A Cross-Protective Vaccine Against 4b and 1/2b Listeria monocytogenes

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Listeria monocytogenes (Lm) is a foodborne zoonotic pathogen that causes listeriosis with a mortality rate of 20–30%. Serovar 4b and 1/2b isolates account for most of listeriosis outbreaks, however, no listeriosis vaccine is available for either prophylactic or therapeutic use. Here, we developed a triple-virulence-genes deletion vaccine strain, and evaluated its safety, immunogenicity, and cross-protective efficiency. The virulence of NTSN\textsuperscript{actA/plcB/orfX} was reduced 794-folds compared with the parental strain. Additionally, it was completely eliminated in mice at day 7 post infection and no obvious pathological changes were observed in the organs of mice after prime-boost immunization for 23 days. These results proved that the safety of the Lm vaccine strain remarkably increased. More importantly, the NTSN\textsuperscript{actA/plcB/orfX} strain stimulated higher anti-Listeriolysin O (LLO) antibodies, induced significantly higher expression of IFN-γ, TNF-α, IL-17, and IL-6 than the control group, and afforded 100% protection against serovar 4b and 1/2b challenges. Taken together, our research demonstrates that the triple-genies-deletion vaccine has high safety, can elicit strong Th1 type immune response, and affords efficient cross-protection against two serovar Lm strains. It is a promising vaccine for prevention of listeriosis.

Keywords: Listeria monocytogenes, attenuated vaccine, cellular immune response, cross protection, humoral immune response

INTRODUCTION

Listeria monocytogenes (Lm) was first isolated by Murray, Webb, and Swann from dead laboratory rabbits and guinea pigs in 1926 (Murray et al., 2005). It is a gram-positive, foodborne bacterium that causes severe listeriosis in immunosuppressed populations, including the elderly, pregnant women, and newborns (Buchanan et al., 2017). Listeriosis is associated with miscarriage, gastroenteritis, sepsis, and meningitis, with a mortality rate of 20–30% (Hernandez-Milian and Payeras-Cifre, 2014; Schutte et al., 2019). Listeriosis has remained a problem in recent years, listeriosis outbreaks were reported in South Africa (Smith et al., 2019), the United States (Angelo et al., 2017), the European Union (Calderon-Gonzalez et al., 2017; Duranti et al., 2018), and other countries and regions.
The emergence of hypervirulent strains, as well as extensively drug-resistant and multi-drug resistant clinical isolates, has brought new challenges to prophylactic and therapeutic treatment of listeriosis (Camargo et al., 2015; Jahan and Holley, 2016). The field has advanced our understanding of *Lm* physiology and infection process considerably (Mostowy and Cossart, 2012; Rollion and Cossart, 2017).

*Lm* is a facultative intracellular pathogen that can escape from phagocytic vesicles into the host cytoplasm, where it activates NFKB pathways, and modulates the type I interferon response. Thus, *Lm* regulates the host’s innate and adaptive immune responses and decreases host resistance to systemic infection (Stockinger et al., 2004; Opitz et al., 2006; Boneca et al., 2007). In the process of host-*Lm* interactions, *Lm* virulence factor listeriolysin O (LLO) can dissolve phagocytic vesicles to assist bacteria escaping into the cytoplasm of phagocytes (Nguyen and Portnoy, 2020). *Lm* antigens were efficiently presented to the MHC-I and MHC-II processing pathways, thus presenting antigenic peptides to either CD4+ T cells or CD8+ T cells (D’Orazio, 2019). The activated T cells differentiate and generate the adaptive immune response, inducing the production of the Th1 cytokines IFN-γ, TNF-α, and IL-12 (Nomura et al., 2002; Berg et al., 2005). The activated CD8+ cytotoxic lymphocytes secrete perforin, granzyme, and granlysin and so on, thus killing the infected cells along with intracellular *Lm* (Ito et al., 2015; Lin and Flynn, 2015). IL-17 is a pro-inflammatory factor, involved in regulating immune response to promote multiple chemokines and inflammatory cytokines, it is essential in defense of the host against the pathogen (Freches et al., 2013; Chamoun et al., 2018). IL-10 is an immunosuppressive and anti-inflammatory substance, plays a vital role in enhancing secretion of anti-inflammatory modulators and specifically inhibiting the cellular immune response (Murai et al., 2009). The variant production between pro-inflammatory cytokines and anti-inflammatory cytokines reflects the biases of immune response against pathogen. *Lm* mediates efficient cellular immune responses and memory T cell responses, thus consistently inducing robust protective immunity. Therefore, *Lm* as a vaccine vector of delivering foreign antigens has been intensively studied, in particular for tumor therapy vectors (McLaughlin et al., 2013; Crittenden et al., 2015; Ding et al., 2019). Although the *Lm* vectored vaccine have inspiring therapeutic effect in clinic, until now, no vaccine is available to protect high-risk populations against listeriosis.

Vaccines is an effective way to prevent and treat listeriosis. Among available *Listeria* vaccines, live attenuated vaccines are highly preferred, since they are highly immunogenic, stimulate robust cellular immunity, and provide longer immunoprotection. Knocking out *Lm* virulence and virulence-associated genes is the primary strategy to reduce its virulence. Several virulence genes are required for a successful *Lm* systemic infection, which are harbored in the *Listeria* pathogenic island 1 (LPI1-1). These genes enable *Lm* to invade cells (*prfA*), escape from phagocytic vesicles (*hly, mpl, plcA, plcB*), survive in the cytoplasm (*orfX*), and facilitate polar movement and cell-to-cell spread (*actA*) (de las Heras et al., 2011). Studies have shown that knocking out LPI-1 genes can significantly reduce *Lm* virulence (Yin et al., 2008; Vasanathkrishnan et al., 2015). For example, the *LmAΔactA* strain is unable to spread from cell to cell, significantly reducing its virulence compared to the wild-type strain (Saklani-Jusforgues et al., 2003). Our previous research reported the development of a serovar 1/2a *LmAΔactA/plcB* strain, which provided effective protection against the wild-type strain challenge (Yin et al., 2008). A triple mutant strain *LmdΔada* with deletion *actA, dal*, and *dat* generated protective immunity against a lethal challenge (Thompson et al., 1998). Encouragingly, the researchers developed the *ΔinlBΔactA* vaccine for which Phase I safety trials indicated no adverse effects in humans (Johnson et al., 2011). However, the bottleneck for the development of an attenuated *Listeria* vaccine is maintaining the balance between safety and virulence. Variations of the ideal attenuated vaccines, those with high safety and robust protective immunity, are on the way.

Based on somatic and flagellar antigens, fourteen serotypes have been identified for *Lm*, comprising 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, 4h, and 7 (Jamshidi and Zeinali, 2019; Yin et al., 2019). As we know, more than 80% of listeriosis in animals and about 50% of listeriosis in humans are caused by serotype 4b *Lm*, which usually manifests as severe miscarriage or neurological symptoms (Czuprynski et al., 2002; Miya et al., 2008; Yao et al., 2018). Serotype 4b strain is primary cause of human and animal listeriosis (Kathariou et al., 2006; Walecka-Zacharska et al., 2013). In this study, we used the serotype 4b strain *Lm* NTSN, which was isolated from a sheep listeriosis outbreak, and constructed a mutant strain with the deletion of three neighboring genes in LPI1-1, *actA, plcB*, and *orfX*. Using this triple-genes-deletion mutant strain, we characterized the cellular and humoral immunity as well as protective immunity induced in a murine model of *Lm* systemic infection.

**MATERIALS AND METHODS**

**Bacterial Strains and Materials**

The NTSN (4b serotype *Lm*) and DH5α (*Escherichia coli*) strains were obtained from the Jiangsu Key Laboratory of Zoonosis. The Ye32 (1/2b serotype *Lm*) was isolated from mutton in Yangzhou, Jiangsu, and was stored in the Jiangsu Key Laboratory of Zoonosis. The pAULA vector was a gift from professor Trinad Chakraborty (University of Giessen, Germany). Brain heart infusion (BHI) broth was purchased from Becton-Dickinson (United States).

**Animals and Ethics Statement**

6 weeks old female BALB/c and C57BL/6 mice were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All animal experiments were performed according to the Laboratory Animal-Guideline for Using Animals in the Education of the Chinese Association for Laboratory Animal Sciences (CALAS). The animal study was reviewed and approved by Yangzhou University Institutional Animal Ethics Committee (Yangzhou, China).
Construction of the Triple-Genes-Deletion Mutant

To achieve the actA, plcB, and orfX deletion strain, the recombinant plasmid pAULA-actA-U/orfX-D was constructed with the primers listed in Table 1. The upstream homology fragment, actA-U, consisted of the 447 bp sequence of mpl and the insertion sequence (IS), located between actA and the 198 bp mpl. The downstream homology fragment, orfX-D, consisted of NTSN_0217 (333 bp) and a 179 bp IS. The ClonExpress™ II One Step Cloning Kit (Vazyme, Nanjing, China) was used to ligate actA-U and orfX-D fragments with linearized pAULA plasmid. Following identification of the desired clone, the recombinant pAULA plasmid was electroporated into NTSN competent cells. The mutant strain NTSN ΔactA/plcB/orfX was obtained according to previously described methods (Yin et al., 2008).

Confirmation of the Triple-Genes-Deletion Mutant

RT-PCR

NTSNΔactA/plcB/orfX and wild-type NTSN were overnight cultured at 37°C, then collected by centrifugation. RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Germany). The cDNA of triple-genes-deletion and wild-type strain was obtained according the PrimeScript RT reagent kit protocol (Takara, China). The primers listed in Table 1 were used to amplify fragments of actA, plcB, orfX, and gyrB.

Western Blotting

The cell wall proteins of NTSN ΔactA/plcB/orfX and NTSN were extracted and analyzed by SDS-PAGE, followed by Western blot. Monoclonal antibodies against either LLO or ActA were used as the primary antibody, while HRP-(horseradish peroxidase)-labeled goat anti-mouse IgG was used as the secondary antibody, and diaminobenzidine (DAB) was the HRP substrate for Western blot analysis.

Phospholipase C Activity

Based on a previous report, we made egg yolk agar charcoal plates to verify phospholipase C activity of our strains. Here, 0.5 g activated charcoal was added to 100 mL of BHI agar, the pH adjusted to 6.5, and then autoclaved (Yeung et al., 2005). Afterward, 8 mL of bacteria-free chicken egg yolk was added to the molten medium (45°C) and the mixture was poured into Petri dishes. Wild-type (WT) and triple-genes-deletion strains were streaked on the plate surface and incubated at 37°C for 48 h.

Genetic Stability of the Triple-Genes-Deletion Mutant

A single colony of NTSN ΔactA/plcB/orfX was inoculated in BHI liquid and cultured at 37°C for 20–24 h as the first passage. This step was repeated until the 30th generation had been passaged.

Detection of Deleted Genes

The bacterial genome extraction kit was used to extract the genomes of each strain subline. Those genomes were prepared as the template for PCR amplification with outer primers (SW-F/R) and inner primers (SN-F/R) (Table 1). Wild-type NTSN was used as a positive control to demonstrate if deleted genes were restored in either the original NTSN ΔactA/plcB/orfX strain, or in strain sublines at 5, 15, and 30 generation passages.

Genetic and Biochemical Identification of the Serially Passaged Strains

We inoculated the original NTSN ΔactA/plcB/orfX strain and strain sublines at 5, 15, and 30 generation passages on BHI plate and incubated them at 37°C for 12–16 h. A single colony was inoculated in PBS to obtain a bacterial suspension. The optical density (OD) of the bacterial suspension was adjusted to McFarland 0.50–0.63 using a turbidimeter instrument. The 1.8 mL suspension of each strain was added to a VITEK 2 gram-positive biochemical identification card and put into the automatic biochemical identification instrument (bioMérieux, France) to analyze 43 biochemical indicators of Lm.

Evaluation the Virulence of NTSN ΔactA/plcB/orfX

Determination of LD50

Six weeks old BALB/c female mice were randomly divided into six groups (5 mice/group) and infected by NTSN ΔactA/plcB/orfX and NTSN. The NTSN ΔactA/plcB/orfX and NTSN were inoculated in BHI broth and cultured for 12 h at 37°C. The culture was diluted 1:40 with fresh broth and then transferred to 10 mL of fresh BHI liquid medium and cultured until it reached an OD600 value of 0.8. The LD50 of NTSN was determined by intraperitoneal injection. The remaining bacteria were plated on BHI agar for bacterial colony counts. The mice were monitored for next 14 days to determine the median lethal dose (LD50).

Assessment of Bacterial Loads

Six weeks old BALB/c female mice were randomly divided into three groups (30 mice/group) and infected abdominally with either NTSN ΔactA/plcB/orfX (5 × 106 CFU), NTSN wild-type (5 × 105 CFU), or PBS (150 µL). On day 1, 3, 5, 7, and 9 post-infection, the spleens and livers were removed from each group, homogenized, and cultured for bacterial colony counts.

Six weeks old C57BL/6 and BALB/c female mice were randomly divided into four groups (6 mice/group) and infected abdominally with NTSN ΔactA/plcB/orfX (5 × 105 CFU) or NTSN wild-type (5 × 105 CFU), or PBS (150 µL). Six weeks old C57BL/6 and BALB/c female mice were randomly divided into four groups (6 mice/group) and infected abdominally with NTSN ΔactA/plcB/orfX (5 × 105 CFU) or NTSN wild-type (5 × 105 CFU). On day 3 post infection, the spleens and livers were harvested and homogenized. Next, the samples were cultured for bacterial colony counts. Mice were weighed before infection and after infection for 3 days post-infection to compare the changes in body weight.

Histopathological Analysis

Six weeks old BALB/c female mice were randomly separated into two groups (6 mice/group) and immunized subcutaneously with either NTSN ΔactA/plcB/orfX (5 × 105 CFU) or PBS (150 µL) followed, by a booster on day 14 post-vaccination (Table 2). The mice were sacrificed 14 days after the booster immunization. The spleen, liver, kidney, heart, lungs, and brain of each mouse...
### TABLE 1 | Primers used in this study.

| Name | Sequence (5′–3′)a | Application |
|------|------------------|-------------|
| acA + | AGGACGTTGTAAAAGAGGAGCCAG | Mutant constructed |
| acA − | CGCTCGGCTTCATTCAAAATTCTTTATTCCTGCTCTGAT | Mutant constructed |
| orfX + | ATCCAGGATTGGAGAATTGAATTTGGAATGGAACAGGACG | Mutant constructed |
| orfX − | TTACGCCAAGCTTGCATGCCTGCAG | Mutant constructed |
| SN-F | GTACGGGATGAGTCTACACCACAA | Mutant verification |
| SN-R | TTACGCCAAGCTTGCATGCCTGCAG | Mutant verification |
| SW-F | GTTCCTTAACCTCTCTGTCA | Mutant verification |
| SW-R | ACAAGCCTTATAAAACCACCAA | Mutant verification |
| actA-R | TAACTCAGGTAGCTCGGTAGCAT | RT-PCR |
| pbF | GCGGATGAGTCTACACCACAA | RT-PCR |
| pbB-R | GCGGATGAGTCTACACCACAA | RT-PCR |
| orfX-F | ATCCCTACAGCTTACAGGG | RT-PCR |
| orfX-R | GTTCCTTAACCTCTCTGTCA | RT-PCR |
| gyvF-B | TAACTCTTTCCCAGACTCTTGG | RT-PCR |
| gyvB-R | CGTAATCCGGTTTGCCCT | RT-PCR |
| IFN-α-F | GGAACTGGCAAAAGGATGGT | qRT-PCR |
| IFN-α-R | ACGCTTATTGTTGATGCGATG | qRT-PCR |
| L-17-F | AACATGAGGACGCCGACGAC | qRT-PCR |
| L-17-R | GGACCGAGGTGAGTCTATGC | qRT-PCR |
| TNF-α-F | TCTCTATCCACTCTTCTTGG | qRT-PCR |
| TNF-α-R | ACGCTTATTGTTGATGCGATG | qRT-PCR |
| IL-10-F | ATCCCTGATTGATTGAGTCC | qRT-PCR |
| IL-10-R | GACACCTCGGTCTTGAGAG | qRT-PCR |
| IL-4-F | TACAGCAGGAGGAAACACC | qRT-PCR |
| IL-4-R | CGAACGGCGCAAAGAGTC | qRT-PCR |
| GAPDH-F | CAAATACGGGTAGTCTCC | qRT-PCR |
| GAPDH-R | CGTAATCCGGTTTGCCCT | qRT-PCR |

a The actA + and orfX- primers contain homologous fragments of the pAULA vector which sequences marked by an underscore.

### TABLE 2 | Mouse immunization and challenge procedures via intraperitoneal (i.p) injection.

| Groups | Number of immunization bacteria (CFU/mouse) | Dose of immunization (µL/mouse) | Number of challenge bacteria (CFU/mouse) | Dose of challenge (µL/mouse) |
|--------|---------------------------------------------|---------------------------------|------------------------------------------|----------------------------|
| A      | PBS/0                                       | 150                             | 2.5 × 10⁶                                | 150                        |
| B      | PBS/0                                       | 150                             | 7 × 10⁵                                  | 150                        |
| C      | 5 × 10⁶                                     | 150                             | 2.5 × 10⁶                                | 150                        |
| D      | 5 × 10⁶                                     | 150                             | 7 × 10⁵                                  | 150                        |
| E      | PBS/0                                       | 150                             | –                                        | –                          |
| F      | 5 × 10⁶                                     | 150                             | –                                        | –                          |
| G      | 5 × 10⁶                                     | 150                             | –                                        | –                          |

A, The PBS inoculated group challenged by Yc32; B, The PBS inoculated group which challenged by NTSN wild-type strain; C, The NTSN ΔactA/plcB/orfX immunized group challenged by Yc32 strain; D, The NTSN ΔactA/plcB/orfX immunized group which challenged by NTSN wild-type strain; E, C57BL/6 mice were inoculated by PBS for cellular immune response determination; F, C57BL/6 mice were immunized by NTSN ΔactA/plcB/orfX for cellular immune response determination; G, C57BL/6 mice were inoculated by NTSN for cellular immune response determination.

were prepared for histopathological sections, and subjected to HE staining at WuXi AppTec Co., Ltd.

### Detection of Antibodies in the Serum of Immunized Mice

Six weeks old BALB/c female mice in each group (6 mice/group) were immunized subcutaneously with NTSN, NTSN ΔactA/plcB/orfX or PBS, and given a boost with the same dose 14 days subsequent the first immunization (Table 2). The humoral immune response induced by the inoculation of NTSN ΔactA/plcB/orfX in mice was evaluated by indirect enzyme-linked immunosorbent assay (ELISA). Blood samples were collected at day 7, 14, 21, and 28. LLO was diluted in NaHCO₃ buffer (10 mmol/L, pH 9.6) and antibody-coated at a final concentration of 0.64 µg/mL. HRP-labeled goat anti-mouse IgG1 and IgG2a (1:5,000; Sigma, United States) were used as secondary antibody. The absorbance rate at OD₄₅₀nm was
detected by multifunctional microplate detector (BioTek, Berten, United States) and a P (Sample OD_{450}/N (negative control OD_{450}) greater than 2.1 was considered positive.

**Cellular Immune Response of Prime-Boost Immunization**

Six weeks old C57BL/6 mice were randomly divided into three groups (5 mice/group) and subcutaneously immunized with 0.1 LD_{50} NTSNΔactA/plcB/orfX, wild-type NTSN or PBS (Table 2). On day 7 after the second immunization, spleen of C57BL/6 mice were harvested and extracted total RNA using RNAprep Pure Tissue Kit (Tiangen, China), then transcribed to cDNA using PrimeScript RT reagent Kit (Takara, China). Next, the transcriptional expression of IL-4, IL-6, IL-10, IFN-γ, TNF-α, and IL-17A cytokines were determined by quantitative Real-Time PCR (qRT-PCR). The primers of cytokines used by qRT-PCR are shown in Table 1. The cDNA template was diluted 4 times with RNA free ddH2O. Cycling conditions were 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, and 60°C for 30 s. The Real-time PCR reactions were performed on 7500 Real Time PCR System (Applied Biosystems qPCR; Thermo Fisher Scientific, United States). Expression levels [relative quantification (RQ)] were assessed using $2^{-\Delta\Delta C_{T}}$ method.

**Challenge With Hypervirulent Lm Strains**

The immunization efficacy of NTSNΔactA/plcB/orfX was further evaluated by a challenge experiment. Six weeks old female C57BL/6 mice were randomly divided into four groups (6 mice/group), where two groups were intraperitoneally inoculated with PBS, and other two with the mutant strain (Table 2). All groups were prime-boost immunized in 14 days intervals. On day 14 after the second immunization, they were challenged with 10 LD_{50} Lm NTSN or Lm Yc32 (Table 2). Mice were monitored daily for 2 weeks post-challenge.

**Software of Statistical Analysis**

Statistical analyses and statistical drawing were performed with Prism 8 (GraphPad Software, version 8). One-way ANOVA pairwise compares the means from two or more groups in pairs to the relative means of control group; two-way ANOVA with Tukey’s or Sidak’s multiple comparisons test was used to compare the means of two groups. Statistically significant differences are: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Statistically non-significant (ns) was denoted when p-value was > 0.05. The primers are designed by Primer Premier 5.0, which produced Premier Biosoft International, Canada.

**RESULTS**

**The Mutant Strain Was Successfully Constructed**

The positive clones were selected under ethryromycin and temperature selection pressures following the electroporation of pULA actA-U/orfX-D into the competent parental strain NTSN. The deletion of all three genes were verified by RT-PCR with no amplification of the 3 fragments specific to actA (1,035 bp), plcB (511 bp), and orfX (302 bp) (Figure 1A). The deletion of plcB was further demonstrated by the loss of phospholipase activity on YAC medium, where a transparent zone was absent (Figure 1C). The knockout of actA was further verified by Western blotting, where the corresponding 97 kDa band was absent (Figure 1B). Taken together, these results confirmed successful construction of the NTSNΔactA/plcB/orfX strain via homologous recombination technology.

**The Triple-Genes-Deletion Mutant Was Genetically Stable**

The genetic stability of the mutant strain was evaluated by serial passages. The genes actA, plcB, and orfX were unable to be detected at the 5th, 15th, and 30th generations indicating that the mutant strain was stable (Supplementary Figures S1A,B). Additionally, the genetic stability of the mutant strain was further determined by biochemical characterization. These results showed that the 5th, 15th, and 30th generations of the NTSNΔactA/plcB/orfX strain had biochemical characteristics consistent with the first original generation, while lost the phospholipase activity comparison with the wild-type NTSN (Supplementary Table S1). In brief, these results indicated that the vaccine candidate was genetically stable.

**The Triple Genes Deletion Reduced Lm Virulence**

The LD_{50} of the mutant strain was 794-folds higher than that of the wild-type Lm NTSN, indicated that its virulence in mice was remarkably decreased (Table 3). The virulence of the mutant strain was further evaluated by determining bacteria loads in organs of infected mice via intraperitoneal inoculation. Even an infection dose 100-folds higher than the parental strain resulted bacteria loads in spleen and liver significantly lower than the control group at each time point (P < 0.001). Additionally, the mutant was cleared by day 7 (spleen) or 9 (liver), suggesting a significant reduction in the colonization ability of the mutant strain (Figures 2A,B). Histopathology did not observe any pathological changes in the liver, spleen, heart, lungs, or brain of mice vaccinated with NTSNΔactA/plcB/orfX (Figure 3), suggesting that the virulence of the mutant was dramatically reduced. It suggested that the virulence of the mutant was significantly reduced, and it had a certain potential safety in the application of humans and animals.

The susceptibility of Lm strains to BALB/c and C57BL/6 mice was compared with intraperitoneal infection at same dose. On day 3 post infection, the colonization ability of the triple-gene-deletion mutant in the spleen and liver either BALB/c and C57BL/6 mice were significantly lower than parental NTSN (P < 0.001) (Figures 2C,D). Body weight of BALB/c and C57BL/6 mice immunized with triple-genes-deletion group didn’t change (P > 0.05), while mice infected by parental NTSN significantly changed (P < 0.01, BALB/c mice; P < 0.05, C57BL/6 mice) (Figures 2E,F). This experiment verified that BALB/c mice were more susceptible to Listeria strains than C57BL/6 mice, and
also demonstrated that the triple-gene-deletion mutant greatly reduced the ability to colonize and infect to two types of mice.

A Strong Humoral Response Was Induced

We used an indirect ELISA to detect the presence of antibody against LLO in the serum of vaccinated mice. From the 7th day post-immunization, anti-LLO antibodies were significantly higher in mice immunized with the triple-genes-deletion mutant strain than those in the control group (7 days, $P \geq 0.05$; 21 and 28 days, $P < 0.001$) (Figure 4A). Antibody levels increased successively and reached their highest levels at day 21 post-immunization. The above results indicated that the mutant strain stimulated a strong humoral immune response in mice.

To understand the biases of the immune response induced in mice vaccinated with the mutant strain, the IgG subtypes, including IgG1 and IgG2a, were determined by indirect ELISA and the ratio of IgG2a to IgG1 was calculated. The ratio of IgG2a/IgG1 exceeded 1 (Figure 4B) after the primary immunization. Importantly, the ratio of IgG2a/IgG1 increased to greater than 2 after the prime-boost immunization. This result confirmed that the NTSN$\Delta$actA/plcB/orfX mutant strain induced a Th1 immune response in mice.

Cellular Immune Response Was Elicited

The primary function of both CD4$^+$ and CD8$^+$ T cells is the secretion of proinflammatory cytokines IFN-$\gamma$, TNF-$\alpha$, IL-6, etc. (Carpenter et al., 1994; Fan et al., 2015). The transcription levels of cytokines in the spleen of mice administration with Listeria strains were assessed by qRT-PCR. In Figure 5, qRT-PCR results showed that the transcription levels of IFN-$\gamma$, IL-17, TNF-$\alpha$, IL-6, in NTSN$\Delta$actA/plcB/orfX

| Strain        | NTSN ($1 \times 10^4$) | NTSN$\Delta$actA/plcB/orfX ($1 \times 10^7$) |
|---------------|-------------------------|---------------------------------------------|
| Dose (CFU)    | 60                      | 12                                          |
|               | 12                      | 2.4                                         |
|               | 2.4                     | 80                                          |
|               | 80                      | 16                                          |
| Mortality     | 4/5                     | 4/5                                         |
|               | 4/5                     | 2/5                                         |
|               | 2/5                     | 4/5                                         |
|               | 4/5                     | 4/5                                         |
| LD50          | $6.31 \times 10^4$      | $5.01 \times 10^7$                         |
Listeria Vaccine Confers Cross-Protection

FIGURE 2 | Bacterial loads and body weight changes in infected mice. Over time 6 mice in each group were abdominally injected with either NTSN\textDelta\textit{actA}/\textit{plcB}/\textit{orfX} ($5 \times 10^5$ CFU), NTSN ($5 \times 10^3$ CFU), or PBS (150 mL). (A) Polyline with round black dots indicated the number of viable bacteria in the liver enumerated of the BALB/c mice infected by NTSN at 1, 3, 5, 7, and 9 days post-infection; While polyline with square black dots represented the BALB/c mice infected by NTSN\textDelta\textit{actA}/\textit{plcB}/\textit{orfX}; And polyline with triangular black dots represented the BALB/c mice were injected by PBS. (B) Polyline with round black dots indicated the number of viable bacteria in the spleen enumerated of the BALB/c mice infected by NTSN at 1, 3, 5, 7, and 9 day post-infection; While polyline with square black dots represented the BALB/c mice infected by NTSN\textDelta\textit{actA}/\textit{plcB}/\textit{orfX}; And polyline with triangular black dots represented the BALB/c mice were injected by PBS. The log CFUs/g represented the mean of six mice per group. Each dot represented an organ from one infected mouse. Error bars represented SD, $n$ = 3 independent experiments. Statistical analyses were carried out by Tukey's multiple comparisons test: **** $P < 0.0001$. (C) The round black dots indicated to the bacterial load in the liver of BALB/c mice post NTSN infected 3 days; The square black dots indicated to the bacterial load in the liver of BALB/c mice post NTSN\textDelta\textit{actA}/\textit{plcB}/\textit{orfX} infected 3 days; and the equilateral triangle black dots presented the bacterial load in the liver of BALB/c mice post NTSN\textDelta\textit{actA}/\textit{plcB}/\textit{orfX} infected 3 days (Log CFU/organ). (D) The round black dot refers to the bacterial load in the liver of C57BL/6 mice post NTSN\textDelta\textit{actA}/\textit{plcB}/\textit{orfX} infected 3 days; and the equilateral triangle black dots indicated the bacterial load in the liver of C57BL/6 mice post NTSN\textDelta\textit{actA}/\textit{plcB}/\textit{orfX} infected 3 days (Log CFU/organ). Body weight changes post infected mice at 3 days compared them with the pre-infection for statistical analysis. The log CFUs/organ represented the mean of seven mice per group. Each dot represents an organ from one infected mouse. Error bars represented SD, $n$ = 3 independent experiments. Statistical analyses were carried out by Tukey's multiple comparisons test: **** $P < 0.0001$, *** $P < 0.001$, * $P < 0.05$, compared to the corresponding control group.
immunized mice were significant higher than control group ($P < 0.01; P < 0.001$). However, IL-4 and IL-10 transcription levels were no significant difference between the group immunized by the mutant strain and the control group ($P > 0.05$). These results indicated that triple-deletion mutant strain could induce a Th1 type immune response.
The Vaccine Candidate Conferred Cross-Protection

The protective efficacy of NTSNΔactA/plcB/orfX in mice was evaluated by challenges with either the wild-type strain, or 1/2b strain Yc32. As shown in Figure 6, vaccination with NTSNΔactA/plcB/orfX provided 100% protection against challenges with either the serovar 4b strain NTSN or 1/2b strain Yc32. However, the PBS control vaccination group provided 0% protection against challenges by either serovar 4b or 1/2b strain (Figure 6). These results indicated that the vaccine-candidate strain NTSNΔactA/plcB/orfX could provide cross-protective immunity to mice against challenges with either serovar 4b or 1/2b strain.

DISCUSSION

In 1881, Pasteur invented an attenuated anthrax vaccine against Bacillus anthracis, which has been the basis of the classic vaccine ever since. Lm is one of the most deadly foodborne pathogen, causing listeriosis. Vaccine immunization is one of the most effective strategy to prevent listeriosis infections. Researchers have developed a diverse array of Listeria vaccine platforms, e.g., Listeria live attenuated vaccine (McLaughlin et al., 2013), live
VECTOR VACCINE (YIN ET AL., 2017; ZENG ET AL., 2020), INACTIVATED VACCINE (JAZANI ET AL., 2010), INACTIVATED BUT METABOLICALLY ACTIVE (KBMA) VACCINE (LAUER ET AL., 2008), IRRADIATED VACCINE (DONG ET AL., 2009), BACTERIAL GHOSTS (BGs) (WU ET AL., 2017), SUBUNIT VACCINE (ANSARI ET AL., 2012; RODRIGUEZ-DEL RIO ET AL., 2015), NANO-VACCINE (CALDERON-GONZALEZ ET AL., 2017), AND PEPTIDES-LOADED DC VACCINE (KONO ET AL., 2012; JENSEN ET AL., 2013). HOWEVER, THERE IS NOT YET A LISTERIA VACCINE THAT HAS BEEN APPROVED BY THE FDA DEPARTMENT ON THE MARKET (CORRY AND CHU, 2014). SUBUNIT VACCINES AND INACTIVATED VACCINES HAVE THE ADVANTAGE OF HIGH SAFETY, WHEREAS THEY INDUCE LIMITED IMMUNOGENICITY, PARTICULARLY, CELLULAR IMMUNE RESPONSE (ANSARI ET AL., 2012; LUO AND CAI, 2012; CHENG ET AL., 2014). LIVE ATTENUATED LM IS CONSIDERED AS A PROMISING VACCINE VECTOR (MC LAUGHLIN ET AL., 2013), THE CLINICAL APPLICATIONS OF LISTERIA-VECTORED VACCINES HAVE A BRIGHT FUTURE (MACIAG ET AL., 2009). AT THE SAME TIME, THE POTENTIAL RISK TO IMMUNODEFICIENT POPULATION (KONO ET AL., 2012) MUST NOT BE OVERLOOKED. THEREFORE, WE HAVE FOCUSED ON THE SAFETY
of live attenuated vaccines. In this study, we constructed a triple-virulence-genes deletion vaccine strain with homologous recombination and tested its safety in a murine infection model. The safety of the vaccine strain increased 794-folds above its parental strain. Additionally, it was completely eliminated at day 9 post-immunization with the doses 100-times higher than the LD$_{50}$, and there was no obvious pathological change in any organ. In brief, the safety of the mutant strain was dramatically improved.

*Lm* has a unique intracellular lifecycle, which escapes from phagocytosis by host macrophages, thereby it enters the host cytoplasm and replicates (Pizarro-Cerda and Cossart, 2018). An efficient adaptive immune response is required to provide protective immunity, thus the ideal *Listeria* vaccine should effectively stimulate both cellular and humoral immune responses. Cytokines play an important role in the host's activation of the immune system (Zharkova et al., 2017), i.e., pro-inflammatory cytokines IFN-$\gamma$ and TNF-$\alpha$, which have major roles in inducing Th1 immune responses to protect against cellular pathogen (Allie et al., 2013; Romagnoli et al., 2017). IL-17 is a strong pro-inflammatory factor which is related with multiple disease, involved in inducing cellular immune responses (Amatya et al., 2017). And the IL-6 is produced to promote T cell differentiation, regulate cellular immune response, and clear the intracellular *Lm* (Hoge et al., 2013). Our results showed that significantly higher expression levels of pro-inflammatory cytokines IFN-$\gamma$, TNF-$\alpha$, IL-10, and IL-17 were elicited post-immunization with the vaccine candidate,
while the levels of anti-inflammatory cytokine IL-4 and IL-10 were unchanged compared with the control group. IL-10 is one of the most important anti-inflammatory cytokines generated during infectious diseases (Peñaloza et al., 2018). This mutant vaccine candidate increased levels of IFN-γ, IL-17, IL-6, and TNF-α, along with decreased levels of IL-4, IL-10 lead to the differentiation of helper T cells into the Th1 type (Haring et al., 2005; Tan et al., 2018). Thus our results suggest that our vaccine candidate induces Th1-type cellular response, contributes to the host response to the intracellular bacteria, and ultimately improves the cellular immune response of mice to eliminate the intracellular pathogen Lm.

Increasing evidence suggests that humoral immunity is important to defend against intracellular pathogens (Mahdy et al., 2019; Lew et al., 2020). Recent evidence suggests that the antibody response to *Mycobacterium tuberculosis* (M.tb) antigens may play a more significant role in impairing the extra-pulmonary dissipation of M.tb and antibody-mediated enhancement of mycobacteria phagocytosis by macrophages. Thus, it contributes to control intracellular M.tb (Lu et al., 2016). Lm is a gastrointestinal pathogen, thus the characterization of humoral immunity elicited by this attenuated vaccine was explored. In our results, the anti-LLO antibodies were significantly higher in the vaccine-candidate group, and peaked on day 21. Listerolysin O (LLO) harbors a variety of B and T cell epitopes and is an important protective antigen against Lm. Our results suggest that candidate vaccine strain could induce a strong humoral immune response to LLO. IgG2a and IgG1 are used as indicators for Th1 and Th2 responses, respectively (Tao et al., 2017), thus the vaccine-induced T-cell phenotype can be evaluated by the ratio of IgG2a/IgG1. Our results verified that vaccination significantly increased the levels of serum IgG2a, and decreased IgG1 levels, such that the ratio of IgG2a/IgG1 was more than 2 after the booster immunization. This further confirms that the vaccine candidate NTSNΔactA/ΔplcB/orfX can induce both humoral and cellular immune responses in mice.

According the characterization of the somatic and flagellar antigens, Lm strains are classified to 14 serotypes, of which serotype 4b strain is responsible for the majority of recorded, invasive listeriosis outbreaks, followed by the 1/2b and 1/2a serotypes (Wiedmann et al., 1996; Walecka-Zacharska et al., 2013). Linde et al. (1995) developed an attenuated bivalent (1/2a and 4b) vaccine by metabolic drift mutation, which afforded 95% protection in mice against a lethal dose of the parental strain. However, a vaccine against the serotype 4b and 1/2b strains infection has not been reported. Our study shows that the vaccine not only affords 100% immunoprotection against the serotype 4b strain in mice, but also 100% protection against the 1/2b strain. These two strains, 4b and 1/2b, have the same flagellum antigen b and different somatic antigens, which are 4 and 1, respectively (Liu, 2006). If the common flagella antigens playing crucial role in cross-protective immunity needs to be further elucidated. Our cross-protective vaccine against 4b and 1/2b serotypes provides a new strategy to prevent and control listeriosis.

### CONCLUSION

In conclusion, the highly attenuated *Lm* vaccine strain NTSNΔactA/ΔplcB/orfX has high safety and induces significant cellular and humoral immune response. It can not only afford protective immunity against serotype 4b strain challenges, but also serotype 1/2b strain, thus prevent infection by multiple pathogenic serotypes of *Lm*. Therefore, this vaccine strain is a potential candidate for controlling *Listeria* infection in human.

### DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

### ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Ethics Committee of Yangzhou University.

### AUTHOR CONTRIBUTIONS

YY and XJ designed the experiments. TZ and FM performed the experiments and analyzed the results. HY, ZL, YF, GL, JL, and XS were involved in animal experiments. JC and CM were involved in immune related experiments. HY, ZL, YF, GL, JL, and XS were involved in animal experiments. JC and CM were involved in immune related experiments. NY and XJ designed the experiments. TZ and FM wrote the manuscript. All authors read and approved the final manuscript.

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### SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.569544/full#supplementary-material
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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