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Impact of Gene Network Alterations Induced by Deletion of Key Virulence Factors in ST87 *Listeria monocytogenes* on Placental Infection

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ABSTRACT: *Listeria monocytogenes* is a globally recognized foodborne pathogen. The ST87 *L. monocytogenes* strains have become the most prevalent clinical isolates in China and are closely associated with perinatal listeriosis. This study aims to investigate the essential roles of key virulence factors in the *L. monocytogenes* ST87 strain during placental infection, focusing on the clinically isolated epidemic ST87 strain from China and constructing gene deletion mutants of *inlA*, *hly*, *inlP*, and *actA*. The roles of these genes in placental infection were systematically investigated using 2D and 3D trophoblast cell models (HTR-8/SVneo, JEG-3), non-pregnant and pregnant ICR mice, and transcriptomic analysis. The results showed that the Δhly mutant strain completely lost its virulence in mice models but showed a tendency of enhanced virulence in cell models; the $\Delta actA$ strain mutant exhibited reduced virulence in both animal models and placental cell models; the $\Delta inlA$ mutant strain displayed no significant change in virulence in animal models, yet its adhesion and invasion abilities decreased in cell models; the $\Delta inlP$ mutant strain only reduced the bacterial load in fetuses. By combining transcriptomic analysis with virulence phenotype analysis, it was found that the placental infection by ST87 *L. monocytogenes* depends on the synergistic effect of *hly* and *actA*, while *inlA* and *inlP* play auxiliary roles in the adhesion process. In addition, transcriptomic analysis identified potential genes associated with key virulence determinants of ST87 *L. monocytogenes*, including lytic polysaccharide monooxygenase, a MucBP domain-containing protein, and the alcohol dehydrogenase PduQ. This study provides a theoretical basis for elucidating the mechanism of gestational infection by the ST87 strain of *L. monocytogenes* and formulating intervention strategies.

Keywords: *Listeria monocytogenes*; ST87; Placental infection; Virulence genes; Transcriptomics.

1. Introduction

Listeria monocytogenes is a globally recognized foodborne pathogen that poses a serious threat to public health [1]. *L. monocytogenes* can be transmitted to humans through food, causing mild gastroenteritis in healthy individuals, meningitis and sepsis in immunocompromised children and the elderly, or leading to miscarriage in pregnant women [2]. Although most cases are considered sporadic, the mortality rate of

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listeriosis is 20-30%, making it one of the pathogenic foodborne pathogens with high hospitalization rates [3, 4]. Multilocus sequence typing (MLST, ST) based on seven housekeeping genes is a commonly used typing method for *L. monocytogenes* [5]. The ST types of *L. monocytogenes* vary across different regions. Studies have shown that ST1, ST2, ST3, ST4, ST6, and ST8 strains have been strongly associated with clinical cases in American and European countries [6, 7]. ST121 and ST9 are the two most prevalent STs in Switzerland [8]. The widespread transmission of ST2 strains accounted for a large number of clinical cases in Sicily, Italy from 2018 to 2020 [9]. In China, ST9, ST87, ST8, and ST155 are the most frequently detected STs in food-derived isolates, whereas ST87, ST8, and ST5 predominate among clinical strains [10]. A large-scale meta-analysis further demonstrated that ST87 accounts for approximately 13% of all clinical isolates in China, ranking as the most prevalent ST in clinical settings, and was detected in 12% of perinatal cases with available clinical information [10]. Notably, genomic analysis of ST87 isolates collected in China between 2001 and 2015 revealed that this sequence type is widely distributed across diverse food products and food-associated environments (account for 87.3%), including aquatic foods, cooked foods, poultry, raw meat, and vegetables, while a substantial proportion of isolates originated from human clinical cases (account for 12.7%) [11]. Collectively, these findings underscore that ST87 represents one of the predominant sequence types of *L. monocytogenes* circulating in China. Consistent with these findings, recent multicenter genomic surveillance in Japan revealed that CC87 represents one of the major clonal complexes causing invasive listeriosis, following CC1 and CC3, and is part of a distinct East Asian population structure of *L. monocytogenes* [12]. Phylogenetic analyses further suggested that CC87 strains in East Asia emerged through international transmission events during the late 20th century and have since undergone regional expansion [12].

During the process of *L. monocytogenes* invading the human body and infecting host target organs, multiple virulence factors play roles [13]. *hly*, which encodes the pore-forming protein listeriolysin O (LLO), influences the virulence of *L. monocytogenes*. For human cells, *L. monocytogenes* secretes LLO to promote bacterial internalization by forming pores in the plasma membrane, leading to endocytosis into cells [14]. After *L. monocytogenes* is internalized into host cells, LLO acts at its main site of action (the phagosomal membrane) and damages the lipid membrane, thereby affecting cells [15]. Internalin protein A (InlA) encoded by gene *inlA* is the earliest identified virulence factor associated with *L. monocytogenes* invasion of host cells [13]. It specifically binds to the host cell membrane receptor E-cadherin and plays a key role in crossing intestinal and placental barriers [16]. Actin A (ActA) encoded by gene *actA* is involved in bacterial intracellular migration and intercellular spread, and is also associated with bacterial adhesion to host cells [17]. However, investigations by Song et al. [13] showed that the presence or absence of *actA* had no significant effect on strain virulence. Internalin P (InlP) encoded by *inlP* is a novel virulence factor for listeriosis, with strong tropism for the placenta. It is conserved in highly virulent *L. monocytogenes* strains but absent in non-pathogenic *Listeria* species in humans [18]. InlP secreted by *L. monocytogenes* targets cytosolic afadin to specifically promote *L. monocytogenes* transcytosis across the basal face of epithelial monolayers, which may contribute to crossing the basement membrane during placental infection [19].

Most existing studies have focused on the characterization of the functions of single genes such as *inlA*, *hly*, *inlP*, and *actA* in laboratory model strains (e.g., EGDe, 10403S) [13,16,19], while research on clinically prevalent strains is relatively scarce. Although these genes are crucial for bacterial adhesion, invasion, and immune evasion, their individual contributions to strain virulence and host-pathogen interactions in ST87 isolates are unclear. Additionally, the transcriptional and phenotypic changes in ST87 gene - mutant strains, especially in 3D cell models and *in vivo* infection scenarios, have not been fully characterized, which limits our understanding of their pathogenic pathways.

Therefore, in this study, we constructed gene-mutant ST87 *L. monocytogenes* strains ($\Delta inlA$, Δhly , $\Delta inlP$, $\Delta actA$) and evaluated their growth characteristics, serum resistance and virulence-related phenotypes. The virulence heterogeneity of these gene mutant strains in different research models (2D/3D cell models, pregnant mouse models) was evaluated, and the impact of gene mutant strains on the relative genes of the bacterial strains was investigated using transcriptomics. By integrating *in vitro* and *in vivo* research methods, this study aims to clarify the multifaceted roles of these genes in the pathogenic process of the ST87 strain and provide new targets for the intervention of listeriosis.

2. Materials and methods

2.1 Strains preparation

The wild-type *L. monocytogenes* isolate MRL 300119 was obtained in 2021 from a pregnant woman clinically diagnosed with listeriosis in Shanghai. It was identified as an ST87 strain through whole-genome sequencing (WGS) and multilocus sequence typing (MLST). Using the aforementioned *L. monocytogenes* isolate MRL 300119 as the parental strain, the gene deletion mutants $\Delta actA$, Δhly , $\Delta inlA$, and $\Delta inlP$ were constructed via homologous recombination. The primer sequences used in this construction are provided in Table S1. After 10 consecutive passages of each target gene mutant strain, the agarose gel electrophoresis results of the PCR amplification products of the target gene are shown in Fig. S1, with the parental strain simultaneously set as the control. During the experiment, no gene complementation occurred in the mutant strains, and all strains have genetic stability. The avirulent *Listeria innocua* ATCC 33090, which is a standard strain (ST139), was obtained from the China Center of Industrial Culture Collection (CICC, <http://www.china-cicc.org/>) and used as a negative control in subsequent experiments. All strains were stored at -80 °C in tryptic soy broth with 0.6% yeast extract (TSB-YE, Hopebio, Qingdao, China) with 50% glycerol (v/v). For activation, bacteria were transferred into TSB-YE medium using an inoculation loop and incubated at 37 °C for 18 - 24 h. The bacterial suspension was then spread onto tryptic soy agar with 0.6% yeast extract (TSA-YE; Hopebio, Qingdao, China) and incubated at 37 °C for 18 h. The TSA-YE plates were used as working plates and stored at 4 °C for later use. Before the experiment, a single colony placed on the TSA-YE plate was transferred into of TSB-YE medium and incubated overnight at 37 °C for 18 - 24 h, obtaining a working bacterial liquid with a concentration of $10^8 - 10^9$ colony-forming units [CFU]/mL.

2.2 Determination of growth ability

The *L. monocytogenes* suspension prepared in section 2.1 was diluted to 10^2 - 10^3 CFU/mL, and then culture it in TSB-YE medium at 37 °C. Perform drop plate counting every 2 h between 0 - 24 h. The bacterial concentration at 24 h was taken as the maximum bacterial concentration ($N_{\max}$) in the stationary phase. The Microrisk Lab (<https://microrisklab.shinyapps.io/english/>) platform was used to fit the counting results with the Baranyi model to obtain the maximum specific growth rate ($\mu_{\max}$) and lag phase (λ) of the strain [20].

2.3 Surface zeta potential evaluation

The *L. monocytogenes* suspension prepared in section 2.1 was used. The surface zeta potential of *L. monocytogenes* supernatant samples was also measured using a dynamic light scattering Zetasizer (Nano ZS90, Malvern Instruments, Worcester, UK) [21].

2.4 Serum resistance determination

To evaluate the serum resistance of *L. monocytogenes*, fetal bovine serum (FBS, Haixing Biosciences, Suzhou, China) was utilized as described previously [22, 23]. Briefly, 2 mL of *L. monocytogenes* solution (concentrated at 10^8 - 10^9 CFU/mL, as detailed in Section 2.1) was centrifuged at $8000 \times g$ for 2 min. The supernatant was discarded, and the bacterial pellet was resuspended in 1 mL of 1% phosphate-buffered saline (PBS). Next, 1 mL of the bacterial suspension was mixed with 1 mL of FBS, and the initial absorbance at 620 nm (N_0) was measured. The mixture was then statically cultured at 37 °C for 24 h, after which the absorbance at 620 nm (N_{24}) was recorded. A control group using 1% PBS followed the same procedure. The serum resistance percentage was calculated using the formula:

$$\text{Serum resistance percentage (\%)} = \frac{N_{24} - N_0}{N_{24(\text{Control})} - N_{0(\text{Control})}} \quad (1)$$

2.5 Evaluation of virulence with in vivo mice models

2.5.1 Laboratory Animal Housing Conditions

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Mingqian Institute of Microbiology (Approval No.: ZAC25-0603), and were conducted in strict compliance with national ethical regulations and the Guide for the Care and Use of Laboratory Animals [24]. Female ICR mice (5 - 6 weeks old) and pregnant mice (on the 14th day of pregnancy) were purchased from Shanghai Jiesijie Laboratory Animal Co., Ltd. (Shanghai, China), The laboratory animal use license number is SCXK (shanghai) 2023-0004. The mice were housed in cages (6 mice per group), maintained in a temperature-controlled environment ($22 \pm 2^\circ\text{C}$) with $55 \pm 15\%$ humidity and a 12 h light/dark cycle. They were allowed to acclimatize to the facility for at least 1 week before infection.

2.5.2 Determination of the death curve

For the ICR mice infection experiment, 1 mL *L. monocytogenes* solution (with a concentration of 10^8 - 10^9 CFU/mL, as described in section 2.1) was centrifuged at $8000 \times g$ /min for 2 min. The bacterial cells were resuspended and diluted to 10^6 - 10^7 CFU/mL with PBS. Then, 100 μL the diluted bacterial solution was

used to infect the mice by via tail vein injection. The selection of this dosage was based on prior preliminary experimental findings. Intravenous administration of 10^5 – 10^6 CFU has been shown to effectively establish stable organ infection *in vivo*. Furthermore, at this inoculum size, both healthy ICR mice and pregnant mice remain viable for at least the first 48 hours post-injection, thereby enabling reliable collection of organ and blood samples. The mortality of the mice was observed every 12 h within 7 days.

2.5.3 Determination of bacterial load in organs

ICR mice and pregnant mice were infected using the method described in Section 2.5.2. After 48 hours (with no mice showing mortality and different strains have shown significant differences in organ bacterial load), mice were euthanized by cervical dislocation. Organs including liver, spleen, and brain (approximately 1 g each) were immediately dissected. For pregnant mice, placentas and fetuses were also collected (four placentas with corresponding fetuses per pregnant mouse). Considering the heterogeneity in the number of fetuses among pregnant mice and the characteristic V-shaped arrangement of intrauterine fetuses, two fetuses were randomly selected from each side of the intrauterine fetal cluster of each pregnant mice, and the placental tissues corresponding to the fetuses were synchronously collected. Tissues were homogenized in 1 mL of PBS using a tissue homogenizer (Wuhan Servicebio Technology CO., LTD, Wuhan, China) at 60 HZ for 30 S, equipped with disposable 4 mm stainless-steel beads. The organ homogenates were gradient-diluted with normal saline (0.85%, w/v) and inoculated onto *L. monocytogenes*-selective PALCAM Agar (Qingdao Hopebio Biotechnology Co., Ltd.) for colony counting.

2.6 Evaluation of virulence with *in vitro* cell models

2.6.1 Cell culture

Human placental choriocarcinoma (JEG-3) cells (CM-H072, generation 10-20) and human chorionic trophoblast (HTR-8/SVneo) cells (CM-H561, generation 10-20) were obtained from Gaining Biology (Shanghai, China). JEG-3 cells were cultured in Mini-mum Essential Medium (MEM; Haixing Biosciences, Suzhou, China) containing 10% FBS, and penicillin-streptomycin solution (100 μ g/mL) (Gibco, Invitrogen, California) [25], while HTR-8/SVneo cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Haixing Biosciences, Suzhou, China) containing 10% FBS, and penicillin-streptomycin solution (100 μ g/mL) (Gibco, Invitrogen, California) [26]. Cells were seeded at a density of 2 – 4×10^4 cells/cm² in the aforementioned medium for each passage. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% relative humidity.

2.6.2 Cytotoxicity assays

The survival rate of JEG-3 cells was determined as a cytotoxicity index using the cell counting Kit-8 (cck-8; Abbkine, Wuhan, China) described by [15,27] and made some modifications. Briefly, approximately 3 – 5×10^3 cells were seeded into each well of a 96-well plate and incubated at 37 °C for 24 h. The activated *L. monocytogenes* culture prepared in 2.1 was centrifuged at $8,000 \times g$ for 2 min, resuspended in MEM medium and then diluted to a final bacterial suspension with a concentration of 10^6 CFU/mL. 20 μ L of

diluted bacterial solution was added to each well and co-cultured with the JEG-3 cells for 24 h. 10 μ L of cck-8 was added to each well and incubated at 37 °C for 2 h before measuring the absorbance value at 450 nm (A_{450}) using a microplate reader (Read-Max1900, Shanpu Biotechnology Co., Ltd, Shanghai, China). The PBS was used as a blank control, and *L. innocua* bacterial solution was used as a negative control. Each set of experiments was repeated 5 times. The percentage of cytotoxicity is calculated as follows:

$$\text{Cytotoxicity}\% = \left[1 - \frac{A_{450 \text{ tested sample}} - A_{450 \text{ blank}}}{A_{450 \text{ control sample}} - A_{450 \text{ blank}}} \right] \times 100\% \quad (2)$$

2.6.3 Analysis of *L. monocytogenes* Adhesion and Invasion Ability based 2D cell model

HTR-8/SVneo and JEG-3 cells prepared by section 2.6.1 were seeded into 12-well plates ($2-4 \times 10^5$ cells/ well), and infection experiments were performed once the cell confluence exceeded 80%. In order to induce the differentiation of JEG-3 cells, when the confluence of JEG-3 cells reaches more than 50%, they are stimulated with 2 μ M forskolin (FSK, Sigma) for 48 h [25]. 1 mL of bacterial suspension prepared as described in section 2.1 was centrifuged at $8000 \times g$ for 2 min, resuspended in MEM/RPMI 1640. The resuspended bacterial solution was plate-counted to determine the initial infection concentration (N_0). Infection of cells was performed with bacteria at an approximate multiplicity of infection (MOI) of 100: specifically, 1 mL of MEM or RPMI 1640 medium and 10 μ L of the bacterial suspension were added to 12-well plates for co-culture (1 h for adhesion and 2 h for invasion).

For the adhesion assay, after co-culture, the medium was then removed, and the cell was gently washed twice with $1 \times$ PBS. Afterward, 0.5 mL of 0.25% trypsin-EDTA and 0.5 mL of 1% Triton X-100 solution were added to lyse the cells for 2-3 min, with repeated pipetting to ensure cell disruption. The cell lysate was plate-counted to determine bacterial concentration (N_1), and the adhesion rate was calculated ($N_1/N_0 \times 100\%$).

For the invasion assay, after co-culture and before cell dissociation, 1 mL of MEM/RPMI 1640 medium containing penicillin-streptomycin (100 μ g/mL) was added to each well and incubated for 1 h to kill extracellular bacteria. Subsequently, cells were dissociated following the same steps described for the adhesion assay, and the bacterial concentration (N_2) was determined. The invasion rate was calculated ($N_2/N_0 \times 100\%$).

2.6.4 Analysis of *L. monocytogenes* Adhesion and Invasion Ability based 3D cell model

HTR-8/SVneo and JEG-3 cells prepared by section 2.6.1 were seeded into 96-well ultra-low attachment plates (Corning, USA) ($3-5 \times 10^3$ cells/ well) to form 3D spheroid model. On the 6th day of cultivation, the cells were in a good spheroid state (the number of cells was approximately 1×10^5). In order to induce the differentiation of 3D JEG-3 cells, On the fourth day of the culture of 3D JEG-3 cell spheres, they are stimulated with 2 μ M forskolin (FSK, Sigma, USA) for 48 h. 900 μ L MEM/RPMI 1640, 10 3D spheroid cells and 100 μ L of the above resuspended bacterial liquid were added into the centrifuge tube and incubate

together at 37 °C for 6 h. The methods for adhesion and invasion assays were performed according to the procedures of 2D cell adhesion and invasion assays described in Section 2.6.3.

2.7 RNA library construction and transcriptomic bioinformation analysis

L. monocytogenes MRL 300119 and mutant strains were prepared as described in section 2.1. Using the method of Gao et al. [23], briefly speaking, the total RNA of all strains was extracted, quantified, and the integrity of total RNA was determined. RNA-seq transcriptome libraries were constructed using total RNA with an integrity value greater than 7.5, and paired-end RNA-seq libraries were sequenced. The sequencing profiles were generated using the cloud-based analysis platform (<https://cloudmajorbio.com/>) provided by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Following the method described by Gao et al. [23], raw sequencing data were filtered to obtain clean, high-quality reads, which were then aligned to the *L. monocytogenes* reference genome. Subsequent analyses included quantification of gene expression levels, determination of isoform abundances, identification of differentially expressed genes (DEGs) between the MRL 300119 wild-type strain and mutant strains, and assessment of statistical significance for DEGs. Principal Component Analysis (PCA) plots, Venn diagrams, sample correlation heatmaps, and DEG volcano plots were generated to visualize relationships among samples. Functional annotation of DEGs was performed using the Diamond software against the Virulence Factor Database (VFDB, <https://www.mgc.ac.cn/VFs/>). Additionally, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were conducted.

2.8 Statistical analysis

The data were presented as mean $\pm$ standard deviation (SD) ($n = 3$). Conducting significance analysis on the data using IBM SPSS Statistics 27 software (SPSS Inc., IBM Corporation, Armonk, New York, USA) analysis of variance (ANOVA) and Tukey's test were employed to compare groups of data. Significance was defined as $P < 0.05$. All experiments were conducted in triplicate.

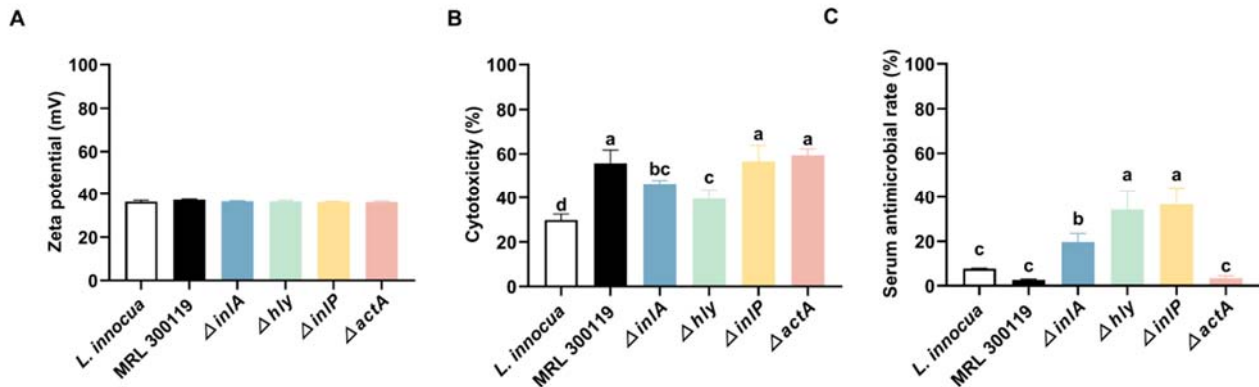
3. Result

3.1 Evaluation of biological characteristic of gene-mutant *L. monocytogenes*

The biological characteristics of wild-type *L. monocytogenes* and gene-mutant strains were analyzed. The results showed that there were no differences in $\mu_{\max}$ and $N_{\max}$ between the gene-mutant strains and the wild-type strain. However, the λ of the $\Delta inlP$ and $\Delta actA$ mutant strains became 2-3 times that of wild-type strain (Table 1). In addition, there were no significant differences in zeta potential between all mutant strains and the wild-type strain ($P > 0.05$) (Fig. 1A). However, compared with the wild-type strain, the placental cytotoxicity of the $\Delta inlA$ and Δhly mutant strains was significantly reduced ($P < 0.05$), while no significant changes were observed in the other mutant strains ($P > 0.05$) (Fig. 1B). In addition, the serum resistance of the $\Delta inlA$, Δhly , and $\Delta inlP$ mutant strains was significantly higher than that of wild-type strain ($P < 0.05$) (Fig. 1C).

Table 1 growth characteristics of *L. monocytogenes*

Strains	$\mu_{\max}$ (log ₁₀ CFU/mL/h)	λ (h)	$N_{\max}$ (log ₁₀ CFU/mL)
MRL 300119	1.06 ± 0.07	0.59 ± 0.84	9.39 ± 0.12
$\Delta inlA$	1.10 ± 0.04	0.49 ± 0.41	9.34 ± 0.06
$\Delta inlP$	1.13 ± 0.05	1.22 ± 0.43	9.31 ± 0.06
Δhly	1.11 ± 0.03	0.48 ± 0.30	9.32 ± 0.04
$\Delta actA$	1.10 ± 0.05	1.59 ± 0.49	9.23 ± 0.07

**Fig. 1** The zeta potential (A), cytotoxicity (B), and serum resistance (C) between wild-type and gene- mutant *L. monocytogenes* strains. Different lowercase letters (a~c) represent significant differences ($P < 0.05$).

3.2. The Pathogenicity of gene-mutant *L. monocytogenes* strains in the ICR mice Model

The pathogenicity of the wild-type strain and gene-deleted strains was analyzed using an ICR mouse model (Fig. 2). The survival of ICR mice infected with *L. monocytogenes* was observed at 168 h (Fig. 2A-E). We observed that all mice infected with the wild-type strain succumbed at 120 h post-infection (Fig. 2A). In contrast, mice infected with the $\Delta inlA$ mutant strain survived until 168 h, whereas those infected with the $\Delta inlP$ mutant strain survived only until 96 h (Fig. 2B & C). However, the survival rate of mice infected with $\Delta actA$ and Δhly strains was 100% within 168 h (Fig. 2D&E). In addition, when observing the status of the mice, we found that the mice infected with the $\Delta actA$ and Δhly strains did not show any abnormalities within the 168 h observation period, which was the same as the blank control group treated with PBS and the negative control group treated with *L. innocua*. In contrast, the mice infected with the wild-type strain as well as the $\Delta inlA$ and $\Delta inlP$ strains showed signs of illness such as weight loss, decreased appetite, loose stools, and ruffled fur at 48 h after infection.

In addition, ICR mice were euthanized and dissected 48 h after being infected with *L. monocytogenes*, and then the bacterial loads in their livers (Fig. 2F), spleens (Fig. 2G) and brains (Fig. 2H) were determined. Compared with the liver and spleen, the bacterial load in the brain was the lowest. The bacterial load in the liver, spleen and brain of the mice infected with the wild-type strain was approximately 8.54, 8.82 and 6.47 log₁₀ CFU/g, respectively. There was no significant difference in bacterial loads in the liver, spleen, and brain between mice infected with $\Delta inlA$ and $\Delta inlP$ strains and those infected with wild-type strain ($P > 0.05$). However, the bacterial loads in the liver, spleen and brain of the mice infected with the $\Delta actA$ strain decreased significantly compared with those of the mice infected with wild-type strain ($P < 0.05$).

Additionally, the bacteria in these organs of the mice infected with the Δhly strain or the negative control *L. innocua* were not detected.

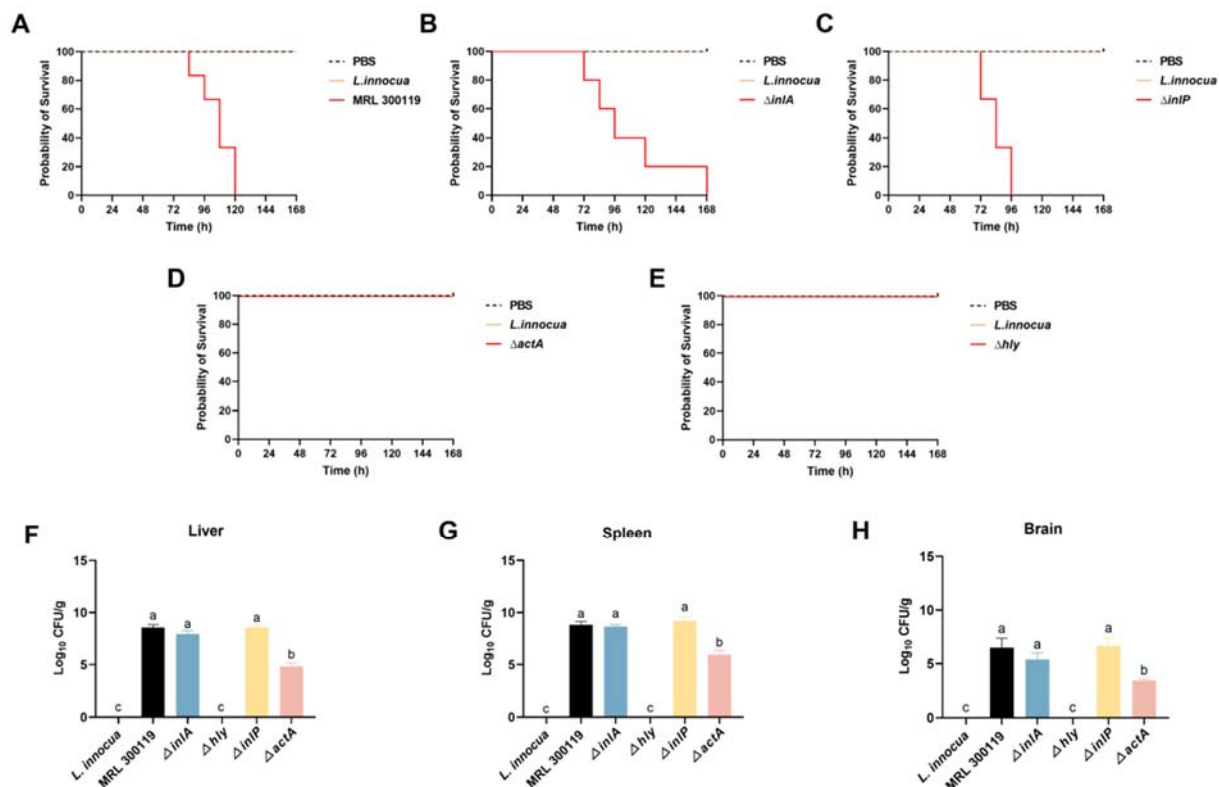


Fig. 2 The survival rate of wild-type and gene-deleted *L. monocytogenes* strains over 168 h based on an infection model of ICR mice (A–E), the bacterial loads in the liver (F), spleen (G) and brain (H) of ICR mice 48 h after infection with wild-type and gene-deleted *L. monocytogenes* strains. Different lowercase letters (a–c) represent significant differences ($P < 0.05$).

3.3. The Pathogenicity of gene-mutant *L. monocytogenes* strains in the pregnant mice model

The pathogenicity of the wild-type *L. monocytogenes* MRL 300119 and gene-mutant strains was analyzed using a pregnant mouse model. Pregnant mice were euthanized and dissected 48 h after being infected with *L. monocytogenes*, and then the bacterial loads in their livers (Fig. 3A), spleens (Fig. 3B), brains (Fig. 3C), placentas (Fig. 3D) and the corresponding fetuses (Fig. 3E) were determined. The bacterial load in the liver of the pregnant mice infected with wild-type strain was approximately $6.08 \log_{10}$ CFU/g, the bacterial load in the spleen was about $6.91 \log_{10}$ CFU/g, the bacterial load in the brain was around $3.31 \log_{10}$ CFU/g, the bacterial load in the placenta ranged from approximately 0 to $6.83 \log_{10}$ CFU/g, and the bacterial load in the fetus was about 0 to $6.09 \log_{10}$ CFU/g. In addition, we found that compared with the pregnant mice infected with wild-type strain, the pregnant mice infected with the gene-deleted strains showed a consistent trend in the bacterial loads of the liver, spleen, brain, placenta, and fetus. There was no significant difference in the bacterial loads of the liver, spleen and brain between the pregnant mice infected with the $\Delta inIA$ and $\Delta inIP$ strains and those infected with wild-type strain ($P > 0.05$). However, the bacterial loads in the liver, spleen, brain, placenta and fetus of the pregnant mice infected with the $\Delta actA$ strain decreased significantly compared with those of the pregnant mice infected with wild-type strain ($P < 0.05$). The bacteria in liver and brain of the mice infected with the $\Delta actA$ strain were not detected. Additionally, the

bacteria in these organs of the mice infected with the Δhly strain or the negative control *L. innocua* were not detected.

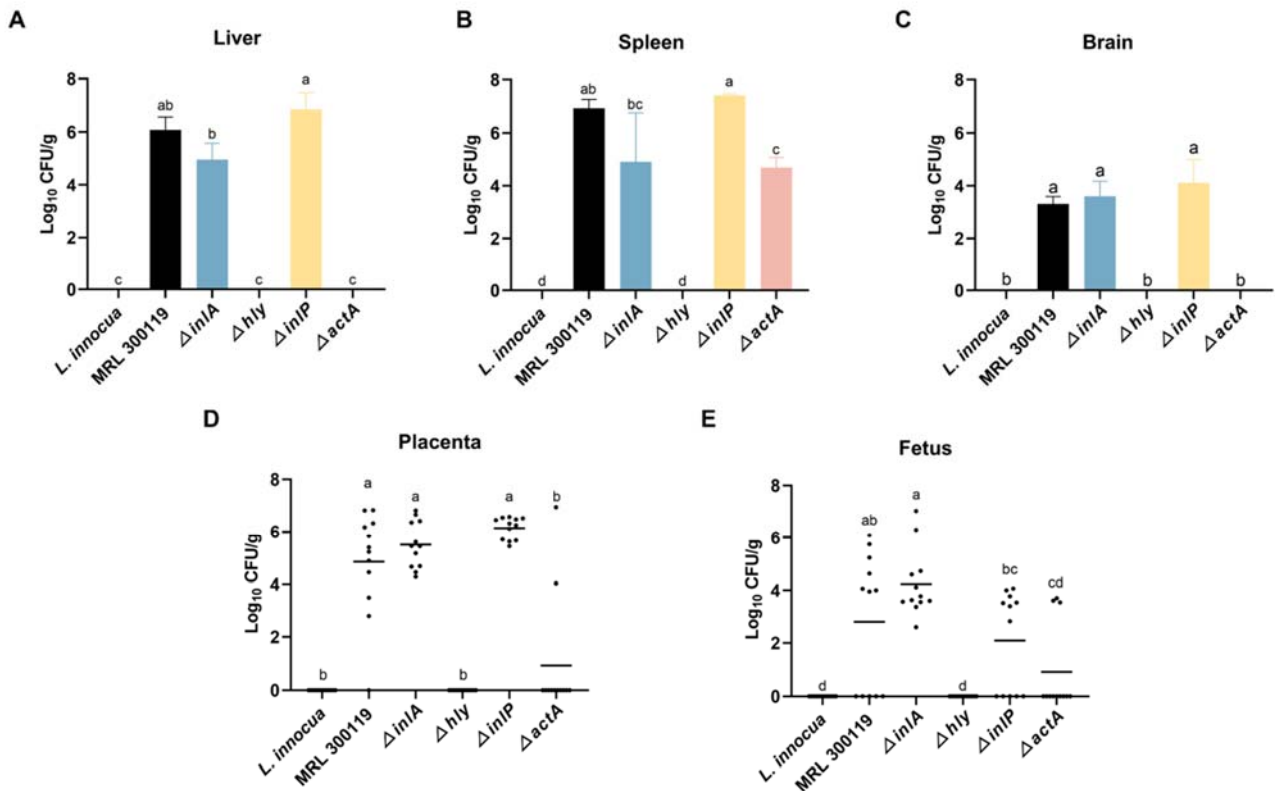


Fig. 3 The bacterial loads in the liver (A), spleen (B), brain (C), placenta (D) and fetus (E) of pregnant mice 48 h after infection with wild-type and gene-deleted *L. monocytogenes* strains. Different lowercase letters (a~d) represent significant differences ($P < 0.05$).

3.4. The difference virulence in vitro of gene-mutant *L. monocytogenes* based on a 2D cell model

The adhesion and invasion abilities of gene-mutant *L. monocytogenes* against JEG-3 cells, forskolin-stimulated JEG-3 cells, and HTR8/SVneo c cells was determined based on a 2D cell model (Fig. 4).

For 2D JEG-3 cells, the results of the adhesion and invasion abilities are similar. Compared with the wild-type *L. monocytogenes*, there were no significant differences in the adhesion and invasion abilities among all the gene-mutant strains ($P > 0.05$) (Fig. 4A&D). However, the adhesion and invasion abilities of $\Delta inlA$ and Δhly showed a downward trend, while those of $\Delta inlP$ and $\Delta actA$ showed an upward trend.

For 2D forskolin-stimulated JEG-3 cells, the results of the adhesion and invasion abilities are similar. Compared with the wild-type *L. monocytogenes*, the adhesion and invasion ability of the $\Delta inlP$ strain was significantly increased ($P < 0.05$), the adhesion ability and invasion of the $\Delta inlA$ strain was significantly decreased ($P < 0.05$) (Fig. 4B&E). In addition, the adhesion ability of the $\Delta actA$ strain was increased but not significantly compared with that of wild-type *L. monocytogenes* ($P > 0.05$) (Fig. 4B), while its invasion ability decreased significantly ($P < 0.05$) (Fig. 4E).

For 2D HTR8/SVneo cells, compared with the wild-type *L. monocytogenes*, the adhesion ability of the $\Delta inlP$ strain was significantly increased ($P < 0.05$), and there was no significant difference in the adhesion

ability between the other mutant strains and wild-type *L. monocytogenes* ($P > 0.05$) (Fig. 4C). Different from the results of the adhesion ability, compared with the wild-type *L. monocytogenes*, the invasion abilities of the $\Delta inlA$ and $\Delta inlP$ strains were significantly decreased ($P < 0.05$), while the invasion ability of the Δhly strain was significantly increased ($P < 0.05$). There was no significant difference in the invasion ability of the $\Delta actA$ strain compared with that of wild-type *L. monocytogenes* ($P > 0.05$) (Fig. 4F).

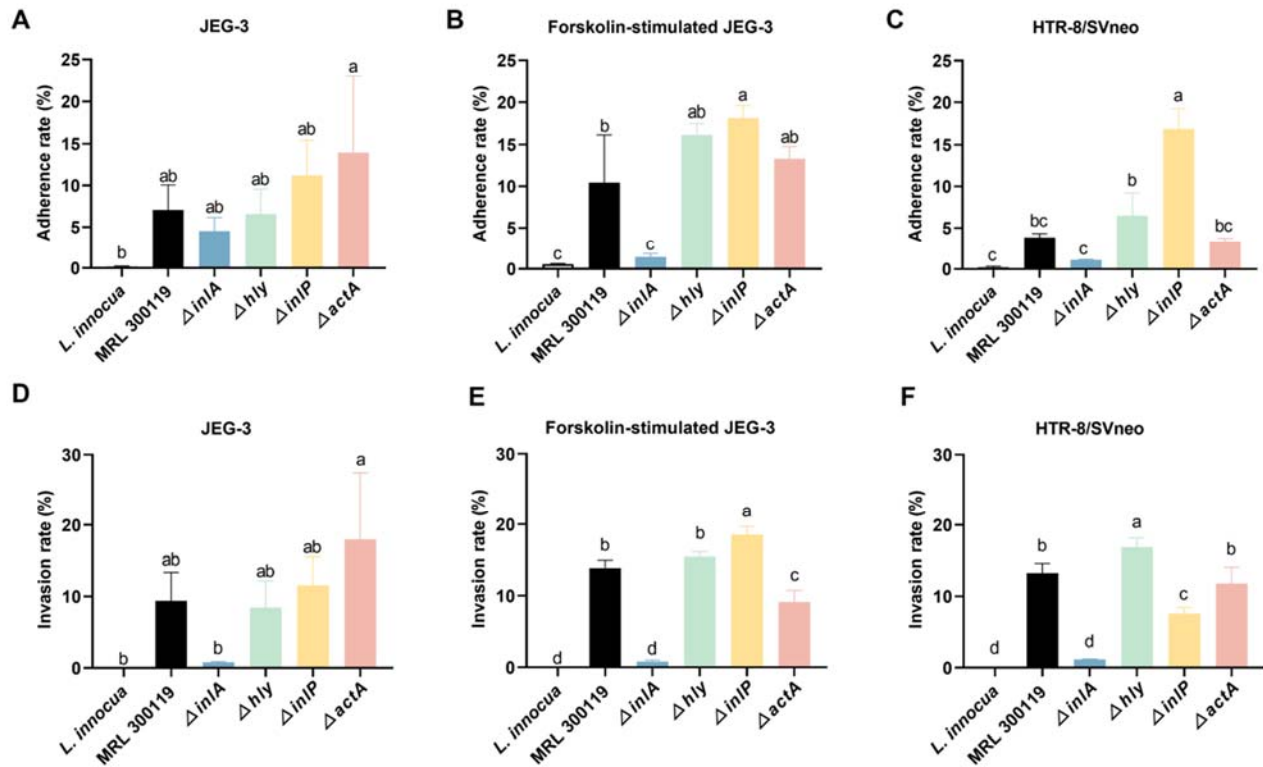


Fig. 4. Determination of the adhesion (A-C) and invasion (D-F) abilities of gene-mutant *L. monocytogenes* to 2D JEG-3 (A&D), forskolin-stimulated JEG-3 cells (B&E) and HTR8/SVneo (C&F) cells. Different lowercase letters (a~d) represent significant differences ($P < 0.05$).

3.5. The difference virulence in vitro of gene-mutant *L. monocytogenes* based on a 3D cell model

The adhesion and invasion abilities of gene-deletion *L. monocytogenes* against JEG-3 cells, forskolin-stimulated JEG-3 cells, and HTR8/SVneo cells was determined based on a 3D cell model (Fig. 5).

For 3D JEG-3 cells, compared with the wild-type *L. monocytogenes*, the adhesion ability of the $\Delta inlA$ and $\Delta inlP$ strains were significantly decreased ($P < 0.05$), and there was no significant difference in the adhesion ability between the other mutant strains and the wild-type *L. monocytogenes* ($P > 0.05$) (Fig. 5A). However, the invasion ability of the $\Delta inlP$ and Δhly strains were significantly increased ($P < 0.05$) compared with the wild-type *L. monocytogenes*, and there was no significant difference in the invasion ability between the other strains and the wild-type *L. monocytogenes* ($P > 0.05$) (Fig. 5D).

For 3D forskolin-stimulated JEG-3 cells, compared with the wild-type *L. monocytogenes*, the adhesion ability of the $\Delta inlA$ strain was significantly decreased ($P < 0.05$), while the adhesion ability of the $\Delta actA$ strain was significantly increased ($P < 0.05$) (Fig. 5B). The adhesion ability of the Δhly and $\Delta inlP$ strain was no significantly difference compared with that of wild-type *L. monocytogenes* ($P > 0.05$) (Fig. 5B).

Different from the results of the adhesion ability, compared with the wild-type *L. monocytogenes*, the invasion ability of the $\Delta inlP$ strain was significantly increased ($P < 0.05$), and there was no significant difference in the invasion ability between the other mutant strains and the wild-type *L. monocytogenes* ($P > 0.05$) (Fig. 5E)

For 3D HTR8/SVneo cells, compared with the wild-type *L. monocytogenes*, the adhesion ability of the $\Delta inlA$ and $\Delta inlP$ strain was significantly decreased ($P < 0.05$), and there was no significant difference in the adhesion ability between the other strains and the wild-type *L. monocytogenes* ($P > 0.05$) (Fig. 5C). Different from the results of the adhesion ability, compared with the wild-type *L. monocytogenes*, the invasion ability of the $\Delta inlA$ and $\Delta actA$ strains were significantly decreased ($P < 0.05$), while the invasion ability of the Δhly and $\Delta inlP$ strain was no significantly difference compared with that of wild-type *L. monocytogenes* ($P > 0.05$) (Fig. 5F).

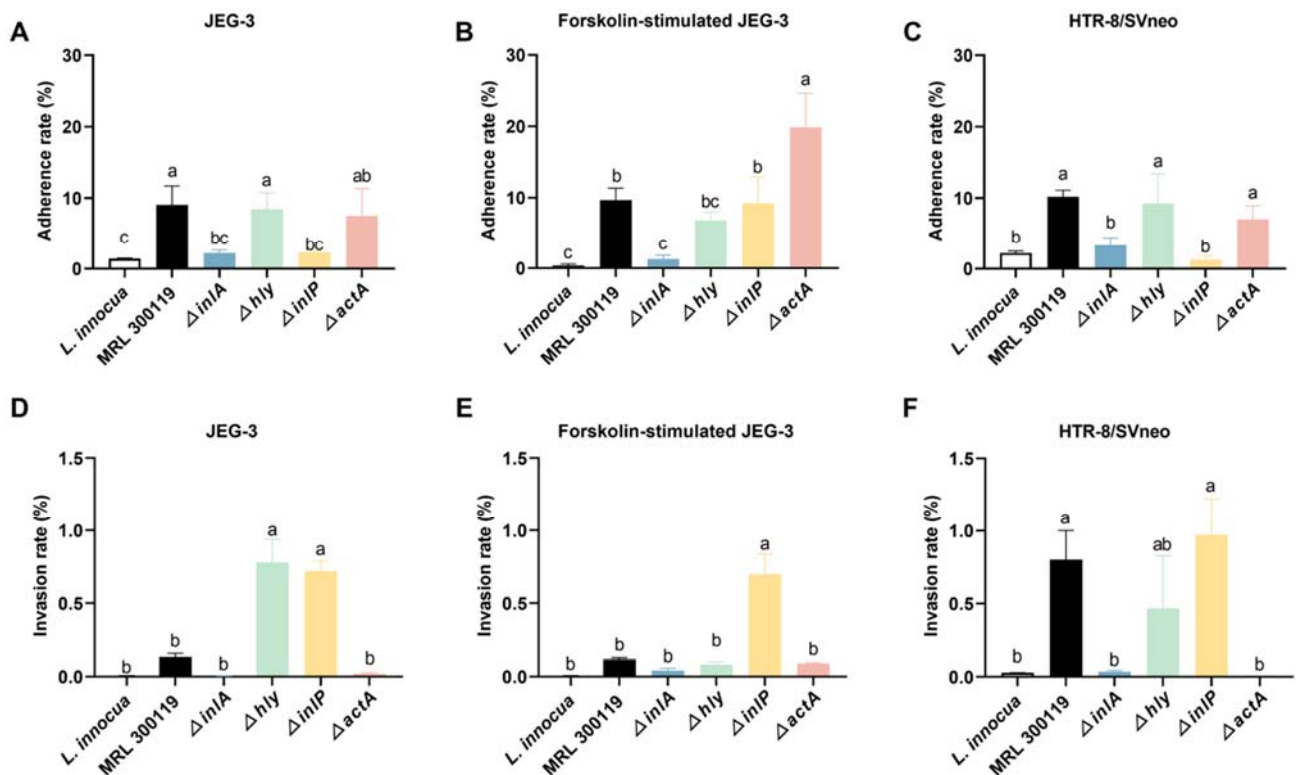


Fig. 5. Determination of the adhesion (A-C) and invasion (D-F) abilities of gene-deletion *L. monocytogenes* to 3D HTR8/SVneo (A&D), JEG-3 cells stimulated by forskolin (B&E) and JEG-3 (C&F) cells. Different lowercase letters (a~c) represent significant differences ($P < 0.05$).

3.6. Transcriptomic analysis

3.6.1 DEGs analysis of gene-mutant *L. monocytogenes*

A differential analysis was performed on the transcriptomes of gene-mutant *L. monocytogenes* strains and the wild-type ST87 *L. monocytogenes* strain MRL 300119. Expression detection of the mutant genes was performed on the four gene-mutant strains. As shown in Fig. S2, the corresponding gene deletion were indeed observed in the different gene- mutant strains.

The PCA plot showed that the five strains exhibited distinct clustering patterns: $\Delta inlP$ was closest to the wild-type *L. monocytogenes*, while $\Delta inlA$ and $\Delta actA$ were located farther away (Fig. 6A). Gene expression correlation analysis further revealed that the gene expression of the four gene-mutant strains was highly correlated with that of the wild-type strain. Among them, $\Delta inlP$ showed the strongest correlation with the wild-type strain in gene expression (correlation coefficient 0.988), while $\Delta inlA$ showed the weakest correlation (correlation coefficient 0.968) (Fig. 6B). These results indicated that the transcriptional divergence between $\Delta inlP$ and the wild-type strain was minimal, whereas $\Delta inlA$ and $\Delta actA$ exhibited more pronounced differences in gene expression compared with wild-type *L. monocytogenes*. The DEGs of $\Delta inlA$, Δhly , $\Delta inlP$, and $\Delta actA$ mutant *L. monocytogenes* strains and the wild-type strain were analyzed. The Venn diagram showed that, using the wild-type *L. monocytogenes* as a control, the mutations in the four genes (*inlA*, *hly*, *inlP*, and *actA*) altogether had 49 overlapping DEGs (Fig. 6C). In $\Delta inlA$, 592 genes were up-regulated and 366 genes were down-regulated (Fig. 6D). In Δhly , 328 genes were up-regulated and 78 genes were down-regulated (Fig. 6E). In $\Delta inlP$, 191 genes were up-regulated and 90 genes were down-regulated (Fig. 6F). In $\Delta actA$, 482 genes were up-regulated and 567 genes were down-regulated (Fig. 6G).

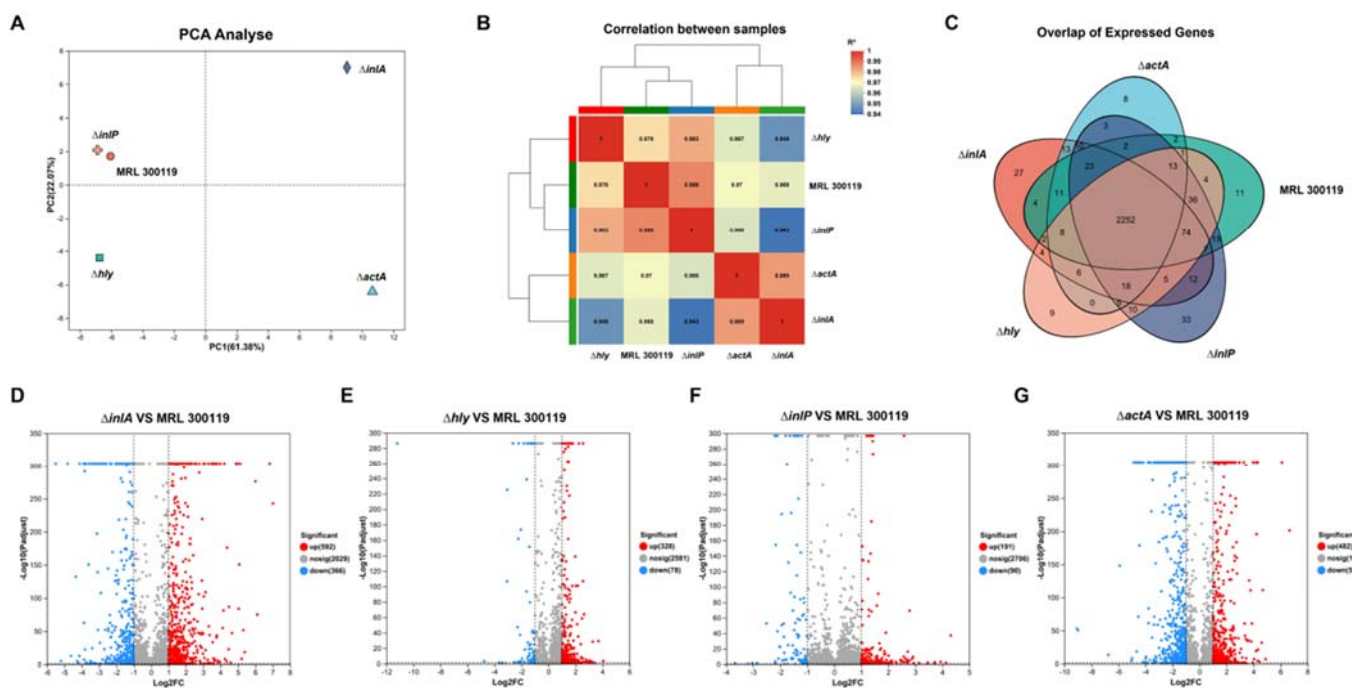


Fig. 6 (A) PCA analysis of gene-mutant *L. monocytogenes* strains and the wild-type strain. (B) Correlation analysis between gene-mutant *L. monocytogenes* strains and the wild-type strain. (C) Overlap of DEGs between gene-mutant *L. monocytogenes* strains and the wild-type strain. Volcano plot for global comparison of DEGs between the wild-type *L. monocytogenes* strain and (D) $\Delta inlA$, (E) Δhly , (F) $\Delta inlP$, (G) $\Delta actA$.

3.6.2 VFDB analysis

Analysis was conducted on virulence factors that promote the self-infection of *L. monocytogenes* and the induction of specific host diseases (Fig. 7). Compared with the wild-type strain, more DEGs related to virulence factors were identified in $\Delta inlA$ (Fig. 7A) and $\Delta actA$ (Fig. 7D) among all gene-mutant strains.

Specifically, in the $\Delta inlA$ mutant strain, factors related to nutritional/metabolic processes (45 upregulated, 26 downregulated genes), immune modulation (33 upregulated, 16 downregulated genes), adherence (15 upregulated, 10 downregulated genes), exotoxin (11 upregulated, 4 downregulated genes), and motility (4 upregulated, 11 downregulated genes) exhibited significant changes (Fig. 7A). In the $\Delta actA$ mutant strain, factors related to nutritional/metabolic processes (33 upregulated, 37 downregulated genes), immune modulation (24 upregulated, 29 downregulated genes), adherence (19 upregulated, 7 downregulated genes) and exotoxin (7 upregulated, 8 downregulated genes) exhibited significant changes (Fig. 7D). However, the Δhly and $\Delta inlP$ mutant strains showed significant changes in virulence factors only in the nutritional/metabolic processes. Specifically, in the Δhly mutant strain, 26 genes were upregulated and 10 genes were downregulated (Fig. 7B). And in the $\Delta inlP$ mutant strain, 24 genes were upregulated and 8 genes were downregulated (Fig. 7C).

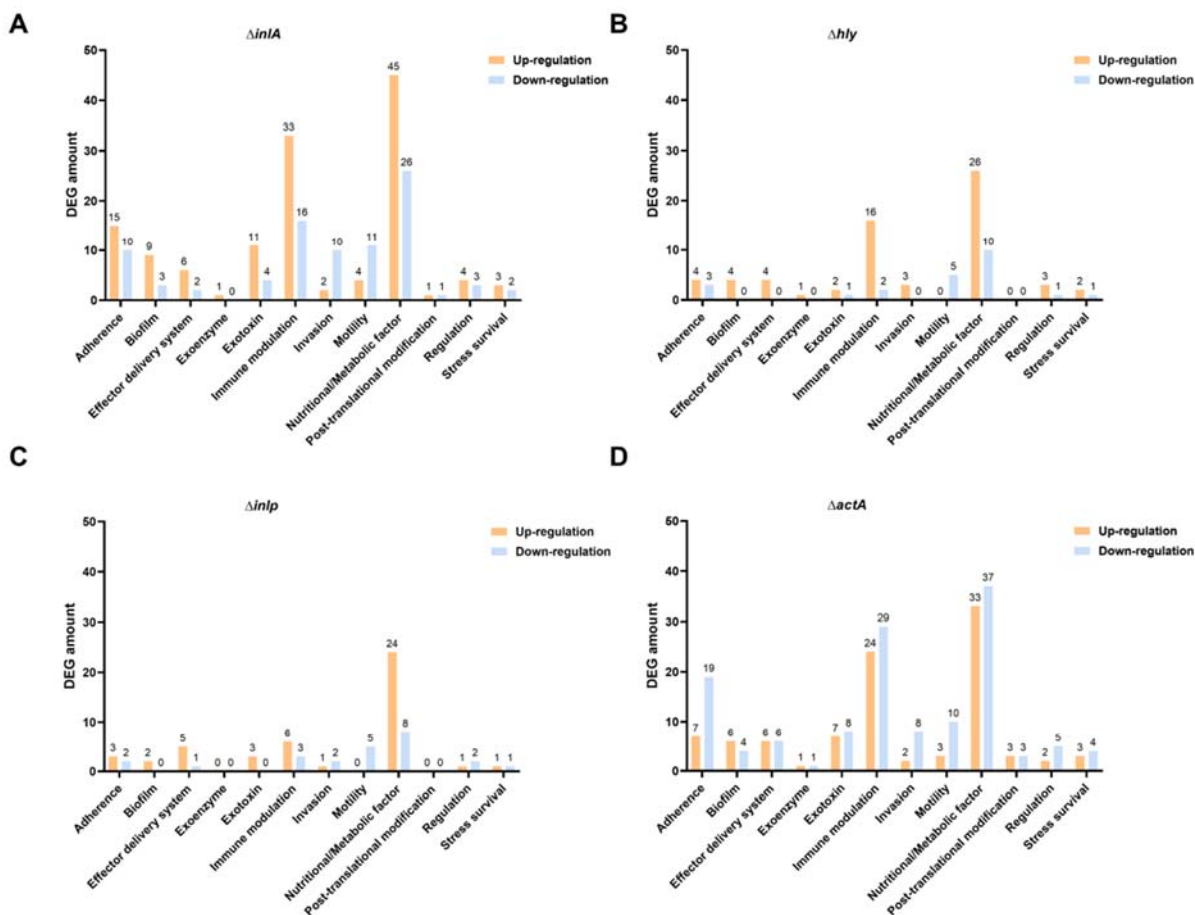


Fig. 7 Hierarchical classification of Virulence Factor analysis for gene-mutant *L. monocytogenes* strains (A) $\Delta inlA$, (B) Δhly , (C) $\Delta inlP$, (D) $\Delta actA$.

DEGs analysis was performed on adhesion- and invasion-related virulence factors, and the heatmap is shown in Fig. 8. From the dendrogram, $\Delta inlA$ and $\Delta actA$ were closer in gene expression patterns and clustered into one branch; Δhly , MRL 300119, and $\Delta inlP$ showed similarity in some gene expressions and clustered into another branch (Fig. 8A&B).

For adhesion-related DEGs, in the Δhly strain, there were 7 significantly altered genes ($P\text{-adjust} < 0.05$). The four significantly upregulated genes were *pduQ* encoding iron-containing alcohol dehydrogenase, *pduP* encoding aldehyde dehydrogenase EutE, *adhE* encoding adhesion-mediating acetaldehyde/alcohol dehydrogenase LAP, and the gene encoding LPXTG cell wall anchor domain-containing protein. The three significantly downregulated genes were *groEL* encoding chaperonin GroEL, *osmC* encoding organic hydroperoxide resistance protein, *dnaK* encoding chaperone protein DnaK.

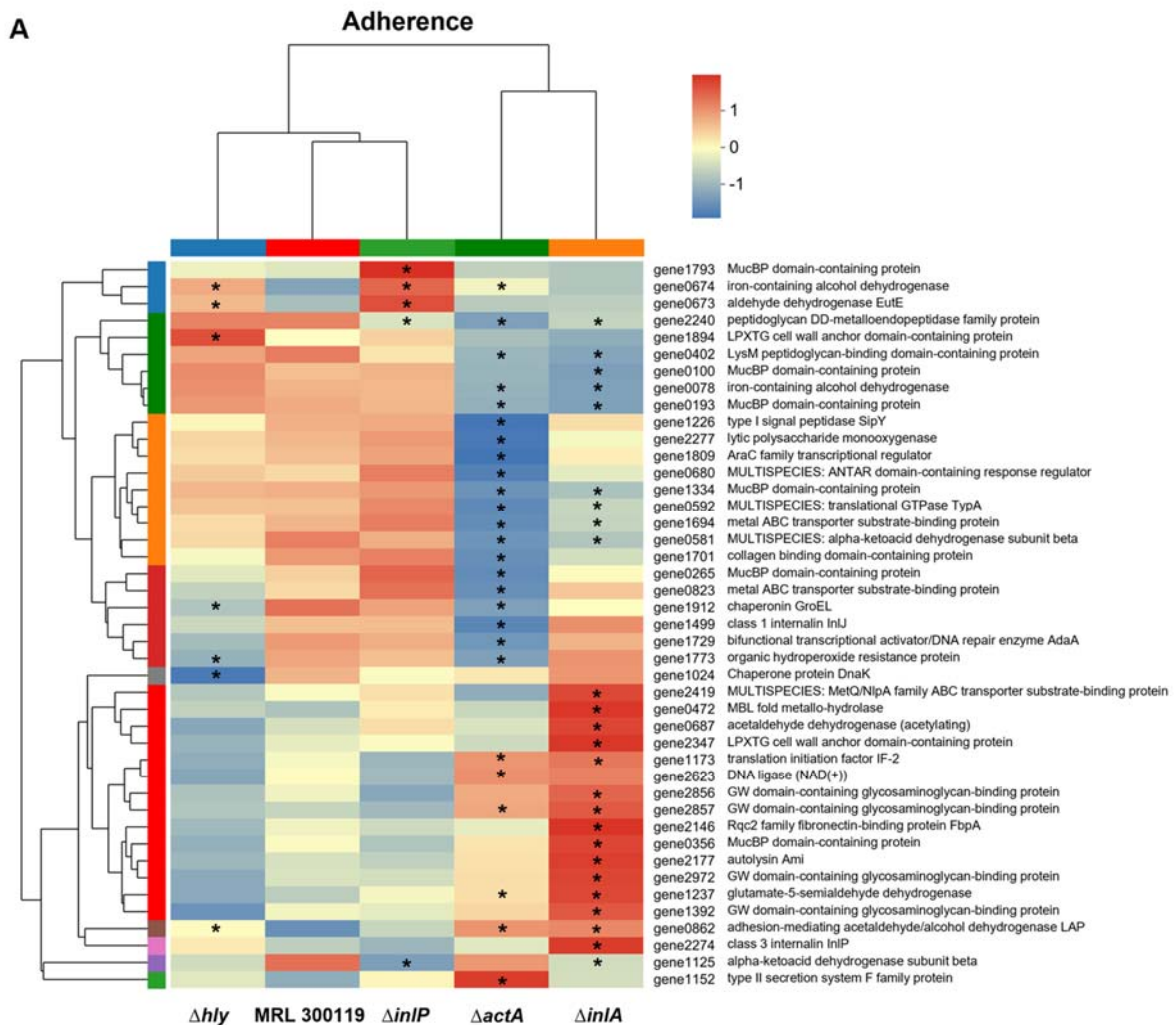
In the $\Delta inlP$ strain, there were 5 significantly changed genes ($P\text{-adjust} < 0.05$). There were 5 significantly altered genes ($P\text{-adjust} < 0.05$). The two significantly downregulated genes were alpha-ketoacid dehydrogenase subunit beta and peptidoglycan DD-metalloendopeptidase family protein, the three significantly upregulated genes were MucBP domain-containing protein, iron-containing alcohol dehydrogenase, aldehyde dehydrogenase EutE.

In the $\Delta actA$ strain, there were had 26 significantly changed genes ($P\text{-adjust} < 0.05$). Among the 19 significantly downregulated genes, the proteins they primarily encode include chaperonin GroEL, organic hydroperoxide resistance protein, alpha-ketoacid dehydrogenase subunit beta, LysM peptidoglycan-binding domain-containing protein, MucBP domain-containing protein, collagen binding domain-containing protein, bifunctional transcriptional activator/DNA repair enzyme AdaA, type I signal peptidase SipY, lytic polysaccharide monooxygenase, AraC family transcriptional regulator, ANTAR domain-containing response regulator, metal ABC transporter substrate-binding protein, class 1 internalin InlJ, iron-containing alcohol dehydrogenase, translational GTPase TypA, peptidoglycan DD-metalloendopeptidase family protein. Among the 7 significantly downregulated genes, the proteins they primarily encode include DNA ligase (NAD(+)), translation initiation factor IF-2, GW domain-containing glycosaminoglycan-binding protein, type II secretion system F family protein, glutamate-5-semialdehyde dehydrogenase, adhesion-mediating acetaldehyde/alcohol dehydrogenase LAP, and iron-containing alcohol dehydrogenase.

In the $\Delta inlA$ strain, there were 25 significantly changed genes ($P\text{-adjust} < 0.05$). Among the 15 significantly upregulated genes, the proteins they primarily encode include LPXTG cell wall anchor domain-containing protein, translation initiation factor IF-2, GW domain-containing glycosaminoglycan-binding protein, Rqc2 family fibronectin-binding protein FbpA, MucBP domain-containing protein, MetQ/NlpA family ABC transporter substrate-binding protein, MBL fold metallo-hydrolase, autolysin Ami, glutamate-5-semialdehyde dehydrogenase, class 3 internalin *InlP*, and adhesion-mediating acetaldehyde/alcohol dehydrogenase LAP. Among the 11 significantly upregulated genes, the proteins they primarily encode include metal ABC transporter substrate-binding protein, alpha-ketoacid dehydrogenase subunit beta, translational GTPase TypA, peptidoglycan DD-metalloendopeptidase family protein, LysM peptidoglycan-binding domain-containing protein, MucBP domain-containing protein, iron-containing alcohol dehydrogenase.

For invasion-related DEGs, in the Δhly strain there were 3 significantly upregulated genes ($P\text{-adjust} < 0.05$), encoding GW domain-containing class 2 internalin InlB, class 1 internalin *InlA* and

resuscitation-promoting factor. In the $\Delta inlP$ strain, there were 3 significantly changed genes ($P\text{-adjust} < 0.05$), encoding glucosaminidase domain-containing protein and C40 family peptidase (downregulated genes), cell invasion LPXTG protein Vip (upregulated gene). In the $\Delta actA$ strain, there were 10 significantly changed genes ($P\text{-adjust} < 0.05$), Among the 8 significantly downregulated genes, the proteins they primarily encode include LPXTG cell wall anchor domain-containing protein, leucine-rich repeat domain-containing protein, metal ABC transporter substrate-binding protein, C40 family peptidase, internalin N-terminal domain-containing protein, glucosaminidase domain-containing protein, and resuscitation-promoting factor. Among the 2 significantly upregulated genes, the proteins they primarily encode leucine-rich repeat domain-containing protein and peptidoglycan hydrolase Auto. In the $\Delta inlA$ strain, there were 12 significantly changed genes ($P\text{-adjust} < 0.05$), among the 10 significantly downregulated genes, the proteins they primarily encode include class 1 internalin InlC2, GW domain-containing class 2 internalin InlB, class 1 internalin *InlA*, LPXTG cell wall anchor domain-containing protein, leucine-rich repeat domain-containing protein, metal ABC transporter substrate-binding protein, internalin N-terminal domain-containing protein, glucosaminidase domain-containing protein, C40 family peptidase, and resuscitation-promoting factor. Among the 2 significantly upregulated genes, the proteins they primarily encode invasion associated endopeptidase and peptidoglycan hydrolase Auto.



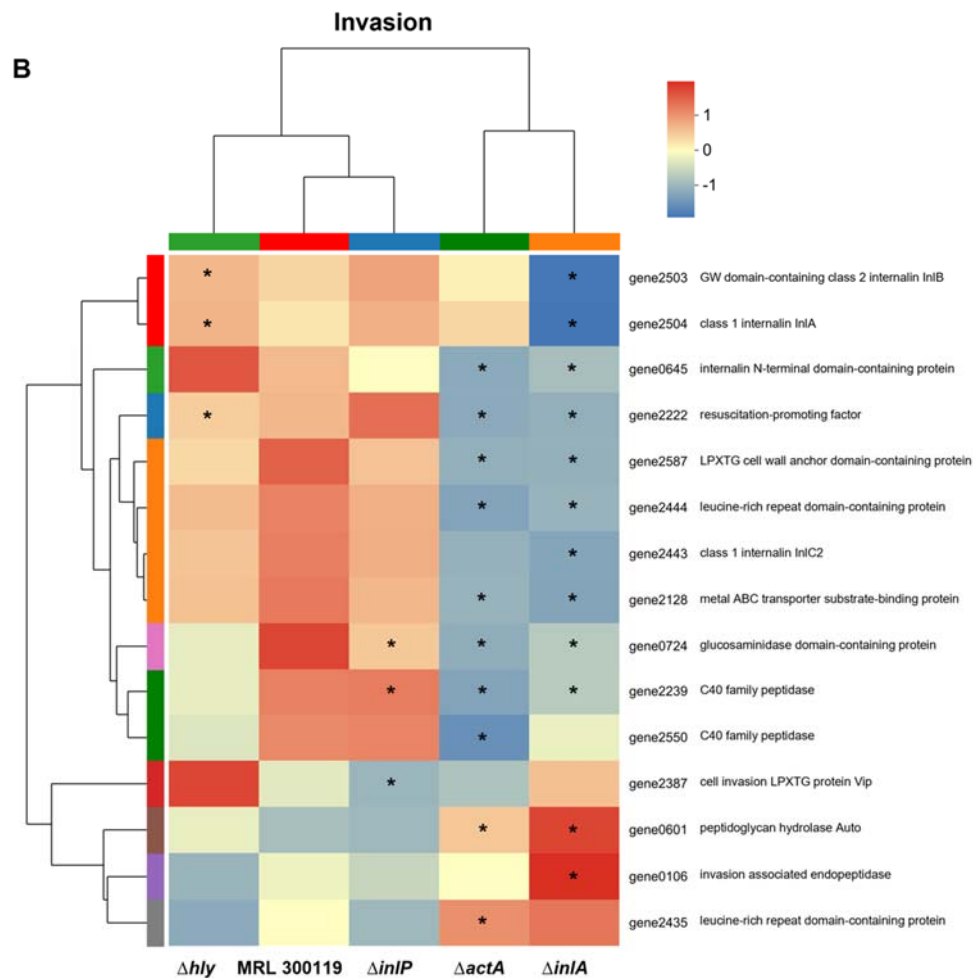


Fig. 8 Heatmap of gene expression for adhesion (A) and invasion (B) - related genes in wild - type *L. monocytogenes*, gene-mutant *L. monocytogenes* $\Delta inIA$, Δhly , $\Delta inIP$, and $\Delta actA$. Each row represents the expression pattern of a single gene, and each column corresponds to a sample. Expression levels are indicated by colored markers, with green representing the highest level of expression and orange representing the lowest level of expression. The dendrogram on the left shows gene clustering. The dendrogram at the top is for sample clustering, where colored blocks represent different groups, and the sample names are at the bottom. * indicates that the expression of the corresponding gene in the corresponding strain is significantly different from that in MRL 300119 ($P\text{-adjust} < 0.05$).

3.6.3 GO analysis and KEGG analysis

GO analysis was used to investigate the distribution of DEGs in the gene functions of *L. monocytogenes*. The GO enrichments of DEGs from all gene deletion strains were sorted by their $P\text{-adjust}$ values (Fig. S3). For $\Delta inIA$ mutant strains, 16 of the top 20 enriched GO terms were related to biological processes (BP), with 6 significantly enriched terms (all BP), including the most significant amino acid biosynthetic process ($P\text{-adjust} < 0.001$) (Fig. S3A). Δhly mutant strains had 19 significantly enriched GO terms (all BP), with carbohydrate transport being the most significant ($P\text{-adjust} < 0.01$), and multiple tryptophan/indole-related processes also highly enriched (Fig. S3B). $\Delta inIP$ mutant strains had three significantly enriched terms, including two molecular function (MF) terms (transmembrane transporter activity and transporter activity, $P\text{-adjust} < 0.01$) and one cellular component (CC) term (plasma membrane) (Fig. S3C). All top 20 GO terms for $\Delta actA$ mutant strains were MF-related but not significantly enriched (Fig. S3D).

KEGG metabolic pathways are classified into seven major categories: Metabolism (M), Genetic Information Processing (GIP), Environmental Information Processing (EIP), Cellular Processes (CP), Organismal Systems (OS), Human Diseases (HD), and Drug Development (DD). Enriched KEGG pathways were ranked by P-adjust values (Fig. S4). In $\Delta inlA$ mutant strain, 18 of the top 20 pathways were M-related, with Histidine metabolism being the only significantly enriched pathway ($P\text{-adjust} < 0.05$) (Fig. S4A). Δhly mutant strain had 14 M-related pathways, with Phosphotransferase system (PTS, EIP) as the sole significant pathway (Fig. S4B). $\Delta inlP$ mutant strain and $\Delta actA$ mutant strain showed no significantly enriched pathways; $\Delta inlP$ had 13 M-related pathways, while $\Delta actA$ had 13 M-related pathways (Pyrimidine metabolism being the most enriched). All other pathways across strains were non-significant ($P\text{-adjust} > 0.05$) (Fig. S4C&D).

4. Discussion

This study analyzes the virulence of wild-type ST87 *L. monocytogenes* and its key virulence gene mutants ($\Delta inlA$, Δhly , $\Delta inlP$, $\Delta actA$) both *in vitro* and *in vivo*, complemented by transcriptomic analysis at the genetic level. ST87 type *L. monocytogenes*, a dominant epidemic strain isolated from clinical samples in China^[10], is recognized as a hypervirulent ST type based on population prevalence data^[28], showing distinct genetic and phenotypic differences from other common ST types (e.g., ST8, ST9, ST121)^[2, 13]. Genetically, all ST87 strains harbor a conserved ST87-specific gene cluster containing a Restriction-Modification (RM) system with multifunctional roles in virulence and harsh environment adaptation^[11]. Additionally, all ST87 strains carry LIPI-4, a 6-gene cluster encoding the cellobiose family phosphotransferase system (PTS), which has been confirmed as a virulence factor associated with maternal-neonatal and central nervous system infections^[29]. Disruption of genes within LIPI-4 downregulates the transcription of several *L. monocytogenes* virulence genes (e.g., *hly*, *inlB*, *inlC*, *motA*, *sigB*), thereby affecting strain virulence^[30]. Previous studies have also shown that LIPI-4 is closely linked to enhanced virulence and placental infection^[31]. Phenotypically, ST87 strains do not exhibit significantly higher growth capacity, biofilm formation, motility^[2], hemolytic activity^[15], or *Galleria mellonella* larval lethality^[15] compared to other ST types, and show relatively lower intestinal barrier penetration^[13]. Notably, ST87 strains demonstrate strong resistance to simulated gastrointestinal stimuli, with enhanced virulence toward *G. mellonella* larvae and upregulated virulence gene expression after gastrointestinal stress^[23]. Moreover, ST87 strains exhibit potent adhesion and invasion capabilities toward placental trophoblast cells^[13], and clinical ST87 isolates show higher virulence than food-derived counterparts^[13], highlighting the elevated infection risk once ST87 enters the human body.

The responses of pregnant mice and ICR mice to ST87 mutants generally showed consistent trends. The Δhly mutant completely lost virulence in both models, with 100% survival in ICR mice, undetectable bacterial loads in organs, and no bacteria found in all maternal organs and fetuses, confirming the absolute necessity of LLO for systemic infection. Notably, previous studies have shown that the placenta serves as a favorable protective niche for bacteria, where even ΔLLO *L. monocytogenes* mutants and *L. innocua* can

persist at stable low levels for days via phospholipase-mediated escape, slow intracellular growth, and extracellular replication in the placenta, despite significantly reduced bacterial loads compared to wild-type EGD-e (ST35) in guinea pigs [32]. In contrast, this study found that the ST87 Δhly mutant completely lost its virulence in *in vivo* experiments, highlighting the unique importance of LLO for ST87 *L. monocytogenes* to breach placental defense barriers. However, Bakardjiev et al. [33] did not observe significantly reduced infection by the LLO mutant of wild-type *L. monocytogenes* 10403S (ST85) in pregnant guinea pigs, suggesting that infection mechanisms of different ST types of *L. monocytogenes* may vary in pregnant mouse models. The $\Delta actA$ mutant showed reduced bacterial loads in both pregnant mouse placentas and ICR mouse livers, consistent with previous studies [32, 33]. Moreover, the reduction in bacterial loads in all maternal organs (including placenta and fetus) of pregnant mice was more pronounced than that in ICR mice, indicating that ActA-mediated actin motility is central to cross-tissue dissemination and facilitates maternal-fetal transmission of *L. monocytogenes*.

The $\Delta inlA$ mutant did not show significantly reduced virulence compared to wild-type *L. monocytogenes* in both models, consistent with previous findings that InlA is not involved in fetal-placental invasion by *L. monocytogenes* in mouse models [32]. The $\Delta inlP$ mutant showed no obvious virulence difference from wild-type *L. monocytogenes* in the ICR mouse model. However, in the pregnant mouse model, bacterial loads in fetuses were significantly reduced compared to the wild-type strain, while no differences were observed in maternal liver, spleen, brain, or even placenta. Studies have shown that InlP increases placental bacterial burden in guinea pigs and mice, with minimal effects on other maternal organs, potentially (1000-fold) promoting placental infection [18]. Our results showing higher bacterial loads in placentas but lower loads in fetuses of the $\Delta inlP$ mutant suggest that InlP is most critical for disrupting trophoblast monolayers and accessing fetal interstitium, consistent with findings from human placental organ culture studies [19]. The extension of λ of $\Delta inlP$ and $\Delta actA$ mutants may increase the infection and colonization process of the strain in the body. In this study, only $\Delta actA$ showed reduced colonization in ICR mice and pregnant mice at 48 h post-infection, while $\Delta inlP$ did not exhibit this phenotype. Since bacterial loads in organs at different time points were not determined, subsequent experiments will quantify organ bacterial loads at multiple time points post-infection and compare colonization efficiency to clarify the impact of prolonged λ on proliferation rate.

ST87 mutant strains exhibited differential virulence regulation between animal and placental cell models. Unlike animal models, Δhly mutant strains did not show reduced virulence in 2D/3D cell models compared to wild-type strains, similar to findings by Phelps Christopher, Vadia [34]. However, it completely lost virulence in animal models, likely because LLO deficiency impaired *L. monocytogenes* escape from *in vivo* immune surveillance, whereas cell models lack a complete immune network. The $\Delta actA$ mutant showed attenuated virulence in cell models (especially 3D models), consistent with significantly decreased bacterial loads in pregnant mouse placentas and fetuses, confirming ActA's decisive role in intercellular dissemination [32]. The $\Delta inlA$ mutant showed decreased adhesion/invasion rates in 2D and 3D cell model,

verifying InlA-mediated initial adhesion via E-cadherin [34]. It is worth noting that the virulence of $\Delta inlP$ mutant increases in the placental cell model, while in the pregnant mouse model, its virulence is only attenuated in fetuses. The difference between these two results suggests that InlP induces actin rearrangement in trophoblast cells by binding to the basement membrane afadin protein, thereby forming transcellular channels [19].

The results of transcriptomic analysis of four gene mutant strains showed that the four gene deletion strains differed from the wild-type strain in the expression of virulence genes, but did not significantly affect their GO and KEGG metabolic pathways. Among them, $\Delta inlA$ and $\Delta actA$ mutant strains exhibited greater differences from the wild-type *L. monocytogenes*. The analysis and prediction of key virulence genes in ST87 *L. monocytogenes* strain are shown in Fig. 9.

As an important virulence factor of *L. monocytogenes*, ActA primarily participates in the intracellular movement and intercellular spread of bacteria by mediating actin polymerization. Consistent with most studies [32, 33], the *actA* gene-mutated *L. monocytogenes* in this study showed attenuated virulence in both cell models and animal models. However, the specific role of the gene network changes caused by *actA* deficiency in placental cells or placental model animals has not been clearly reported. Our transcriptome results showed that ActA deficiency significantly downregulated a series of metabolism-related genes, including *pduP* (encoding aldehyde dehydrogenase PduP), *yqhD* (encoding iron-containing alcohol dehydrogenase), genes encoding lytic polysaccharide monooxygenase, genes encoding glucosaminidase domain-containing protein, *typA* (encoding translational GTPase TypA), *pdhB* (encoding pyruvate dehydrogenase), and genes encoding C40 family peptidase. Studies have shown that deletion of *PduP* causes *Klebsiella pneumoniae* to produce metabolites toxic to itself during metabolism, potentially leading to bacterial death [35]. Deletion of PduQ, an alcohol dehydrogenase similar to PduP, results in slower growth of *Salmonella* in stressful environments compared to the wild-type [36]. Therefore, downregulation of metabolism-related genes may lead to energy depletion during *in vivo* traversal of the $\Delta actA$ mutant, thereby inhibiting bacterial virulence.

Additionally, deletion of a lytic polysaccharide monooxygenase CbpD in *Pseudomonas aeruginosa* prevents the establishment of lethal systemic infections, which is associated with enhanced bacterial clearance *in vivo* [37]. Other studies have found that wild-type *Pseudomonas aeruginosa* CbpD impairs the terminal complement cascade in human serum, independent of its inhibition of proinflammatory responses *in vivo* or *in vitro* [37]. However, there are few reports on the relationship between lytic polysaccharide monooxygenase and virulence in *L. monocytogenes*. Among the four gene mutants, only the $\Delta actA$ mutant showed significant downregulation of the gene encoding lytic polysaccharide monooxygenase. The relationship between the two and the specific mechanism of lytic polysaccharide monooxygenase in *L. monocytogenes* virulence require further investigation. *TypA* is involved in the virulence of *Pseudomonas* [38], while deletion of *pdhB* affects *in vitro* growth and cell invasiveness [39]. Furthermore, deletion of *actA* led to downregulation of several internalin family genes (e.g., *inlA*, *inlB*, *inlC*, *inlJ*), the *znuA* gene

(encoding a metal ABC transporter substrate-binding protein), and the *lepB* gene (encoding type I signal peptidase SipY). Studies have shown that deletion of *znuA* affects the *in vivo* virulence of *Brucella abortus* [40]. In *L. monocytogenes* with SipY deletion, the efficiency of signal peptide cleavage for InlA and InlB is reduced, resulting in a more than 50% decrease in bacterial adhesion to host cells and significantly attenuated virulence in a mouse infection model [41]. Thus, the downregulation of multiple genes caused by *actA* mutant leads to reduced virulence of the $\Delta actA$ mutant, but the specific mechanism remains to be studied.

The $\Delta inlA$ mutant strain showed a significant decrease in adhesion and invasion abilities in the 2D/3D JEG-3 and HTR8/SVneo placental cell models. Transcriptome results indicated that the deletion of *inlA* also led to the downregulation of some genes that seemed to be the same as those in the $\Delta actA$ mutant strain, such as *pduP*, *yqhD*, *znuA*, *pdhB*, and *inlB*. Therefore, it is speculated that the mechanism by which the virulence to placental cells is weakened after *inlA* deletion may be similar to that of the $\Delta actA$ mutant strain. No attenuation of virulence was observed in animal experiments, which from the transcriptome results may also be related to the upregulation of some genes. For example, FbpA is an important adhesion molecule of *L. monocytogenes*, especially for hepatocytes [42], while the MucBP domain-containing protein is involved in mucin binding, promotes bacterial colonization of host mucous membranes, and is related to the intestinal colonization of *Salmonella* [43]. The autolysin Ami promotes the adhesion of *L. monocytogenes* to eukaryotic cells through its cell wall anchoring [44]. Therefore, although *inlA* is deleted in the $\Delta inlA$ mutant strain and some metabolism-related genes are downregulated, many adhesion-related genes show an upregulation trend. Thus, it is speculated that these adhesion-related genes may compensate for its invasive effect on the placenta in ICR mice and pregnant mouse models.

The Δhly mutant strain only showed attenuated virulence to the placenta in ICR mouse and pregnant mouse models. Transcriptome results indicated that genes such as *groEL*, *osmC*, *dnaK*, and resuscitation-promoting factor were significantly downregulated. The fully conserved molecular chaperones *groEL* and *dnaK* in bacteria can further cooperate to assist proteins in completing folding and play an important role in protein targeting pathways [45]. It can be seen that the upregulated expression of GroEL can help bacteria maintain protein homeostasis, thereby enhancing their viability. In addition, GroEL is a potential antibacterial target, and the expression of GroEL is weakened in bacteria with attenuated virulence [46]. Moreover, *dnaK* has also been proven to be involved in the expression of virulence genes in *L. monocytogenes* [47]. Therefore, the deletion of the gene *hly* leading to the downregulation of genes expressing these proteins may collectively weaken the immune escape ability of *L. monocytogenes*, making it phagocytosed by immune cells, which is consistent with the results of mouse experiments. However, there is a difference between the placental cell infection situation and the animal experiment results. The adhesion and invasion abilities of the Δhly mutant strain to placental cells are not different from those of the wild strain, which may be due to the increased expression levels of its *inlB* and *inlA* genes. Therefore, we

speculate that once *L. monocytogenes* escapes from the immune system and reaches the target organ, *inlB* and *inlA* may play a more important role in *L. monocytogenes*.

$\Delta inlP$ mutant strain did not show a significant attenuation of virulence compared with the wild-type *L. monocytogenes*. We only found that the bacterial load in fetuses in the pregnant mouse model was significantly lower than that of the wild-type strain. The InlP encoded by the *inlP* gene is a novel virulence factor of *L. monocytogenes*. Studies have shown that it helps in crossing the basement membrane during placental infection^[18] and seems to be most helpful in disrupting the trophoblast monolayer and entering the fetal interstitium. Based on its transcriptomic analysis, we found that the deletion of *inlP* did not lead to significant changes at the transcriptional level. Only *pduP*, the gene encoding glucosaminidase domain-containing protein, and the gene encoding cell invasion LPXTG protein Vip were downregulated. In addition, there were even upregulations of *pduQ*, the gene encoding MucBP domain-containing protein, and the gene encoding C40 family peptidase. The involvement of MucBP domain-containing protein in mucin binding and promotion of bacterial colonization of host mucous membranes seems to explain the enhanced virulence in some cell models.

In summary, this study confirms that placental infection by ST87 depends on the synergistic action of *hly* and *actA*, with *inlA* and *inlP* assisting invasion through adhesion. Novel targets related to *L. monocytogenes* infection were identified, such as lytic polysaccharide monooxygenase, MucBP domain-containing protein, alcohol dehydrogenase PduQ, and the *lepB* gene encoding type I signal peptidase SipY. These findings provide new insights into the mechanism of gestational *L. monocytogenes* infection specific to China and lay a theoretical foundation for the development of anti-ST87 drugs. Future studies could combine proteomics to analyze the interaction network between ST87 and host placental cells, or use organoid models to simulate the three-dimensional immune microenvironment of the placental barrier. Furthermore, this study did not explore the immune evasion mechanism of ST87 in the context of gestational immunosuppression; subsequent research could use single-cell sequencing to analyze the immune cell atlas of infected placentas, providing targets for the development of ST87-specific vaccines for pregnancy.

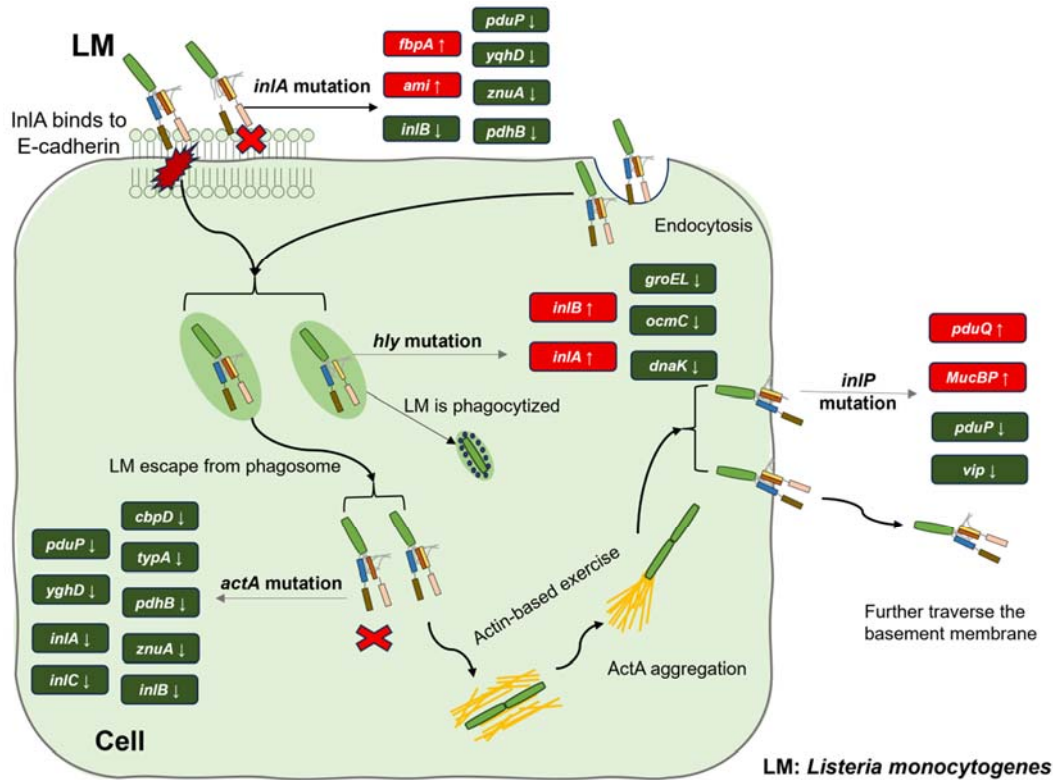


Fig.9 Analysis and prediction of key virulence genes of *L. monocytogenes* ST87 in placental infection.

5. Conclusion

This study systematically analyzed the roles of key virulence genes (*inlA*, *hly*, *inlP*, and *actA*) of the China-specific epidemic ST87 *L. monocytogenes* strain in placental infection. We found that placental infection by ST87 *L. monocytogenes* strain depends on the synergistic effect of *hly* and *actA*, with *inlA* and *inlP* playing auxiliary roles. *hly* is an essential gene for ST87 systemic infection in the ICR mouse and pregnant mouse models used, and its deletion leads to a complete loss of virulence in the strain in these animal models. Based on the results of transcriptome analysis, it was speculated that *actA* affects bacterial energy metabolism and intercellular spread by regulating metabolism-related genes including *pduP* and *yqhD*, and internalin family genes including *inlA* and *inlB*, making it a core factor for placental invasion and mother-to-fetus transmission. *inlA* mainly mediates cell adhesion through E-cadherin; its deletion reduces virulence only in human trophoblast models, but not mice model. *inlP* has a weak impact on overall virulence and mainly assists in penetrating the placental barrier, with its deletion only reducing the bacterial load in fetuses. In addition, the regulatory network revealed by transcriptomics shows that the differentially expressed genes between $\Delta inlA$, $\Delta actA$ and the wild-type strain are the most significant, involving adhesion, metabolism, and virulence regulation. In addition, transcriptomic analysis identified potential genes associated with key virulence determinants of ST87 *L. monocytogenes*, including lytic polysaccharide monooxygenase, a MucBP domain-containing protein, and the alcohol dehydrogenase PduQ. These genes may serve as potential novel targets for controlling infection of ST87 *L. monocytogenes*. Future research can combine proteomics to analyze the interaction network between ST87 *L. monocytogenes* and host placental

cells, or use human organoid models to simulate the three-dimensional immune microenvironment of the placental barrier.

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Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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