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Prevalence, antibiotic resistance and virulence feature of *Listeria monocytogenes* isolated from bovine milk in Yunnan, Southwest China

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ABSTRACT

The prevalence of *Listeria monocytogenes* in bovine milk from different regions of Yunnan Province, Southwest China, was investigated. The isolated strains were genotyped using pulsed-field gel electrophoresis (PFGE), and their virulence and antibiotic resistance potential analysed with whole-genome sequencing (WGS). A total of 8 *L. monocytogenes* strains were isolated from 4 out of 161 samples, with a detection rate of 2.48%. All strains were at least resistant to one antibiotic, and the majority of strains (75%) were multi-antibiotic resistant. The 8 strains were clustered into 3 pulsotypes, and each pulsotype contained only strains isolated from the same geographical area. One strain was selected from each pulsotype for WGS, and it was found that a total of 99 antibiotic resistance genes and 83 virulence genes were detected from the 3 strains, respectively, which indicated that *L. monocytogenes* isolated from bovine milk have strong antibiotic resistance and virulence potential.

1. Introduction

Listeria monocytogenes, a Gram-positive facultative anaerobe, is one of the most prevalent foodborne pathogens worldwide. Listeriosis, caused by *L. monocytogenes,* is a seriously lethal disease that has become a cause of foodborne infections and a global public health problem. *L. monocytogenes* can grow at low temperatures and adhere to or become frequently introduced to food-processing facilities, causing food contamination repeatedly and subsequent listeriosis (Yang, Hoffmann, Allard, Brown, & Chen, 2020). Although the incidence of listeriosis is low compared with other foodborne diseases, it is often accompanied by a high mortality rate, which can reach 20–30% (Lopez-Valladares, Danielsson-Tham, & Tham, 2018). Newborns, pregnant women, and older people with the weakened immune system are at high risk for listeriosis. Studies have shown that some virulent strains of *L. monocytogenes* can cause severe central nervous system infections in immunocompromised populations (Lomonaco, Nucera, & Filipello, 2015).

In recent years, *L. monocytogenes* has been detected in many types of food, including meat, fish, milk, and dairy products (Gillesberg Lassen et al., 2016; Lee et al., 2020; Skowron et al., 2019; Yu et al., 2017). Generally, most pathogens can be sterilised during the pasteurisation process, but pasteurised milk is still at risk of contamination with pathogens due to critical issues such as the tolerance conditions of different pathogens and the standardisation of the pasteurisation process (Gabriel, Bayaga, Magallanes, Aba, & Tanguilig, 2020). It is also noteworthy that reports of various foodborne diseases linked to the consumption of raw milk have increased prominently in recent years (Langer et al., 2012). Generally, the foodborne pathogens in milk have been focused mainly on *Staphylococcus aureus*, while *L. monocytogenes* has been under-investigated.

The abuse of antibiotics increases the selective pressure on animals used for food products. Due to its innate high tolerance, *L. monocytogenes* can survive in various harsh conditions, increasing the probability of *L. monocytogenes* being exposed to antibiotics in the environment and thereby increasing its antibiotic resistance. In addition, the risk of generation and transmission of resistance genes and mobile genetic elements between pathogenic and non-pathogenic bacteria is one of the reasons for the multi-antibiotic resistance of strains (Fischer et al., 2020). Although resistance levels of *L. monocytogenes* have shown an overall increasing trend over the past years, *L. monocytogenes* strains are still considered relatively sensitive to a wide range of antibiotics

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compared with other foodborne pathogens (Conter et al., 2009; Kovacevic, Sagert, Wozniak, Gilmour, & Allen, 2013; Maung et al., 2019). Considering the impact of the potential spread of antibiotic-resistant strains in milk on public health, it is crucial to identify and monitor the antibiotic resistance profiles and virulence levels of *L. monocytogenes* in a timely manner.

The pathogenic ability of *L. monocytogenes* is closely associated with its virulence genes. The truncated inlA gene, which codes for internalin A and plays a significant role in intestinal cell invasion, has been related to a marked weakening in virulence. In the past, the strains encoding this truncation were commonly identified in the food supply (Nightingale et al., 2008), whereas the full-length inlA gene was frequently identified in strains that caused severe diseases and was relatively rare in foods (Althaus et al., 2014). However, recent studies have found that these strains were frequently isolated from food (Henri et al., 2016; Martín et al., 2014; Wang et al., 2015; Wu, Wu, Zhang, Chen, & Guo, 2016). These findings indicate that the highly virulent *L. monocytogenes* were not limited to being isolated from the clinic, but existed universally. However, the previous analysis of the genome of *L. monocytogenes* was mostly focused on clinical rather than foodborne isolates. To evaluate the safety status of *L. monocytogenes* in milk, this study investigated the contamination status and antibiotic resistance of *L. monocytogenes* in bovine milk from Yunnan Province, China. In addition, the antibiotic resistance genes and virulence genes of three strains of *L. monocytogenes* were investigated, providing a basis for the prevention and control of *L. monocytogenes* in milk.

2. Materials and methods

2.1. Sampling

From June 2019 to January 2020, a total of 161 bovine milk samples (each sample at least 500 mL) were collected from standardised cattle farms, dairy cooperatives, individual farmers, and supermarkets in Yunnan Province, southwestern China. Samples were collected aseptically, transported immediately to the laboratory under refrigeration, and stored at 4 °C before testing.

2.2. Isolation of L. monocytogenes

The *L. monocytogenes* strains were isolated according to the National Food Safety Standard-Food Microbiological Examination (GB4789.30-2016, Ministry of Health, China). A total of 25 mL of milk was added to 225 mL of LB1 enrichment solution and incubated at 30 °C for 24 h. Subsequently, 0.1 mL of culture broth was added to the LB2 enriched solution, and incubated at 30 °C for 24 h. A loopful of the enrichment culture was streaked onto Palcam Agar Plate (PALCAM) and *L. monocytogenes* selective agar plates, respectively, and incubated at 37 °C for 24–48 h. Grey-green colonies with sunken black centres, black haloes on PALCAM, and blue-green colonies with opaque rings on *L. monocytogenes* chromogenic media were identified as suspected strains of *Listeria* species. Colonies matching the morphological characteristics were inoculated with xylose and rhamnose. If negative for xylose and positive for rhamnose, they were transferred to plates with trypticase soy agar medium with 0.6% yeast extract (TSA-YE) and incubated at 37 °C for 24 h for further identification.

2.3. Identification of the suspected L. monocytogenes strains

Preliminary identification of the suspicious isolates was examined for morphological and biochemical characteristics by Gram stain, peroxidase test, Methyl Red and Voges Proskauer (MR-VP) test, kinetic test, and blood haemolysis assay. The suspected *L. monocytogenes* strains were subjected to further molecular identification based on the 16S rRNA gene. The primers were 27F/1492R (27F: 5′-AGAGTTTGATCMTGGCTCAG-3′, and 1492R: 5′-TACGGYTACCTTGTTACGACTT-3′). Analysis was performed on 1380 bp of sequence. Phylogenetic analysis was conducted using Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0.

2.4. Antibiotic susceptibility testing

L. monocytogenes strains were tested for their minimum inhibitory concentrations (MIC) using the broth microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI). Eight antibiotics, including ampicillin (AMP), chloramphenicol (CAM), tetracycline (TET), meropenem (MEM), trimethoprim-sulfamethoxazole (SXT), erythromycin (ERY), vancomycin (VAN), and ciprofloxacin (CIP), were tested (Supplementary material Table S1). The results were interpreted according to CLSI (2016). In this edition, there are no available susceptibility breakpoints for the three antibiotics MEM, VAN, and CIP against *L. monocytogenes*. Therefore, the susceptibility results for these three antibiotics are provided as MIC values. *Enterococcus faecalis* ATCC 29212 and *S. aureus* ATCC 29213 were used for quality control of the test (Cheng et al., 2017). Multiple antibiotic resistance (MAR) was defined as being resistant to three or more antimicrobial classes. The MAR index of each strain was calculated using the formula MAR index = A/B, where “A” is the number of antibiotics to which the strains were resistant and “B” is the total number of antibiotics tested (Meng et al., 2020).

2.5. Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) of *L. monocytogenes* strains was performed according to the PulseNet protocol. Plug mould was digested with 25 U Asc I (Takara, Japan) at 37 °C for 2 h, followed by electrophoresis performed on a CHEF-DRIII system (Bio-Rad Laboratories, Hercules, CA, United States). Electrophoresis was conducted with a switch time of 4–40 s and lasted for 9 h. Xba I-digested genomic DNA of *Salmonella* serotype Braenderup strain H9812 was used as a molecular size marker (McMillan, Moore, McAuley, Fegan, & Fox, 2016). Gels were visualised using a Gel Doc 2000 system (BioRad). The fingerprint pattern of the gel was analysed using the Bionumerics software (version 7.6, Applied Maths, Kortrijk, Belgium). After the background subtraction and gel normalisation, the fingerprint patterns were subjected to typing based on banding similarity with a position tolerance of 1.5%.

2.6. Genomic DNA extraction and whole-genome sequencing

One strain from each PFGE cluster was selected for whole genome sequencing (WGS). The strains were cultured with vigorous shaking in trypticase soy-yeast extract broth (TSB-YE) for 24 h. Genomic DNA was extracted using a bacterial genomic DNA extraction kit following the manufacturer's protocol (Thermo Fisher Scientific, USA). Then, the DNA was sheared into about 500 bp fragments to construct libraries. The prepared libraries were subjected to 2 × 150 bp paired-end sequencing using Illumina NovaSeq 6000, and raw data were analysed and processed by several bioinformatics software packages. Besides, the raw bacterial genomic reads were deposited into the NCBI Sequence Read Archive database (Accession ID: PRJNA755432).

2.7. Genome splicing and annotation

Bioinformatics analysis was performed using genomic raw data generated by the Illumina platform. The Illumina reads were quality

controlled and assembled using Velvet, and then gaps filled with SSPACE and GapFiller (Boetzer & Pirovano, 2012; Hunt, Newbold, Berriman, & Otto, 2014; Yang et al., 2020). Prodigal software was used to find coding genes in bacteria (Liu, Lawrence, Austin, & Ainsworth, 2007). Transfer RNAs (tRNAs) were detected in the genome using the program tRNAscan-SE with the default parameters (Lowe & Eddy, 1997). The rRNA genes were identified by RNAmmer (Lagesen et al., 2007). The coding genes were annotated in the Non-Redundant Protein Sequence Database of the National Centre for Biotechnology Information (NCBI). Moreover, the functions of genes were annotated in the Gene Ontology (GO) database, the proteins encoded by genes were classified on a phylogenetic tree by the Clusters of Orthologous Groups (COG) database, and the pathways were annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Furthermore, the nucleotide sequence of the predicted coding genes was compared with the comprehensive antibiotic resistance database (CARD) and the virulence factor database (VFDB) to obtain the relevant annotation information of the antibiotic resistance gene and the virulence gene of the strain.

2.8. Data analysis

The data record and analysis were done with Microsoft Excel 2016 (Microsoft Corp). Data generated from the Illumina platform were used for bioinformatic analysis. All bioinformatic analyses were performed using the I-Sanger Cloud Platform from Shanghai Majorbio BioTech Co., Ltd. (Shanghai, China).

3. Results

3.1. Identification of the isolates from bovine milk

In the present study, a total of 8 strains of suspected *L. monocytogenes* were obtained from 4 out of 161 samples after preliminary identification by Gram staining, motility testing, biochemical reactions, and haemolysis testing. Further, it was demonstrated that all 8 strains were *L. monocytogenes* based on the phylogenetic analysis of their 16S rRNA genes (Fig. 1). The results suggested that the prevalence of *L. monocytogenes* was detected in milk in Yunnan Province, China, with a detection rate of 2.48%.

3.2. Antibiotic resistance profiles of L. monocytogenes

Eight *L. monocytogenes* strains were tested for their resistance to 8 antibiotics. MIC values were converted into susceptibility categories (susceptible, intermediate, and resistant) according to antibiotic susceptibility testing breakpoints for *L. monocytogenes* described by the CLSI. The resistance profiles of *L. monocytogenes* for 8 antibiotics are presented in Table 1 and Supplementary material Table S1. Eight strains were all sensitive to ampicillin, but the proportion of strains resistant to penicillin, tetracycline, trimethoprim-sulfamethoxazole and erythromycin was 100%, 87.5%, 75%, and 50%, respectively. The distribution of resistance patterns of *L. monocytogenes* strains to the 5 antibiotics is shown in Fig. 2. Only two strains were resistant to one or two antibiotics, respectively. The other six strains (75.0%) were resistant to three or more classes of antibiotics and defined as multiple antibiotic resistance (MAR). Besides, the MAR index values for eight *L. monocytogenes* strains ranged from 0.2 to 0.8, and the MAR of most strains (87.5%) was >0.2 (Table 2).

3.3. PFGE genotyping of L. monocytogenes strains

All *L. monocytogenes* strains were subjected to DNA macrorestriction with the enzyme AscI, and three pulsotypes were generated by PFGE (Fig. 3). The PFGE analysis showed the presence of 3 different pulsotypes. Each pulsotype contained only strains isolated from the same geographic area. Specifically, one pulsotype contained 2 strains from the Dairy Cooperative in Dali; one pulsotype contained 4 strains isolated from individual cattle farms in Shangri-La; and another pulsotype contained 2 strains from standardised cattle farms in Kunming. The PFGE genotyping of *L. monocytogenes* strains in this study showed a clear geographical profile.

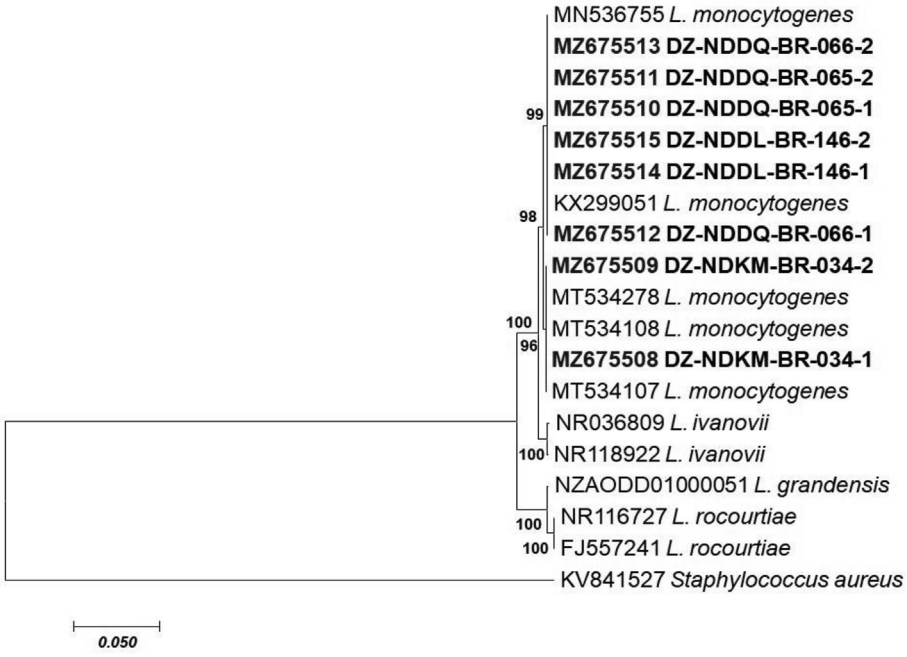

Fig. 1. Phylogenetic tree based on the 16S rRNA gene of *L. monocytogenes* isolates with a neighbour-joining (NJ) algorithm, using *Staphylococcus aureus* (KV841527.1) as an outgroup. The numbers at the branch are bootstrap values of 1000 replicates. The bold fonts indicate the strains isolated from milk in this study.

Table 1
The interpretive categories of *L. monocytogenes* strains for the tested antibiotics.[a]

Class	Antibiotic	Interpretive	Total number (n = 8)
Penicillins	Ampicillin	S	8
		I	–
		R	–
	Penicillin	S	–
		I	–
		R	8
Tetracyclines	Tetracycline	S	1
		I	–
		R	7
Sulfonamide	Trimethoprim-sulfamethoxazole	S	2
		I	–
		R	6
Macrolide	Erythromycin	S	4
		I	–
		R	4
	Vancomycin	0.5	4
		1	4
	Ciprofloxacin	0.5	7
		1	1
	Meropenem	0.06	1
		0.125	7

[a] Abbreviations are: S, susceptible; I, intermediately resistant; R, resistant. Vancomycin, ciprofloxacin, and meropenem have no breakpoints for *L. monocytogenes* according to CLSI standards and therefore MIC values are given (μg mL^{-1}).

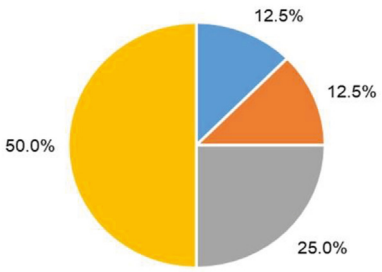

Fig. 2. Distribution of resistance patterns of all *L. monocytogenes* strains (n = 8): ●, resistance to 1 class; ●, resistance to 2 classes; ●, resistance to 3 classes; ●, resistance to 4 classes.

3.4. WGS of representative L. monocytogenes strains

To further analyse the profile of antibiotic resistance and virulence genes in *L. monocytogenes*, one strain from each of the three pulsotypes was selected for WGS. The three strains of *L. monocytogenes* generated a total of 26,736,256 to 30,275,932 reads by WGS. Their genome sequence lengths ranged from 2,885,876 bp to 2,966,764 bp after de novo assembly. The GC content of the three strains ranged from 37.3% to 37.93% (Table 3).

Table 2
Multiple antibiotic resistance (MAR) index of *L. monocytogenes*.

Isolates	MAR index	Antibiotics
NDKM-034-1	0.4	Penicillin, tetracycline
NDKM-034-2	0.2	Penicillin
NDDQ-065-1	0.8	Penicillin, tetracycline, trimethoprim-sulfamethoxazole, erythromycin
NDDQ-065-2	0.8	Penicillin, tetracycline, trimethoprim-sulfamethoxazole, erythromycin
NDDQ-066-1	0.8	Penicillin, tetracycline, trimethoprim-sulfamethoxazole, erythromycin
NDDQ-066-2	0.8	Penicillin, tetracycline, trimethoprim-sulfamethoxazole, erythromycin
NDDL-146-1	0.6	Penicillin, tetracycline, trimethoprim-sulfamethoxazole
NDDL-146-2	0.6	Penicillin, tetracycline, trimethoprim-sulfamethoxazole

The genome sequencing results of three *L. monocytogenes* strains were compared and annotated with the GO, COG, and KEGG databases. GO analysis showed that a total of 1844, 1860, and 1864 genes were annotated in strains NDKM-034-1, NDDQ-065-1, and NDDL-146-1, respectively, accounting for 65.46%, 63.57%, and 62.85% of the total predicted genes. Those genes were classified into three major functional classes, including biological process (BP), cellular components (CC), and molecular function (MF). Among them, the genes associated with MF exhibited the highest abundance, followed by BP and CC. The genes most commonly classified within GO functional subcategories are those related to metabolic processes, catalytic activity, and cellular processes (Fig. 4). According to the COG annotation analysis, strains NDKM-034-1, NDDQ-065-1, and NDDL-146-1 contained 2415, 2373, and 2417 proteins, respectively. Among all genes, 85.73%, 82.54%, and 81.49% were COG annotated. These genes involved in carbohydrate transport and metabolism are essential for maintaining bacterial metabolic and functional properties (Fig. 5). A KEGG functional annotation was performed on *L. monocytogenes* isolated from milk to characterise its gene function further. The number of encoding genes predicted in the *L. monocytogenes* strains NDKM-034-1, NDDQ-65-1, and NDDL-146-1 were 2063, 2103, and 2093, respectively. A total of 37 pathways representing six functions, including cellular processes, environmental information processing, genetic information processing, human diseases, metabolic pathways, and organismal systems, were obtained. The results showed that the annotations for gene function of three strains of *L. monocytogenes* are dominantly centred on metabolic pathways. For all strains, the most relevant genes were predominantly involved in the carbohydrate metabolic pathway (Fig. 6).

3.4.1. Antibiotic resistance genes of L. monocytogenes strains

A total of 99 antibiotic-resistance genes were found in three *L. monocytogenes* strains, and these resistance genes were resistant to 22 different classes of antibiotics (Table 4). Compared with other antibiotic classes, the number of genes with resistance to glycopeptide, macrolide, tetracycline, and the peptide was relatively high, with 13, 13, 12, and 11, respectively. In this study, the results of the resistance phenotypes showed that strain NDKM-034-1 was resistant to penicillin and tetracycline; strain NDDQ-065-1 was resistant to penicillin, tetracycline, and erythromycin cotrimoxazole resistance; and strain NDDL-146-1 was resistant to penicillin, tetracycline, and erythromycin (Table 2). Compared with resistance phenotypes, genes associated with resistance to β-lactams, macrolides, tetracyclines, and sulfonamide antibiotics were detected in this study, indicating that the resistance phenotypes and resistance gene results were generally consistent. Furthermore, 19 of the 99 antibiotic-resistance genes are mutated (Supplementary material Table S2).

3.4.2. Virulence genes of L. monocytogenes strains

Putative virulence genes were identified by the Virulence Factor Database (VFDB). A total of 83 annotated virulence genes was found in the three strains of *L. monocytogenes*, with strain NDKM-034-1 having the most virulence genes (83 genes), followed by strain NDDL-146-1 (69 genes), and strain NDDQ-065-1 having the least (62 genes) (Table 5). Three *L. monocytogenes* strains co-harboured 61 virulence genes. The 21 virulence genes, including *actA, Ami, Aut, Bsh, Hly, Hpt, inlA, inlB, inlC, inlJ, inlK, isdE2, lapB, LM1816_14165, lntA, Mpl, plcA, plcB, prfA, Vip,* and *fliS*, were present only in strain NDKM-034-1. While the *fss3* gene was only present in strain NDDL-146-1, and strain NDDQ-065-1 did not have a unique gene. These results suggest that the virulence genes of *L. monocytogenes* isolated from milk are characterised by diversity.

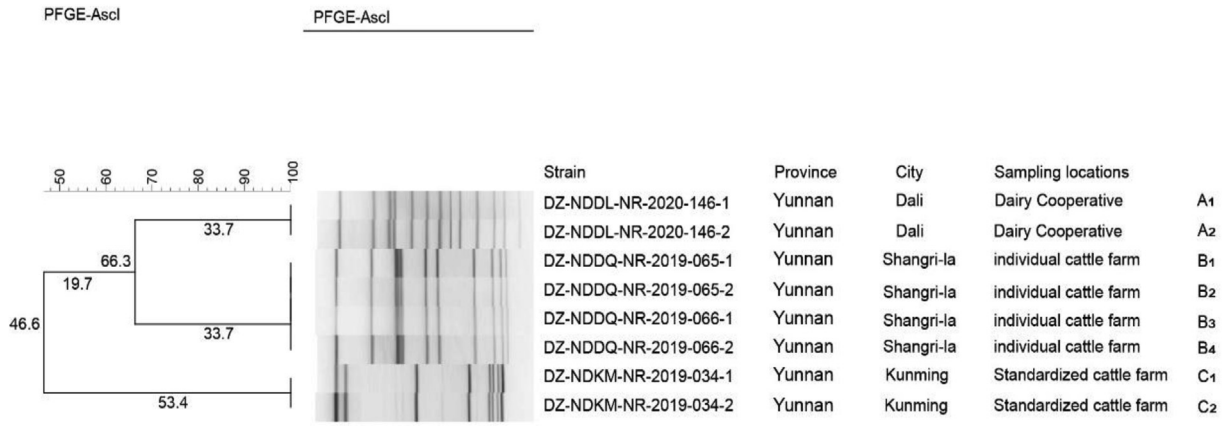

Fig. 3. Dendrogram of PFGE patterns of the 8 *L. monocytogenes* strains collected from milk samples.

Table 3
Assembly data associated with *L. monocytogenes* whole genomes obtained from Illumina paired-end sequencing data.

Strain	No. of reads	Total bases (bp)	Average length (bp)	GC content (%)	N50	tRNA	rRNA	Other ncRNA
NDKM-034-1	30,275,932	2,885,876	169,757.41	37.93	515,231	67	12	93
NDDQ-065-1	27,135,310	2,959,425	147,966.25	37.30	544,274	67	14	101
NDDL-146-1	26,736,256	2,966,764	148,338.20	37.31	489,990	68	12	106

4. Discussion

In recent years, the detection of *L. monocytogenes* in raw and pasteurised milk has been frequently reported, and milk has also been considered to be related to several outbreaks of listeriosis (www.cdc.gov). Although milk had been repeatedly proven to be the main source of foodborne *L. monocytogenes*, the antibiotic resistance and virulence features of *L. monocytogenes* isolated from milk were still unclear. Therefore, it is necessary to monitor *L. monocytogenes* in milk in real time.

In this study, *L. monocytogenes,* isolated from milk in Yunnan province, China, was tested for molecular typing, antibiotic resistance, and virulence genes. The results showed that *L. monocytogenes* was detected in 4 out of the 161 milk samples, for a detection rate of 2.48%. Similar detection rates were obtained in Poland (5.5%) (Skowron et al., 2019), Ethiopia (5.6%) (Seyoum, Woldetsadik, Mekonen, Gezahegn, & Gebreyes, 2015), India (5.8%) (Soni, Singh, Singh, & Dubey, 2013), and Kosovo (2.7%) (Mehmeti, Bytyqi, Muji, Nes, & Diep, 2017). By contrast, the detection rate of *L. monocytogenes* in raw milk was much higher in Egypt (25.0%) (Tahoun et al., 2017). In this research, *L. monocytogenes* was not detected in pasteurised milk, suggesting that pasteurisation appears to be an effective method for reducing *L. monocytogenes* in milk.

The genetic similarity between 8 strains was analysed by PFGE molecular typing and clustering analysis. The PFGE analysis showed the presence of 3 different pulsotypes, and each pulsotype contained only strains isolated from the same geographic area (Fig. 3). Notably, four strains isolated from the same geographic area (Shangri-La) in cluster B also showed 100% similarity, which suggested that they came from the same pollution source. The PFGE genotyping results of isolated strains from three areas showed distinct geographical characteristics, indicating no cross-geographic contamination. In the present study, the genetic diversity of *L. monocytogenes* isolated from southwestern Chinese milk was relatively low, which was consistent with that in milk from the United States and Slovenia (Latorre et al., 2011; Papić, Golob, Kušar, Pate, & Zdovc, 2019).

Antibiotic resistance is considered a worldwide health problem, especially for foodborne pathogens, which can directly threaten human health (Prabakusuma et al., 2022). Since *L. monocytogenes* is a cold-tolerant bacterium with high lethality, it is necessary to monitor the antibiotic resistance of *L. monocytogenes* isolated from milk. This study showed that most strains had relatively high levels of resistance against tetracycline, erythromycin, and trimethoprim-sulfamethoxazole. Even the rate of resistance to penicillin was up to 100% (Table 2). A similar result was obtained for resistance to *L. monocytogenes*, which was detected in buffalo milk (Terzi Gulel, Gucukoglu, Cadirci, Saka, & Alisarli, 2020).

Ampicillin or penicillin is used alone or combined with gentamicin and other aminoglycoside antibiotics as a first-line treatment method for listeriosis, which has been proven to have a good curative effect in the clinic (Hof, 2004). Notably, the resistance of *L. monocytogenes* to ampicillin and penicillin in this study was polarised, with all strains being sensitive to ampicillin but resistant to penicillin. It may be due to the irregular use of antibiotics during dairy farming. In addition, 75% of *L. monocytogenes* were multi-drug resistant, and 87.5% of the strains had MAR values >0.2 (Table 2), indicating that *L. monocytogenes* strains isolated from milk were highly resistant to a wide range of antibiotics. Interestingly, there was a correlation between the PFGE molecular clustering of *L. monocytogenes* strains and their antibiotic resistance. Namely, multi-antibiotic resistant strains were concentrated in clusters B and C, while that of cluster A was relatively low, which was accordant with the results of a previous study (Abdi et al., 2018).

In the present study, 99 different resistance genes were detected in three *L. monocytogenes* strains, including seven β-lactam resistance genes (Table 4). Penicillin is one of the β-lactam antibiotics. Penicillin-binding proteins (*PBPs*) are the natural targets of β-lactam antibiotics. Mutations in the *PBP1a* and *PBP2x* genes are responsible for resistance to beta-lactams (Aslan et al., 2012). Mutations in *PBP1a* and *PBP2x* were detected in three *L. monocytogenes* strains in this study. Moreover, the acquired gene *mecA* encoding a penicillin-binding protein (*PBP2a*) and the chromosomal encoding gene *NmcA* for β-lactamase were also detected. These genes may lead to the resistance of three *L. monocytogenes* strains to penicillin

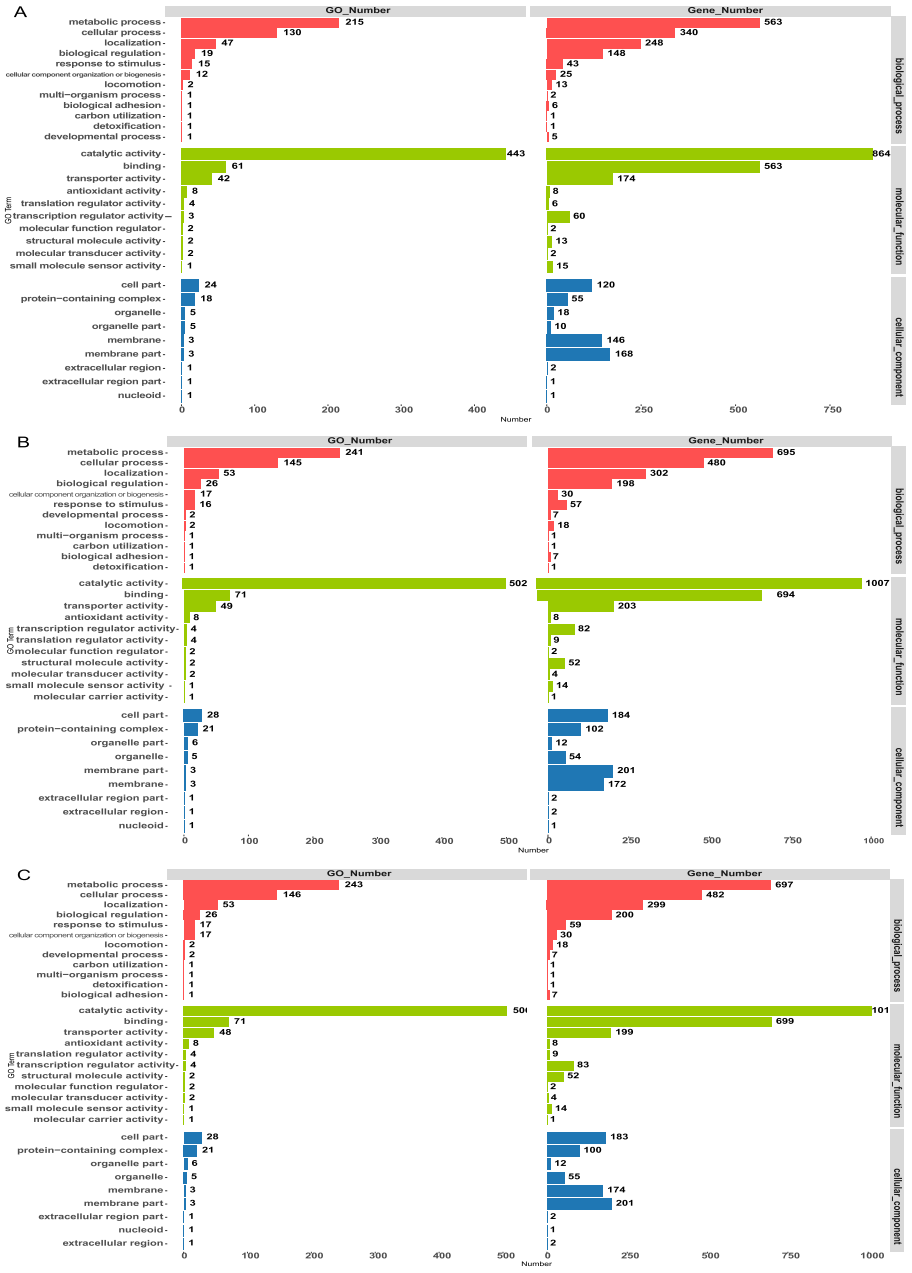

Fig. 4. GO classification of three *L. monocytogenes* strains with three functional classes, including biological process, cellular component, and molecular function: A, *L. monocytogenes* NDKM-034-1; B, *L. monocytogenes* NDDQ-065-1; C, *L. monocytogenes* NDDL-146-1.

(Tables 1 and 4). Generally speaking, patients with an allergic reaction to penicillin are often treated with sulfonamides instead (trimethoprim-sulfamethoxazole) (Spitzer, Hammer, & Karchmer, 1986). In this study, the detection of sulfonamide resistance genes was relatively low, with only *sul4* and *folp* being detected. The two strains (NDDQ-065-1 and NDDL-146-1) resistant to trimethoprim-sulfamethoxazole harboured the *folp* gene. This supports the conclusion of Buwembo, Aery, Rwenyonyi, Swedberg, and Kironde (2013), in which the *folp* point mutations could explain the resistance of some bacteria to sulfonamide. The gene *sul4* is the first mobile sulfonamide resistance gene discovered since 2003 (Razavi et al., 2017). The mobile *sul4* resistance gene, which was present in strain NDKM-034-1, did not show resistance, but *sul4* may provide more opportunities to spread *sul4* resistance.

A total of 12 tetracycline-related resistance genes were found, of which seven genes, including *tetA (60)*, *tetB (60)*, *tetB (P)*, *tetA (48)*, *tetA (46)*, *tetT*, and *tcr* were shared by the three strains. The other five genes, *tet (C)*, *tet (D)*, *tetM*, *tetS*, and *tet (42)*, only existed in one or two strains. Previous studies showed that the primary resistance gene found in *L. monocytogenes* was *tetM* (Granier et al., 2011), which is somewhat different from our results. In this study, all detected tetracycline-related resistance genes are efflux pump genes. Efflux is considered the most prevalent tetracycline resistance mechanism (Nguyen et al., 2014). Tetracycline that has entered the cell can be discharged out of the cell through the efflux pump system, thereby reducing the intracellular concentration of antibiotics and leading to resistance. Therefore, the presence of these tetracycline resistance genes may be responsible for the

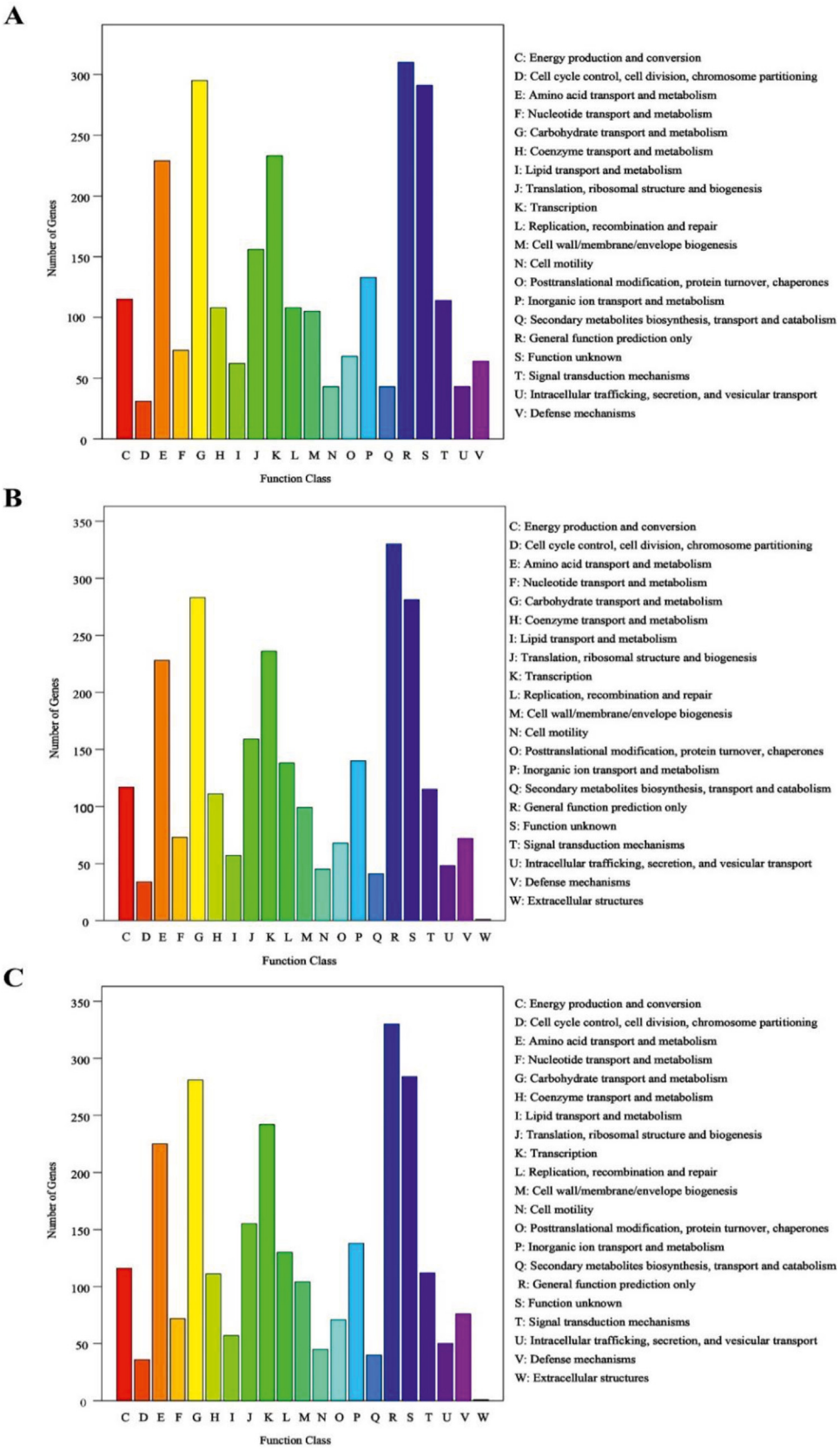

Fig. 5. COG classification of three *L. monocytogenes* strains was associated with 21 functional classes: A, *L. monocytogenes* NDKM-034-1; B, *L. monocytogenes* NDDQ-065-1; C, *L. monocytogenes* NDDL-146-1.

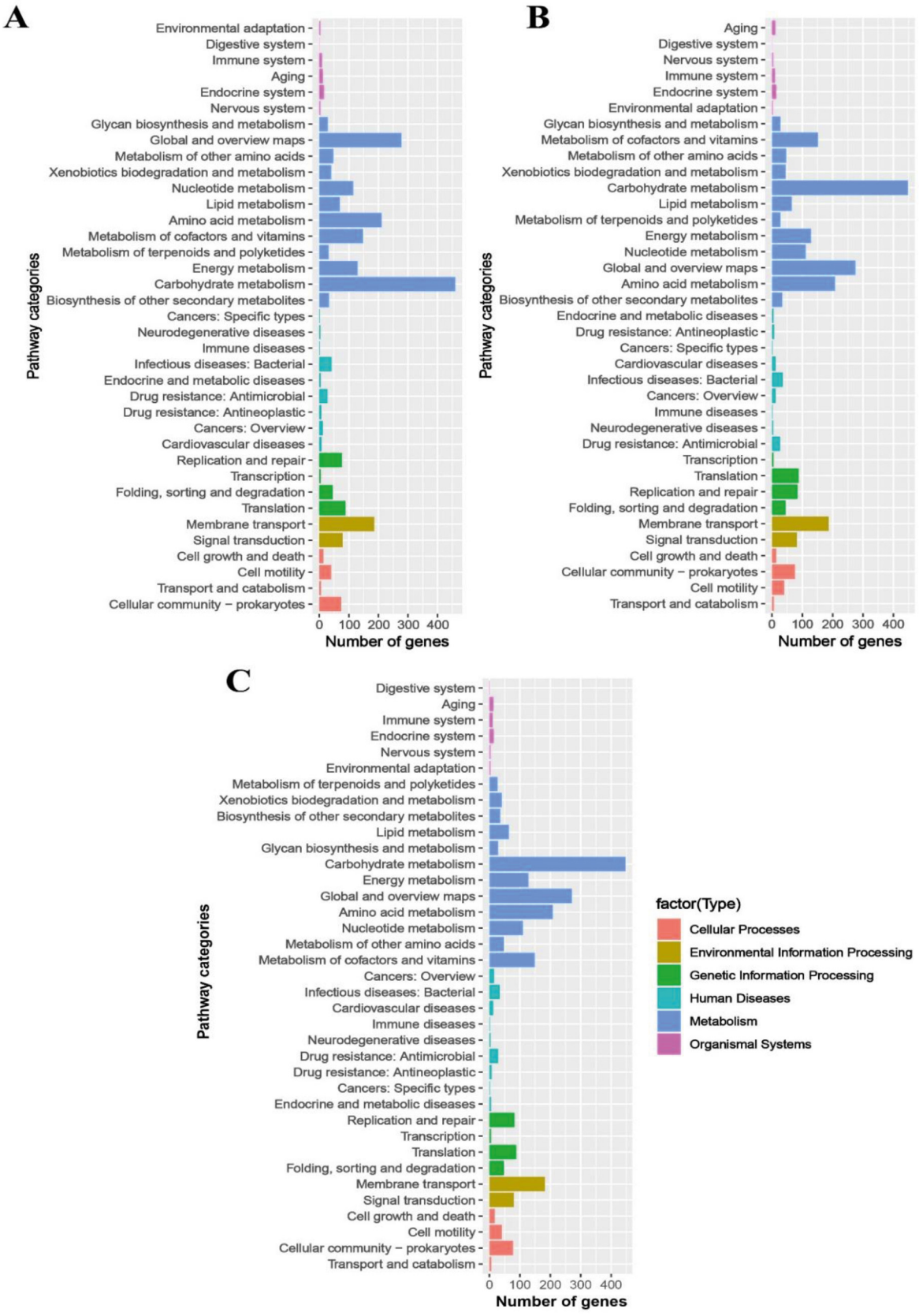

Fig. 6. KEGG classification of three *L. monocytogenes* strains with six functional classes, including organismal systems, metabolism, human disease, genetic information processing, environmental information processing, and cellular processing: A, *L. monocytogenes* NDKM-034-1; B, *L. monocytogenes* NDDQ-065-1; C, *L. monocytogenes* NDDL-146-1.

Table 4
Antibiotic resistance genes in *L. monocytogenes* strains.

Antibiotic classes	Resistance genes [no. of strains]
Aminocoumarin	*novA* [3]
Aminoglycoside	*kdpE* [3], *ANT(9)-Ia* [1], *ANT(6)-Ia* [1], *APH(3′)-IIIa* [1], *baeS* [3], *ykkC* [3], *adeR* [3]
β-Lactam	*NmcR* [3], *PBP2x* [3], *PBP1a* [3], *mecA* [3], *L1 beta-lactamase* [3], *SRT-1* [3], *ACC-3* [2]
Diaminopyrimidine	*dfrG* [3]
Elfamycin	*facT* [3], *EF-Tu* [3]
Fluoroquinolone	*norB* [3], *patB* [3], *arlS* [3], *arlR* [3], *acrR* [3], *marA* [3],
Fosfomycin	*mdtG* [3], *FosX* [3], *PtsI* [3], *UhpT* [1]
Fusidic acid	*fusA* [3], *fusB* [3], *fusE* [3]
Glycopeptide	*vanRM* [3], *vanTE* [1], *D-Ala-D-Ala* [3], *vanHF* [3], *vanHD* [3], *vanYM* [3], *vanRG* [3],*vanSM* [3], *vanRE* [3], *vanTG* [2], *vanRF* [2], *vanRI* [2], *mepA* [3]
Lincosamide	*lmrB* [3], *lmrC* [3], *lmrD* [3], *lnuB* [1], *lsaE* [3], *salA* [3], *lsaA* [3]
Macrolide	*macB* [3], *LpeA* [1], *carA* [3], *oleC* [1], *abeS* [3], *evgS* [1], *efrA* [3], *Erm (34)* [2], *ErmH* [1], *cfrA* [3], *mtrA* [3], *msrC* [3] *ErmB* [1]
Nitrofuran	*nfsA* [3]
Nitroimidazole	*msbA* [3]
Oxazolidinone	*optrA* [3]
Peptide	*bcrA* [3], *PmrF* [3], *mprF* [3], *CdsA* [3], *liaR* [3], *liaS* [3], *cls* [3], *menA* [3], *rpoC* [3], *walK* [3], *rpoB* [3]
Phenicol	*cmrA* [3], *fexA* [2]
Pleuromutilin	*TaeA* [3]
Rifamycin	*rphB* [3]
Streptogramin	*VatI* [3], *vatB* [3], *vgaE* [2], *vgaALC* [3]
Sulfonamide	*sul4* [1], *folp* [2]
Tetracycline	*tetA (60)* [3], *tetB (60)* [3], *tetB (P)* [3], *tetA (48)* [3], *tetA (46)* [3], *tetT* [3], *tcr3* [3], *tet (C)* [1], *tet(D)* [2], *tetM* [2], *tetS* [1], *tet (42)* [2]
Triclosan	*fabG* [3]

development of resistance to tetracycline in these *L. monocytogenes* strains.

In the present study, the number of detected macrolide antibiotic resistance genes was also relatively high, with 13 genes. However, the detection rates of these resistance genes in the three strains varied greatly. The results of this study on antibiotic resistance showed that the three strains were resistant to macrolide antibiotics (erythromycin). Hadorn, Hächler, Schaffner, and Kayser (1993) found that the *ErmB* gene was present in most erythromycin-resistant strains, which was also present in NDDQ-065-1 in our study.

In addition to the genes associated with resistance phenotypes for β-lactam, sulfacycline, tetracycline, and macrolide antibiotics, other resistance genes were also identified. Among them, the glycopeptide antibiotic resistance genes were the most detected, with 13 genes. Among the glycopeptide resistance genes, it is worth noting that *vanRM* is a *vanR* variant of the *vanM* gene cluster. *VanRM* can confer resistance to the glycopeptide antibiotic (vancomycin) by altering the antibiotic target (McArthur et al., 2013). The same goes for the *vanTE*, *vanHF*, *vanHD*, *vanYM*, *vanRG*, *vanSM*, *vanRE*, *vanTG*, *vanRF*, and *vanRI* genes. It has been suggested that partial or complete amplification or transfer of the *vanM* gene cluster leads to increased copy number and expression of the *vanM* gene by an unknown mechanism, which contributes to the transformation of vancomycin-resistant *Enterococcus faecium* into high levels of vancomycin-resistant variants (Zhou et al., 2020). It was also shown that the essential alanine racemase evolved into a vancomycin-resistant enzyme under antibiotic pressure. Although the presence of antimicrobial resistance genes is not always consistent with the antimicrobial resistance phenotype of foodborne pathogens, the existence of related genes may participate in different resistance mechanisms in different environments and affect the resistance phenotype of strains. Vancomycin is one of the last resorts against Gram-positive and drug-resistant pathogens. Therefore, more attention should be paid to the potential existence of such genes.

Another important type of resistance gene detected was the peptide resistance gene. Eleven peptide resistance genes were detected in all three *L. monocytogenes* strains, with a 100% detection rate (Table 4). Additionally, mutations in *liaR*, *liaS*, *cls*, *rpoC*, and *Walk*, known to be responsible for daptomycin resistance (Gómez Casanova, Siller Ruiz, & Muñoz Bellido, 2017), were found in three strains. The point mutation of the *rpoB* gene was also detected, leading to rifampicin resistance (Yang et al., 2010). In the present study, a total of 19 mutation genes were detected (Supplementary material Table S2). These genes were originally found in *Escherichia coli*, *E. faecalis*, *S. aureus*, and *Streptococcus pneumoniae*.

Some bacteria acquire antibiotic resistance through horizontal gene transfer (HGT) of plasmids carrying genetic material encoding antibiotic resistance. HGT is faster than spontaneous mutations and can provide genes required for survival (Charpentier, Polard, & Claverys, 2012). It proved that HGT was important for the proliferation and development of *L. monocytogenes*. Yan et al. (2019) showed that intra- and inter-strain gene horizontal transfer of foodborne *L. monocytogenes* also appeared in China from 2012 to 2015, leading to the development of antibiotic resistance in strains. Other studies have obtained similar results (Bertsch et al., 2014). Despite antibiotic resistance being a major public health concern, it is unknown whether the resistance genes in disease-causing microorganisms are passed from bacteria in the environment or elsewhere (Forsberg et al., 2012). Antibiotic resistance may be easier to acquire through HGT for these *L. monocytogenes* strains isolated from milk, which is worth further exploration.

Virulence genes are essential determinants of bacterial virulence. In the present study, three *L. monocytogenes* strains from milk carried different virulence genes. Specifically, strain NDKM-034-1 carried some genes from the internalin family, including *inlA*, *inlB*, *inlC*, *inlJ*, and *inlK* (Table 5). Internalins in *L. monocytogenes* have a broad spectrum of functions, including facilitating host−pathogen interaction and other virulence-related physiological processes (McGann, Ivanek, Wiedmann, & Boor, 2007). For instance, *inlA* and *inlB* genes play important roles in the process of bacteria invading host cells (Haidar-Ahmad et al., 2016; Sobyanin et al., 2017). The *inlC* gene encodes an avirulence protein that plays a role after the intestinal phase of infection. The *inlJ* gene is directly involved in the passage of *L. monocytogenes* through the intestinal barrier and the subsequent infection phase (Sabet, Lecuit, Cabanes, Cossart, & Bierne, 2005). Some studies have reported that the genome of potentially virulent *L. monocytogenes* contains the *inlC* or *inlJ* genes. In contrast, these genes are not detected in strains that cannot cause mouse death (Liu et al., 2007).

Table 5
Virulence genes detected in three *L. monocytogenes* strains.[a]

Virulence gene	*L. monocytogenes* strain			Virulence gene	*L. monocytogenes* strain		
	NDKM-034-1	NDDQ-065-1	NDDL-146-1		NDKM-034-1	NDDQ-065-1	NDDL-146-1
actA	+	−	−	*fliE*	+	+	+
Ami	+	−	−	*fliF*	+	+	+
Aut	+	−	−	*fliG*	+	+	+
Bsh	+	−	−	*fliH*	+	+	+
Hly	+	−	−	*fliI*	+	+	+
Hpt	+	−	−	*fliM*	+	+	+
inlA	+	−	−	*Flip*	+	+	+
inlB	+	−	−	*fliQ*	+	+	+
inlC	+	−	−	*fliR*	+	+	+
inlJ	+	−	−	*fliS*	+	−	−
inlK	+	−	−	*gtcA*	+	+	+
isdE2	probable	−	−	*hbp2*	+	+	+
lapB	+	−	−	*hdp1*	+	+	+
LM1816_14165	+	−	−	*iap/cwhA*	+	+	+
lntA	+	−	−	*Lap*	+	+	+
Mpl	+	−	−	*Lgt*	+	+	+
plcA	+	−	−	*lisK*	+	+	+
plcB	+	−	−	*lisR*	+	+	+
prfA	+	−	−	*lmo0693*	+	+	+
Vip	+	−	−	*lmo0698*	+	+	+
agrA	+	+	+	*lmo0700*	+	+	+
agrC	+	+	+	*lpeA*	+	+	+
cheA	+	+	+	*lplA1*	+	+	+
cheR	+	+	+	*lspA*	+	+	+
cheV	+	+	+	*motA*	+	+	+
cheY	+	+	+	*motB*	+	+	+
clpC	+	+	+	*oatA*	+	+	+
clpE	+	+	+	*oppA*	+	+	+
clpP	+	+	+	*pdgA*	+	+	+
dltA	+	+	+	*prsA2*	+	+	+
fbpA	+	+	+	*srtA*	+	+	+
flaA	+	+	+	*srtB*	+	+	+
flgB	+	+	+	*Stp*	+	+	+
flgC	+	+	+	*virR*	+	+	+
flgD	+	+	+	*virS*	+	+	+
flgE	+	+	+	*fss3*	−	−	+
flgG	+	+	+	*llsB*	+	−	+
flgK	+	+	+	*llsD*	+	−	+
flgL	+	+	+	*llsG*	+	−	+
flhA	+	+	+	*llsH*	+	−	+
flhB	+	+	+	*llsX*	+	−	+
flhF	+	+	+	*llsY*	+	−	+
fliD	+	+	+				

[a] Definitions: +, ≥95% coverage and >75% identity; probable, ≥36.4% coverage and >75% identity; −, absent. NDKM-034-1, NDDQ-065-1, and NDDL-146-1 contained 83, 62 and 69 virulence genes, respectively.

Other widely reported virulence factors, such as actin assembly-inducing protein (*actA*), transcriptional regulators (*prfA*), and amidase (*Ami*), were present only in strain NDKM-034-1. *Listeriolysin S* (*LLS*) is a haemolytic and cytotoxic virulence factor that is expressed in the gastrointestinal tract after being infected by *L. monocytogenes*. It has been proven to be one of the prerequisites for pathogenicity *in vivo* (Quereda, Meza-Torres, Cossart, & Pizarro-Cerdá, 2017). The genes *llsG*, *llsH*, *llsX*, *llsB*, *llsY*, and *llsD* have been identified as genes necessary for the haemolytic activity of *LLS* (Tavares, Silva, Camargo, Yamatogi, & Nero, 2020). However, these genes were absent in strain NDDH-065, indicating that the virulence of this strain was relatively weak. These results suggested that the three strains have different virulence potentials.

Based on GO analysis, this study found that strains NDKM-034-1, NDDQ-065-1, and NDDL-146-1 have 17, 19, and 19 predicted genes encoding cellular component organisation or biogenesis, respectively. Considering that the clinical treatment of *L. monocytogenes* spread in milk and other dairy products is challenging, it is imperative to monitor the spread of *L. monocytogenes* and the resistance mechanisms of this bacterium along its various food chains. The monitoring and controlling actions include HACCP implementation, good hygiene practices (GHP), good manufacturing practices (GMP) from farm to off-farm, maintaining safe time and temperature control (TCS), and molecular epidemiological assessment using metabolomics approaches (Li et al., 2021; Oliver, Jayarao, & Almeida, 2005; Prabakusuma et al., 2022; Zhao et al., 2021).

5. Conclusion

In this study, the prevalence and the antibiotic resistance of *L. monocytogenes* isolated from milk in Yunnan Province, Southwest China, were characterised, and antibiotic resistance and virulence features of the three *L. monocytogenes* strains were evaluated using WGS. The results showed that some raw milk was contaminated with *L. monocytogenes* in Yunnan, China, and these strains exhibited extensive antibiotic resistance. The results of WGS showed that in addition to detecting resistance genes associated with the antibiotic-resistant phenotypes, other resistance determinants were also present in these *L. monocytogenes* strains. Some resistance genes were possibly obtained from other bacteria, indicating the presence of HGT among these *L. monocytogenes* strains and

other bacteria. The emergence of multi-antibiotic-resistant *L. monocytogenes* and HGT are important public health issues. Therefore, it is necessary to monitor the contamination of *L. monocytogenes* in milk in real time and explore its antibiotic resistance mechanism to ensure milk's safety.

CRediT authorship contribution statement

Rongzhen Su: Investigation, Data curation, Formal analysis, Writing - original draft. Yanlong Wen: Data curation, Writing - review & editing. Adhita Sri Prabakusuma: Writing - review & editing. Xiaozhao Tang: Methodology, Resources. Aixiang Huang: Funding acquisition. Lingfei Li: Conceptualization, Writing - review & editing, Supervision.

Declaration of competing interest

None.

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Appendix A. Supplementary data

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