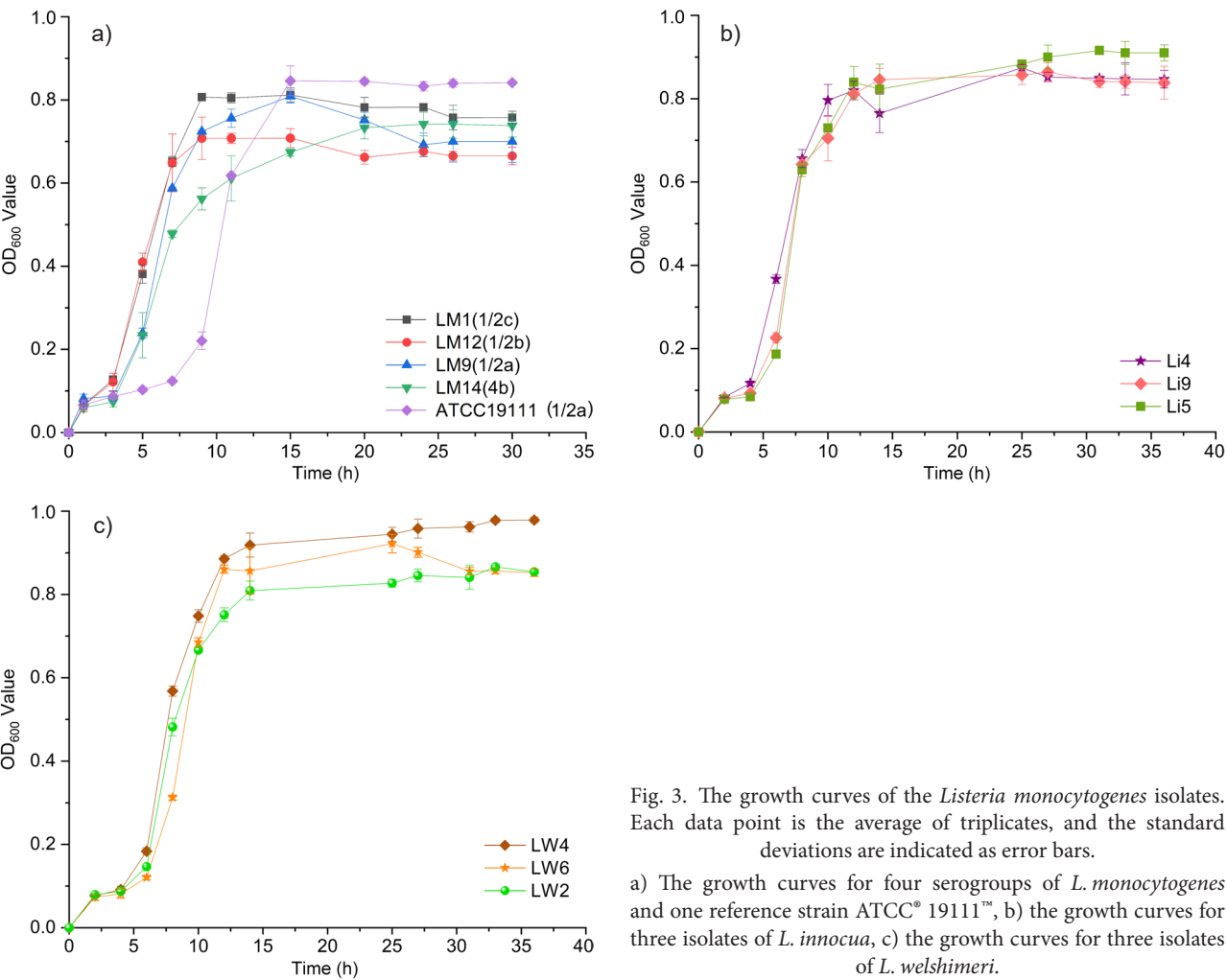


Fig. 3. The growth curves of the *Listeria monocytogenes* isolates. Each data point is the average of triplicates, and the standard deviations are indicated as error bars.
a) The growth curves for four serogroups of *L. monocytogenes* and one reference strain ATCC® 19111™, b) the growth curves for three isolates of *L. innocua*, c) the growth curves for three isolates of *L. welshimeri*.

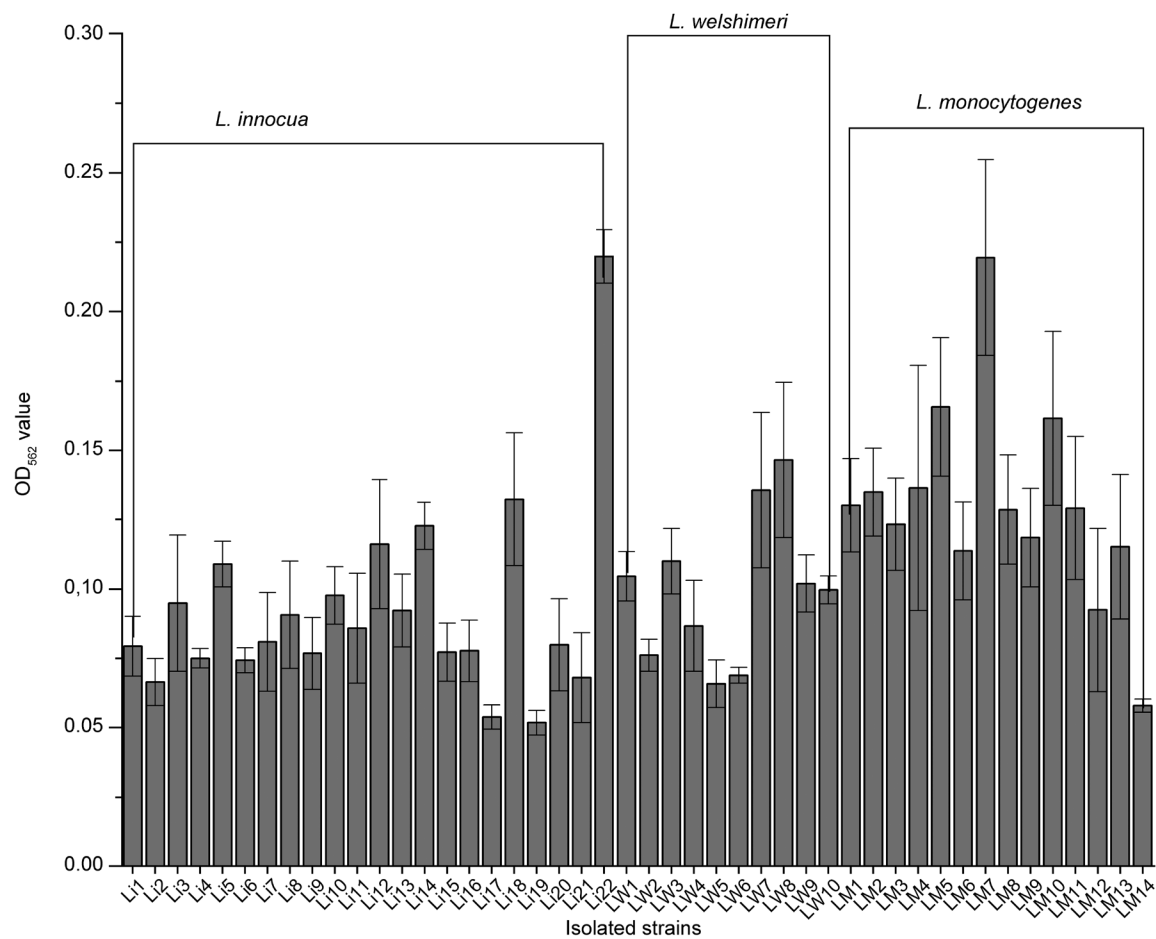


Fig. 4. Biofilm formation by *Listeria* spp. isolates. The absorbance at 562 nm was measured for 22 *L. innocua*, 10 *L. welshimeri*, and 14 *L. monocytogenes* biofilms.

(p -value < 0.05) between different *L. welshimeri* isolates were demonstrated. *L. monocytogenes* isolates formed stronger biofilm than the other two *Listeria* species.

Cluster analysis. Cluster analysis was performed on 16S rRNA sequences of 17 reference strains of *Listeria* spp. downloaded from NCBI and 16 isolates in this study (Fig. 5). The cluster analysis showed that all isolates exhibited a high level of similarity. The 16S rRNA sequence indicated that 33 strains included in genus *Listeria* were comprised of two closely related but distinct clades. The first of the clade included *L. floridensis*, *L. aquatic*, *L. grayi*, and *L. fleischmannii*, the remaining species (including *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri* and *L. welshimeri*) constituted the second clade.

Nevertheless, the *L. grayi* strain formed a subline distinct from the other highly interrelated species, especially from *L. innocua* and *L. welshimeri*. Fourteen species of *L. monocytogenes* isolated in this study were very similar to the *L. monocytogenes* EGD-e and *L. monocytogenes* strain ATCC® 19115™. According to the cluster analysis, all three species of *Listeria* spp. isolated in this study belonged to the same cluster as the corresponding reference strains selected from NCBI.

Discussion

Several reports have shown that *Listeria* spp. is prevalent and distributed in food processing environments in China (Du et al. 2017; Wang et al. 2017). However, the prevalence and distribution of *Listeria* spp. from livestock and poultry meat have been rarely reported in China.

In this study, we collected 1,387 samples from different areas in Gansu province. *L. monocytogenes* isolates were mainly present in pork samples in the market environment, and the prevalence of *L. monocytogenes* was 1.0% (14/1,387). This result differs from other provinces of China (Yan et al. 2016; Chen et al. 2020; Wang et al. 2021). The differences stem from the bacteria's isolation rate and the drug-resistant percentage. Differences may be caused by the distribution of microorganisms, sample sources, sampling methods, storage conditions, and hygiene conditions of retailers. We also isolated ten isolates of *L. welshimeri* (0.7%, 10/1,387) and 150 isolates of *L. innocua* (10.8%, 150/1,387) from the collected samples. The prevalence rate of *L. innocua* was much higher than that of *L. monocytogenes* and *L. welshimeri*. This finding is consistent with that of Locatelli et al.

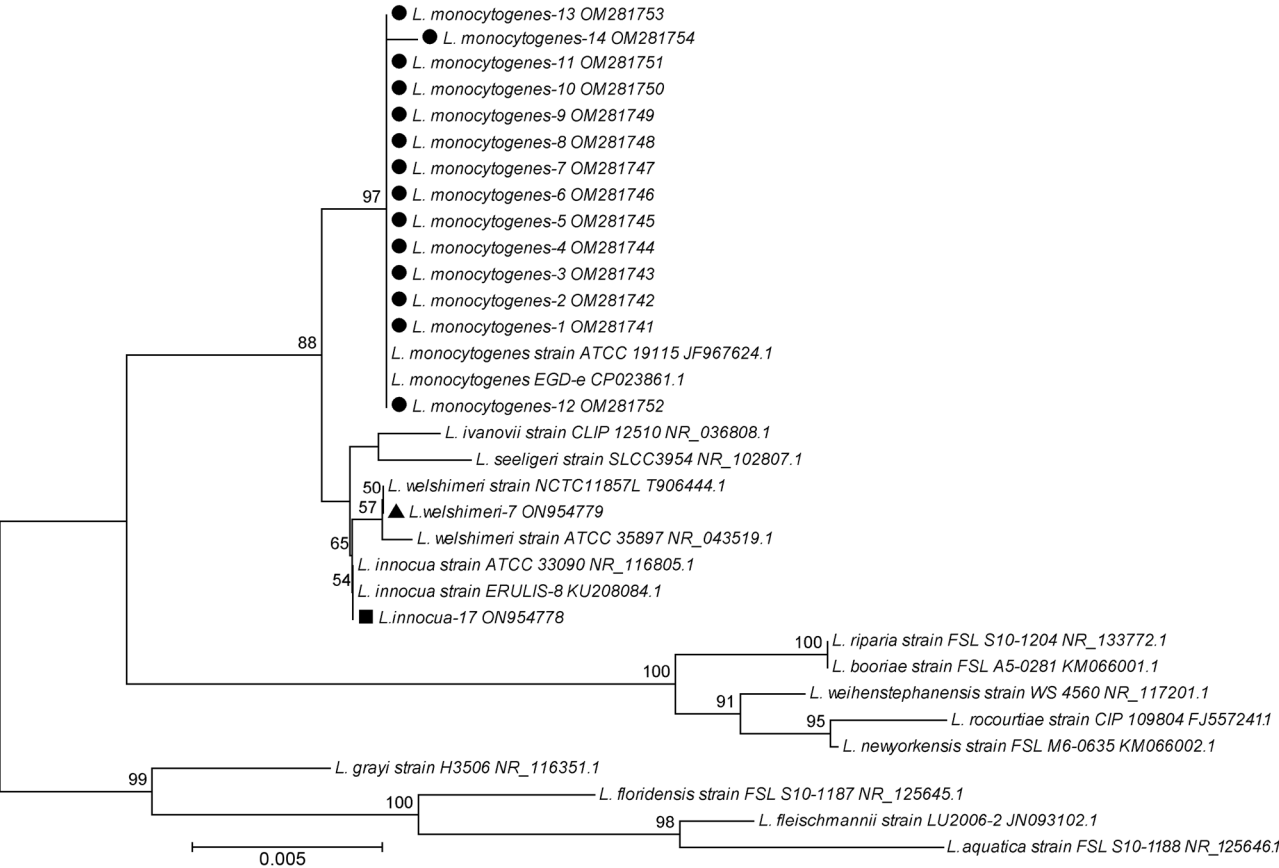


Fig. 5. Cluster analysis of *Listeria* spp. isolates based on the sequences of the 16S rRNA genes. The tree was calculated using the Neighbor-Joining method. Isolates isolated in this study are indicated with circles (*L. monocytogenes*), boxes (*L. innocua*), and triangles (*L. welshimeri*). The scale bar represents 0.005 nucleotide substitutions per character. The numbers in the tree indicate the significance (percent of outcomes) of the branches (bootstrap analysis).

(2017). Although *L. innocua* and *L. welshimeri* are non-pathogenic, the presence of these two species affects the laboratory results on the distribution of *L. monocytogenes* in the natural environment. It is related to the dominant growth of different *Listeria* species in the selective culture medium. Samples with multiple *Listeria* species coexisting during the enrichment step will inhibit *L. monocytogenes* growth (Besse et al. 2010).

Since the first strain of antibiotic-resistant *L. monocytogenes* was isolated in 1988, antibiotic resistance has become more and more serious (Heger et al. 1997). *L. monocytogenes* have special proteins on their cell membranes that can bind penicillin but cannot bind cephalosporins, so they are resistant to cephalosporins (Krawczyk-Balska et al. 2014), which was also confirmed by the results of this study. Meanwhile, all *Listeria* isolated in this study can form biofilms, which warrant bacterial persistence in the environment and antibiotic resistance development. Compared to the antibiotic resistance of *L. monocytogenes* from other places (Li et al. 2016; Fang et al. 2018; Wang et al. 2021), the isolates in this study were resistant to more than five antibiotics, indicating that they were multidrug-resistant isolates. Resistance to penicillin, tetracycline,

erythromycin, and cefoxitin appears to be particularly higher in these isolates, illustrating regional variability in the use of antibiotics.

The 14 isolates of *L. monocytogenes* isolated in this study included four serotypes: 1/2a, 1/2b, 1/2c, and 4b. We combined the results of multiplex PCR and analysis of 16S rRNA sequencing on BLAST to distinguish different isolates. Among the isolated isolates, there were 11 isolates of serotypes 1/2a and 1/2c, the main serotypes of *L. monocytogenes*. Li et al. reported that strains of serotype 4b mainly exist in patients infected with listeriosis. However, this serotype was also detected in animal-derived food in this study, indicating that this serotype is widely distributed (Li et al. 2016). Among the different samples, the chicken meat was the most contaminated with *L. monocytogenes* serotype 1/2a. Most human diseases related to listeriosis, including septicemia, meningitis, and abortion were caused by only three serotypes, 1/2a, 1/2b, and 4b (Datta and Burall 2018). In China, 253 invasive listeriosis cases were reported in 19 provinces from 2011 to 2016, with a fatality rate of 25.7% (Li et al. 2018b).

The 16S rRNA gene is considered a molecular marker in bacterial molecular classification. Orsi and

Wiedmann (2016) divided *Listeria* into two groups based on their genetic relationship. One group included *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, and *L. welshimeri*. Another group included *L. grayi* and some other newly discovered species of *Listeria* (Orsi and Wiedmann 2016). This result is the same as that obtained from our previous study. However, some scholars believe that except for *L. grayi*, the 16S rRNA sequences of other members have a high similarity and thus are not suitable as a genetic marker gene in *Listeria* (Czajka et al. 1993). As a result, the 23S rRNA, *iap*, *ldh*, *prs* and *vclB* were also used as potential genetic markers to illustrate the phylogenetic relationship of *Listeria* (Schmid et al. 2005). With the evolution of the *Listeria* virulence gene cluster, some of the *Listeria* species have lost their pathogenic ability, such as *L. innocua* and *L. welshimeri*. The location of the *L. innocua* and *L. welshimeri* virulence gene cluster breakpoints is also consistent with this view.

Conclusions

This research investigates the prevalence of *Listeria* spp. in livestock and poultry meat in Gansu province for the first time. This study provides strong evidence for the prevalence of *Listeria* species in slaughterhouses and markets. Although the isolation rate of *L. monocytogenes* is low, four serogroups were identified with high multi-drug resistance and biofilm-forming ability. The above phenomena bring great difficulty in controlling *L. monocytogenes* contamination in this area. Therefore, it is necessary to strengthen and standardize the management of processing, transportation, sales of products, rationally use antibiotics, and regular monitor the bacteria to reduce the threat of listeriosis. In addition, ecological symbiosis and phenotypic similarity existed among the three *Listeria* species. *L. innocua* and *L. welshimeri* can be used to predict the physiological behavior and contamination level of *L. monocytogenes*. Our study provides the scientific basis for preventing and controlling listeriosis in higher-risk groups and susceptible animals.

Ethical statement

All experiments were approved by the College of Veterinary Medical, Gansu Agricultural University, China.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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