



OPEN ACCESS

EDITED BY

Mengmeng Zhao,
Foshan University, China

REVIEWED BY

Songbiao Chen,
Henan University of Science and
Technology, China
Consuelo Pia Badilla Pino,
Universidad de Las Américas, Chile

*CORRESPONDENCE

Anchun Cheng
✉ chenganchun@vip.163.com
Xinxin Zhao
✉ xxinzhao@163.com

†These authors have contributed equally to
this work

RECEIVED 29 May 2025

ACCEPTED 04 July 2025

PUBLISHED 12 August 2025

CITATION

Xu R, Ji X, Lian J, Zhu D, Liu M, Wang M, Jia R,
Chen S, Yang Q, Wu Y, Zhang S, Huang J,
Ou X, Sun D, Tian B, He Y, Wu Z, Cheng A and
Zhao X (2025) Roles of SPI-2 T3SS effectors in
virulence of *Salmonella* Choleraesuis and
Construction of a triple-gene mutant vaccine
strain. *Front. Vet. Sci.* 12:1637327.
doi: 10.3389/fvets.2025.1637327

COPYRIGHT

© 2025 Xu, Ji, Lian, Zhu, Liu, Wang, Jia, Chen,
Yang, Wu, Zhang, Huang, Ou, Sun, Tian, He,
Wu, Cheng and Zhao. This is an open-access
article distributed under the terms of the
Creative Commons Attribution License (CC
BY). The use, distribution or reproduction in
other forums is permitted, provided the
original author(s) and the copyright owner(s)
are credited and that the original publication
in this journal is cited, in accordance with
accepted academic practice. No use,
distribution or reproduction is permitted
which does not comply with these terms.

Roles of SPI-2 T3SS effectors in virulence of *Salmonella* Choleraesuis and Construction of a triple-gene mutant vaccine strain

Rui Xu^{1,2,3,4†}, Xiangfei Ji^{1,2,3,4†}, Junqi Lian⁵, Dekang Zhu^{1,2,3,4},
Mafeng Liu^{1,2,3,4}, Mingshu Wang^{1,2,3,4}, Renyong Jia^{1,2,3,4},
Shun Chen^{1,2,3,4}, Qiao Yang^{1,2,3,4}, Ying Wu^{1,2,3,4}, Shaiqi Zhang^{1,2,3,4},
Juan Huang^{1,2,3,4}, Xumin Ou^{1,2,3,4}, Di Sun^{1,2,3,4}, Bin Tian^{1,2,3,4},
Yu He^{1,2,3,4}, Zhen Wu^{1,2,3,4}, Anchun Cheng^{1,2,3,4*} and
Xinxin Zhao^{1,2,3,4*}

¹Institute of Veterinary Medicine and Immunology, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan, China, ²Research Center of Avian Diseases, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan, China, ³Key Laboratory of Animal Disease and Human Health of Sichuan Province, Chengdu, Sichuan, China, ⁴Engineering Research Center of Southwest Animal Disease Prevention and Control Technology, Ministry of Education of the People's Republic of China, Chengdu, Sichuan, China, ⁵Hulunbuir Agricultural and Livestock Product Quality and Safety Center, Hulunbuir, Inner Mongolia, China

Effector protein functions of Type III secretion system (T3SS) encoded by *Salmonella* pathogenicity islands 2 (SPI-2) have not been fully characterized in *Salmonella enterica* serovar Choleraesuis. This study characterized 21 effectors of SPI-2 T3SS of *S. Choleraesuis* in terms of macrophage survival and virulence in mice via construction of various gene mutant strains. Eight effector genes including *sseF*, *sseJ*, *sifB*, *sseK*, *sifA*, *sopD₂*, *steC*, and *steD* contributed to bacterial survival in macrophage cell line RAW264.7; whereas only *sopD₂* also promoted bacterial virulence in mice like other three effector genes *sseL*, *steA*, and *spiC*. The mutant strain, Δ *sopD₂*, Δ *sseL*, Δ *steA*, or Δ *spiC*, led to higher mouse survival compared to the wild-type strain post-oral infection, while their bacterial loads in spleen and liver were not reduced except the Δ *spiC* that was undetectable in mouse tissues. Then, the triple-gene mutant strain Δ *sseL Δ *sopD₂* Δ *steA* was constructed and found to be virulence attenuated with a compromised colonization ability. Finally, immunization of this mutant orally induced robust serum IgG responses and provided 40% protection against lethal *S. Choleraesuis* challenge. Our study highlights the critical role of four SPI-2 T3SS effectors in *S. Choleraesuis* pathogenesis.*

KEYWORDS

S. choleraesuis, SPI-2, type III secretion system, virulence, vaccines

1 Introduction

Salmonella enterica serovar Choleraesuis (*S. Choleraesuis*) is a zoonotic pathogen causing swine paratyphoid, characterized by enterocolitis and septicemia, which imposes substantial economic burdens on global swine husbandry (1, 2). Although *S. Choleraesuis* is adapted to pigs, it is also a major cause of life-threatening septicemia, particularly

in children and immunocompromised individuals in East Asia and Europe (3, 4). Human infections frequently arise from direct contact with infected swine or ingestion of contaminated pork-derived products (1, 5). Due to the excessive use of antibiotics and environmental diversity, the emergence of multidrug-resistant *S. Choleraesuis* strains has become increasingly prevalent (6–8).

Vaccination represents the most cost-effective prophylactic strategy for disease control, effectively reducing antibiotic use and retarding the emergence of antibiotic resistance (9). The live vaccine strain C500 obtained by chemical mutagenesis has been used in China for more than 40 years to prevent paratyphoid fever in piglets (10). However, it still has non-negligible side effects related to residual toxicity, leading to adverse reactions in animals after vaccination and the genetic background of the vaccine is still unclear (11). Notably, there is no available vaccine for human use to date. Therefore, it is urgent and necessary to devise an innovative and efficacious vaccine against this important pathogen.

Understanding the mechanisms underlying bacterial pathogenesis is essential for the development of live attenuated vaccines. The *Salmonella* pathogenicity islands 2 (SPI-2) type III secretion system (T3SS) has been found to be essential for bacterial virulence of *S. Typhimurium* (12). This system promotes bacterial replication within membrane-bound *Salmonella*-containing vesicles (SCV) in host macrophages via production of various effector proteins (13). Loss of some effector genes *sseF*, *sseG*, and *sseM* that maintain bacterial nutrient acquisition within vesicles significantly reduces the replication ability of *S. Typhimurium* in host cells (14, 15). Effectors SpvB and SteC manipulate the actin cytoskeleton, affecting bacterial replication and subsequently impacting bacterial virulence (16, 17). Mutations in SPI-2 T3SS effectors can attenuate virulence, positioning them as promising candidates for live attenuated vaccines (18).

Despite their recognized importance, the specific roles of individual SPI-2 T3SS effectors in *S. Choleraesuis* virulence remain poorly characterized. This study aims to characterize SPI-2 T3SS effectors of *S. Choleraesuis* via construction of various single effector gene deletion strains. The mutant strains were systematically evaluated for their intracellular survival in macrophages, growth curves and swimming, and virulence in mice. Then, a triple mutant strain ($\Delta sseL\Delta sopD_2\Delta steA$) was constructed based on virulence effectors and its protection efficacy was finally evaluated.

2 Materials and methods

2.1 Bacterial strains and growth conditions

A complete list of all bacterial strains and plasmids utilized in the experiments is provided in [Supplementary Tables S1, S2](#). *Salmonella Choleraesuis* CVCC2139 was referred to as the wild-type (WT) strain for genetic manipulation to construct the indicated mutants. The *Escherichia coli* (*E. coli*) SM10 λ pir strain (19) served as the host for transferring suicide plasmids. All bacterial strains were cultured in Luria Bertani (LB) broth or agar at 37°C containing the appropriate antibiotics: 25 μ g/mL chloramphenicol, 100 μ g/mL ampicillin, and 50 μ g/mL 2,6-diaminopimelic acid. For counterselecting mutant constructs

via the *sacB* gene system, NaCl-free LB agar supplemented with 12.5% (w/v) sucrose was used.

2.2 Construction of the *S. Choleraesuis* mutant and complemented strains

Twenty-two *S. Choleraesuis* mutants were generated via allelic exchange, employing the suicide plasmid pRE112 as previously detailed (20). The primer sequences designed for gene deletion or complementation in *S. Choleraesuis* strains are detailed in [Supplementary Table S3](#). To generate the $\Delta sseJ$ mutant, upstream and downstream homologous arms were PCR-amplified using primer pairs DsseJ-1F/1R and DsseJ-2F/2R, respectively. These PCR products were joined by overlap PCR and subsequently cloned and inserted into the suicide vector pRE112 (21) through seamless cloning generating plasmid pRE112- $\Delta sseJ$, which carries a deletion of the entire *sseJ* gene. The pRE112- $\Delta sseJ$ plasmid was subsequently introduced into the WT strain via conjugation. This process involved chloramphenicol-mediated positive selection and *sacB*-mediated sucrose sensitivity screening for the generation of the markerless mutant strain $\Delta sseJ$. Furthermore, to complement *sseJ* gene in the $\Delta sseJ$, the coding sequence of *sseJ* were amplified with the primer *sseJ*-F/R. Then, the PCR product was inserted into the plasmid of pCZb1 (20) via a Seamless Cloning Kit (Sangon Biotech, Shanghai, China), generating plasmid pCZb1-*sseJ*. Following, the recombinant plasmid was transformed into the mutant strain $\Delta sseJ$ to construct the complemented strain named C- $\Delta sseJ$. The same method was applied to constructions of other gene mutants and complemented strains.

2.3 Detection of growth curves of *S. Choleraesuis* strains

The *S. Choleraesuis* WT and mutant strains were inoculated in 5 mL of LB broth at 37°C with shaking at 180 rpm/min overnight. The following day, cultures of each strain were normalized to an OD₆₀₀ of 0.05 and then cultured in LB broth at 37°C with shaking at 180 rpm/min. The OD₆₀₀ of each culture was measured every 2 h for 12 h.

2.4 Swimming assay

The swimming motility phenotypes of wild-type (WT) and mutant bacterial strains were evaluated using a previously described protocol (22). Cultures of each strain were grown in LB broth to an optical density (OD₆₀₀) of 0.6–0.8. Bacteria were harvested by centrifugation, washed twice with PBS, and resuspended in the same buffer. Subsequently, 3 μ L of the bacterial suspension was applied as droplets to LB agar plates containing 0.25% agar. After incubation at 37°C for 6 h, the diameter of the bacterial migration zone was measured to assess swimming motility.

2.5 Adhesion, invasion and intracellular survival of *S. Choleraesuis* in macrophages

RAW264.7 macrophages were plated at a density of 5×10^5 cells per well in 24-well plates containing DMEM (Gibco, NY, USA) supplemented with 10% FBS (Tian Hang, Hangzhou, China) and 1% penicillin-streptomycin. *S. Choleraesuis* WT or mutant strains were added at a multiplicity of infection (MOI) of 100. Following a 30-min incubation in a 5% CO₂ at 37°C incubator to facilitate bacterial adhesion, cell monolayers were washed thrice with PBS to remove non-adherent bacterial cells. The adherent bacteria were then released by lysing the cells with 0.2% Triton X-100, and their numbers were enumerated via serial dilution and colony counting.

For the invasion assay, after the 30-min adhesion step, fresh DMEM was added and cells were incubated for an additional 90 min at 37°C under 5% CO₂. After incubation, the supernatant was discarded, cells were washed twice with PBS and lysed using 0.2% Triton X-100 to enumerate intracellular bacteria.

For intracellular survival analysis, following the invasion step, DMEM supplemented with 10 ng/mL gentamicin was used to eliminate extracellular bacteria. $T = 0$ h was defined as the initial time point following invasion. At designated time points ($T = 0$ h and 24 h), serial dilutions of the resulting cell lysates were then plated onto MacConkey agar (Coolaber, Beijing, China) plates for bacterial enumeration and incubated at 37°C for 24 h to count colony-forming units (CFUs).

2.6 Colonization and virulence of *S. Choleraesuis* mutant strains in mice

Female Kunming mice (6 weeks old) were procured from Dashuo Experimental Animal Ltd. (Chengdu, China) and underwent a 1-week acclimation period before experimental procedures. Bacterial colonization and virulence phenotypes were evaluated using methodologies reported in prior studies (20, 22). Ten mice were orally inoculated with PBS or approximately 1×10^8 CFU of *S. Choleraesuis* WT strain or each mutant strain. Then, spleens and livers were collected from 5 mice at 6 days post-infection. The samples were weighed, ground in PBS, and the bacterial suspensions were serially diluted and spread onto MacConkey agar (Coolaber, Beijing, China) to enumerate viable CFUs, which were expressed as log₁₀ CFU/g. The remaining 5 mice in each group were observed for survival for 1 month after infection.

2.7 Measurement of 50% Lethal dose (LD₅₀) of the Δ sseL Δ sopD₂ Δ steA strain

The LD₅₀ of the Δ sseL Δ sopD₂ Δ steA was determined as previously described (23). 10-fold serial dilutions of the CFU of the Δ sseL Δ sopD₂ Δ steA strain were orally inoculated into groups of Kunming mice ($n = 5$ /per dose). Animals were

monitored daily for 30 days post-infection to assess survival rates. The median lethal dose (LD₅₀) was determined using the Reed-Muench method. To ensure humane endpoints, mice exhibiting severe distress—characterized by labored breathing, tremors, unresponsiveness to tactile stimuli, or inability to access food/water—were humanely euthanized via CO₂ inhalation. Deceased animals were immediately subjected to sterilization, sealed in biohazard bags, and transferred to the Sichuan Agricultural University Laboratory Animal Center for compliant biosafety disposal.

2.8 Immunization and challenge

Female Kunming mice (6–8 weeks old) were randomly divided into three groups ($n = 20$ /group). The experimental group received an oral gavage of 1×10^9 CFU Δ sseL Δ sopD₂ Δ steA in 200 μ L PBS, while the control group received an equal volume of PBS alone. A booster immunization was administered on day 14 using the same protocol. Serum samples were collected via retro-orbital bleeding on days 7 and 21 from six randomly selected mice per group. On day 42, all mice were orally challenged with 10-fold LD₅₀ of *S. Choleraesuis* CVCC2139. Five mice per group were euthanized on day 6 post-challenge, and samples from the spleen and liver were collected for measurement of bacterial loads. Survival of the remaining mice was monitored and recorded daily for 30 days.

2.9 Enzyme-linked immunosorbent assay (ELISA)

Antibody titers against inactivated *S. Choleraesuis* antigens were quantified using an indirect ELISA protocol as previously described (24). In brief, 100 μ L of 10^9 CFU/ml of the heat-killed *S. Choleraesuis* antigens was added to wells of a 96-well ELISA plate coated with antigen with overnight incubation at 4°C. The next day, the plates were washed three times with PBST followed by blocking with 5% BSA (BD, San Diego, CA) in PBS at 37°C for 2 h. Following antigen coating and blocking, the plate was washed three times with PBST. Serum samples, diluted 1:200 in blocking buffer (5% BSA in PBS), were added to each well (100 μ L/well) and incubated at 37°C for 1 h in a humidified chamber. The plate was then washed five times with PBST to remove unbound antibodies. Subsequently, 100 μ L of HRP-conjugated goat anti-mouse IgG (Abclonal, Wuhan, China), diluted 1:5,000 in antibody diluent, was added to each well and incubated at 37°C for 1 h. After five additional PBST washes, the plate was ready for substrate development. 100 μ L of TMB substrate solution (Macgene, Shanghai, China) was added, and the plates were incubated in the dark at 25°C for 10 min. After adding 50 μ L of 2 M H₂SO₄ to stop the reaction, absorbance was measured at 450 nm using a Bio-Rad microplate reader (Bio-Rad, California, USA).

2.10 Ethics statement

All animal procedures were conducted in strict adherence to the Guide for the Care and Use of Laboratory Animals published by China's Ministry of Science and Technology. The study protocol was approved by the Animal Ethics Committee of Sichuan Agricultural University and the Sichuan Laboratory Animal Management Committee (permit number: SYXK2019-187), ensuring compliance with national and institutional welfare guidelines.

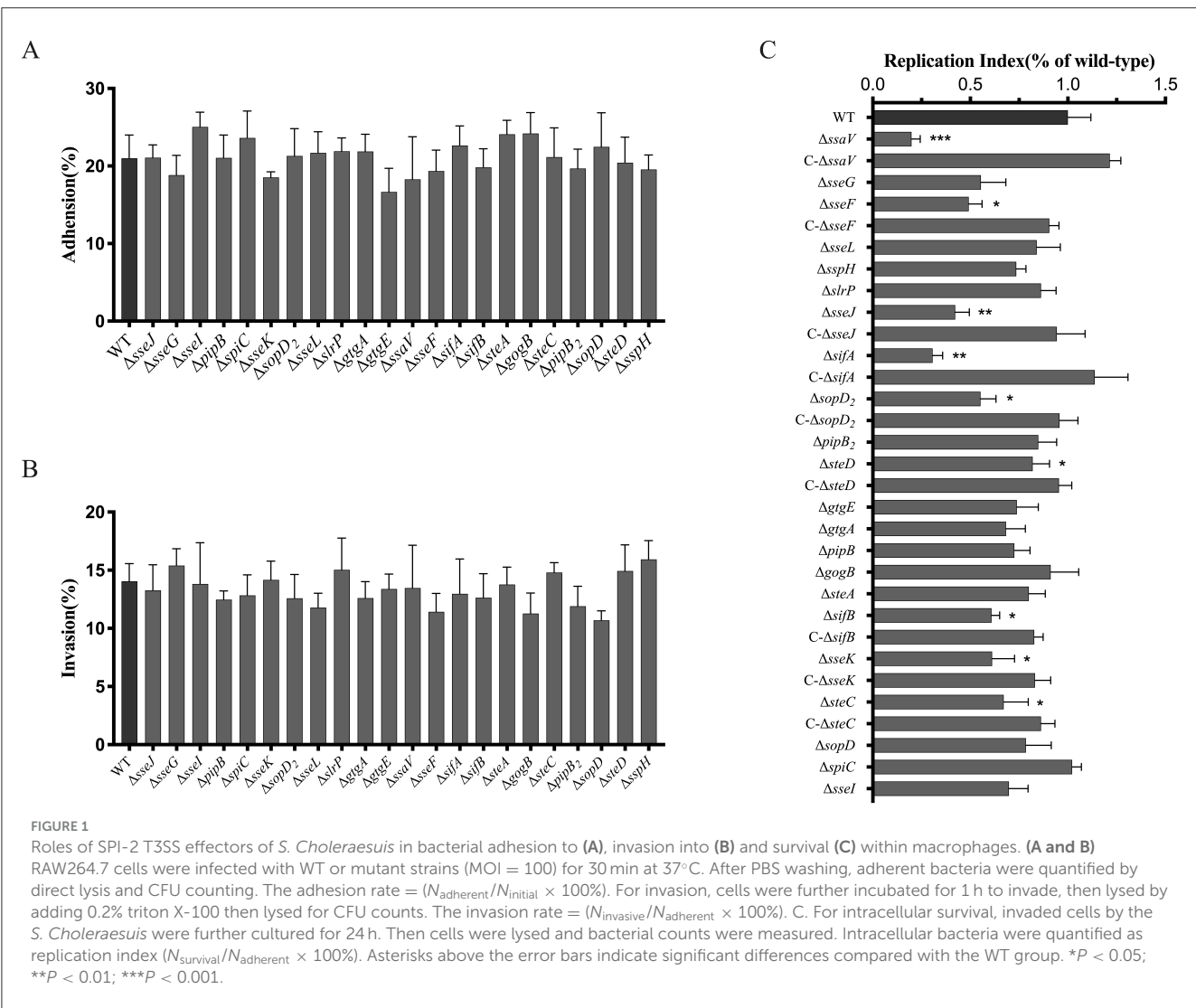
2.11 Statistical analysis

Data are presented as the mean \pm standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* multiple-comparison test with GraphPad Prism software (GraphPad Software, California, USA). Statistical significance was defined as $P < 0.05$. All *in vitro* experiments were independently repeated three times to ensure reproducibility.

3 Results

3.1 Roles of SPI-2 T3SS effectors of *S. Choleraesuis* in bacterial adhesion to, invasion into and survival within macrophages

Twenty-one effector genes (*sseJ*, *sseG*, *slrP*, *sseF*, *gtgE*, *gogB*, *sspH*, *gtgA*, *sifA*, *sifB*, *sseK*, *steA*, *steC*, *sseL*, *sopD*₂, *spiC*, *sseI*, *pipB*, *pipB*₂, *sopD*, *steD*) were screened and each of them was deleted from the WT *S. Choleraesuis* strain CVCC2139. The *ssaV* mutant strain (Δ *ssaV*) was also constructed as a positive control as the *SsaV* is a structural component forming the inner ring of the SPI-2 T3SS injectosome that is involved in effector protein translocation (25). Then, the mutant strains were compared to the WT strain in terms of the ability of bacteria to adhere to, invade and survive within RAW264.7 macrophages. Deletion of the SPI-2 T3SS effector genes neither affected bacterial adhesion to nor changed bacterial invasion into macrophages (Figures 1A, B). Nevertheless, bacterial replication in the mutant strain Δ *ssaV*, Δ *sseF*, Δ *sseJ*,



$\Delta sifB$, $\Delta sseK$, $\Delta sifA$, $\Delta sopD_2$, $\Delta steC$, or $\Delta steD$ was significantly decreased compared to that in the WT strain, while loss of either of the other 13 genes had no adverse effects (Figure 1C). Gene complementation *in trans* in the mutant strains fully or partially restored the WT phenotypes (Figure 1C). This finding indicated that effector genes including *sseF*, *sseJ*, *sifB*, *sseK*, *sifA*, *sopD_2*, *steC*, and *steD* promoted bacterial survival in macrophages.

3.2 Roles of SPI-2 T3SS effectors of *S. Choleraesuis* in bacterial growth and swimming

The 21 *S. Choleraesuis* mutant strains were subjected to detection of growth curves and swimming. Compared to the WT strain, the growth rates of the $\Delta gtgA$, $\Delta steA$, $\Delta spiC$, $\Delta sopD_2$, $\Delta slrP$, $\Delta pipB_2$, $\Delta sopD$, and $\Delta steD$ strains were significantly reduced in LB broth at 37°C (Figure 2A). In contrast, the remaining 14 mutants exhibited similar growth rates to the WT strain (Figures 2B, C). This finding suggested that the effector genes *gtgA*, *steA*, *spiC*,

sopD_2, *slrP*, *pipB_2*, *sopD*, and *steD* promotes the *in vitro* growth of *S. Choleraesuis*. Also, swimming of the $\Delta sseJ$, $\Delta slrP$, $\Delta sifB$, and $\Delta sopD_2$ mutants were significantly enhanced compared to that of the WT strain, whereas the $\Delta pipB_2$ and $\Delta sspH$ strains exhibited reduced motility (Figure 2D). Complementation of *sseJ*, *slrP*, *sifB*, *sopD_2*, *pipB_2* and *sspH* in corresponding mutants restored WT swimming phenotype. Therefore, the effector genes *sseJ*, *slrP*, *sifB*, and *sopD_2* restrain the swimming ability of *S. Choleraesuis*, whereas *sspH* and *pipB_2* positively influence motility.

3.3 Roles of SPI-2 T3SS effectors of *S. Choleraesuis* in bacterial virulence in mice

To determine roles of the SPI-2 T3SS effector in virulence of *S. Choleraesuis*, mice were orally administered with 1×10^8 CFU of the WT strain or each of the mutant strains. The survival of mice was monitored for 1 month. The WT strain led to 40% of survival post-infection, while the five strains including $\Delta spiC$, $\Delta ssaV$, $\Delta sseL$, $\Delta sopD_2$, and $\Delta steA$ caused no death (Table 1). Also, $\Delta gtgA$, $\Delta gtgE$, $\Delta sseF$, $\Delta sifA$, and $\Delta sspH$ resulted in increased

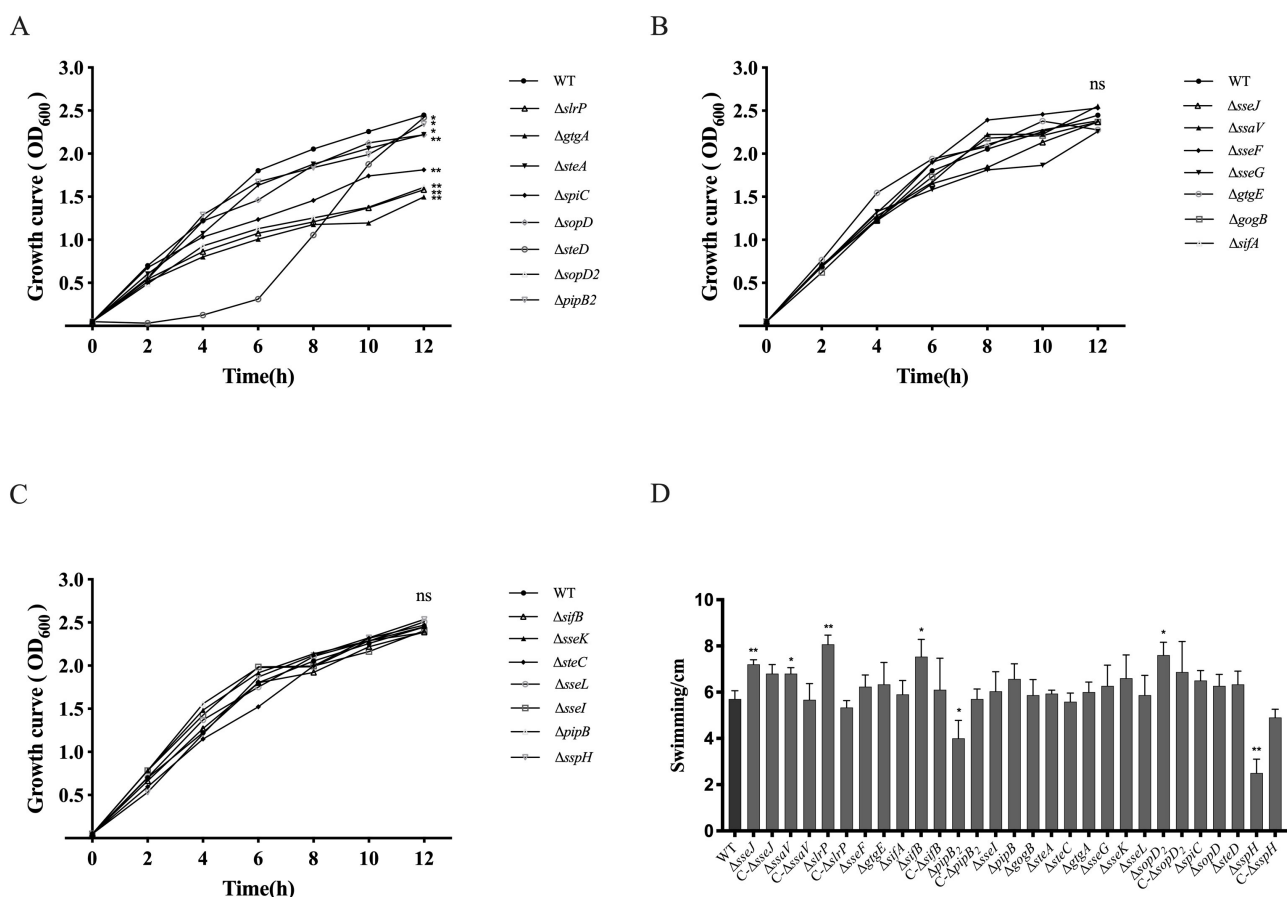


FIGURE 2

Detection of growth curves and swimming of *S. Choleraesuis* strains. (A–C) Growth curves of *S. Choleraesuis* WT and mutant strains were analyzed by measuring OD₆₀₀ every 2 h for 12 h. (D) Swimming assay. The WT strain, 22 mutant strains and complemented strains were cultured in LB to OD₆₀₀ = 0.6–0.8, harvested and suspended in PBS. Three microliters of bacterial suspension were spotted onto the center of a soft agar plate. Then, colony diameters were measured after 6 h of incubation at 37°C. An asterisk above the error bar shows a significant difference from the WT group.

* $P < 0.05$; ** $P < 0.01$.

survival (80%), by contrast, all mice succumbed to the $\Delta steD$ infection. The remaining strains caused a 40% or 60% survival (Table 1). Thus, SPI-2 T3SS effector genes *sseL*, *sopD₂*, and *spiC*, and the gene *ssaV* contributed remarkably to *S. Choleraesuis* virulence in mice, functioning as virulence genes. To further detect the roles of the five virulence genes in bacterial colonization, mice were inoculated with 10⁸ CFU of the WT or mutant strains, then bacterial loads in liver and spleen were measured 6 days post-infection. No bacteria were recovered from the $\Delta spiC$ and $\Delta ssaV$ groups. However, the bacterial loads of $\Delta sseL$, $\Delta sopD_2$, and $\Delta steA$ in liver and spleen tissues were comparable to those of the wild-type strain (Figures 3A, B). This finding suggests that the *spiC* and *ssaV* contribute to the colonization of *S. Choleraesuis* in the liver and spleen of mice, while *sseL*, *sopD₂*, and *steA* are not involved in bacterial colonization.

3.4 Virulence and colonization of the triple mutant strain $\Delta sseL\Delta sopD_2\Delta steA$ of *S. Choleraesuis* mutants in mice

Although the two genes *spiC* and *ssaV* play a significant role in bacterial virulence, their mutant strains lost colonization ability in mice, hinting their poor immunogenicity. To develop a suitable live attenuated strain, the other three virulence genes *sseL*, *sopD₂*, and *steA* were deleted from the WT strain simultaneously, generating a triple mutant $\Delta sseL\Delta sopD_2\Delta steA$. This strain colonized of the spleen and liver at a significantly lower level than the WT strain post oral infection (Figure 4). Also, the LD₅₀ of the triple mutant was $>1.65 \times 10^{10}$ CFU, demonstrating at least a 150-fold reduction in virulence compared with that of the wild-type strain with the LD₅₀ of 1.08×10^8 CFU (Table 2).

3.5 Protection efficacy of the triple mutant strain $\Delta sseL\Delta sopD_2\Delta steA$

To evaluate the vaccine potential of the attenuated strain $\Delta sseL\Delta sopD_2\Delta steA$, mice were orally administered with 10⁹ CFU of the vaccine strain twice with an interval of 14 days and then were challenged orally with a lethal dose of the *S. Choleraesuis* WT strain 28 days post-second immunization. Immunization with the $\Delta sseL\Delta sopD_2\Delta steA$ induced significantly higher serum IgG responses to whole bacterial antigens than with the PBS group on Day 7 and 21 post-immunization (Figure 5A). Following the challenge, the bacterial loads in the spleen and liver of the $\Delta sseL\Delta sopD_2\Delta steA$ group were significantly lower than those in the PBS group (Figures 5B, C). Furthermore, all the mice in the PBS control group died, whereas 40% of the mice in the immunized group survived (Figure 5D). Thus, immunization with the $\Delta sseL\Delta sopD_2\Delta steA$ strain induced a robust antibody response, significantly reduced the tissue loads of the challenge strain, and provided 40% protection efficacy against lethal *S. Choleraesuis* infection.

TABLE 1 Survival rates of mice infected with *S. Choleraesuis* strains.

Strains	Challenge dose (CFU)	Survival	Survival rate
WT	10 ⁸	2/5	40%
$\Delta spiC$		5/5	100%
$\Delta sseL$		5/5	100%
$\Delta sopD_2$		5/5	100%
$\Delta steA$		5/5	100%
$\Delta ssaV$		5/5	100%
$\Delta slrP$		3/5	60%
$\Delta gogB$		3/5	60%
$\Delta sseJ$		2/5	40%
$\Delta sopD$		3/5	60%
$\Delta sseI$		2/5	40%
$\Delta gtgA$		4/5	80%
$\Delta gtgE$		4/5	80%
$\Delta sseG$		2/5	40%
$\Delta sseF$		4/5	80%
$\Delta sifA$		4/5	80%
$\Delta sifB$		3/5	60%
$\Delta sseK$		3/5	60%
$\Delta pipB$		2/5	40%
$\Delta steC$		3/5	60%
$\Delta pipB_2$		2/5	40%
$\Delta steD$		0/5	0%
$\Delta sspH$		4/5	80%
PBS		5/5	100%

4 Discussion

Our comprehensive analysis of SPI-2 T3SS effectors in *S. Choleraesuis* reveals multifaceted roles of individual effectors in intracellular survival, systemic virulence, motility, and growth, offering mechanistic insights into how this pathogen adapts to host defenses and establishes infection.

None of detected SPI-2 T3SS effectors was involved in the adhesion and invasion process of *S. Choleraesuis* to the macrophage RAW264.7, which is in line with previous studies on *S. Typhimurium* (26–29). We also observed notable effects of effectors on bacterial motility and growth. Several mutants exhibited reduced growth rates or altered swimming motility, indicating that SPI-2 effectors also influence global physiological fitness. Such effects may be mediated via metabolic reprogramming or indirect transcriptional regulation. For instance, *pipB₂* has been shown to alter host cytoskeletal tension and organelle dynamics, which may feed back to bacterial stress signaling (30). Reduced motility may compromise their ability to penetrate mucus layers or disseminate systemically, further contributing to attenuation (31).

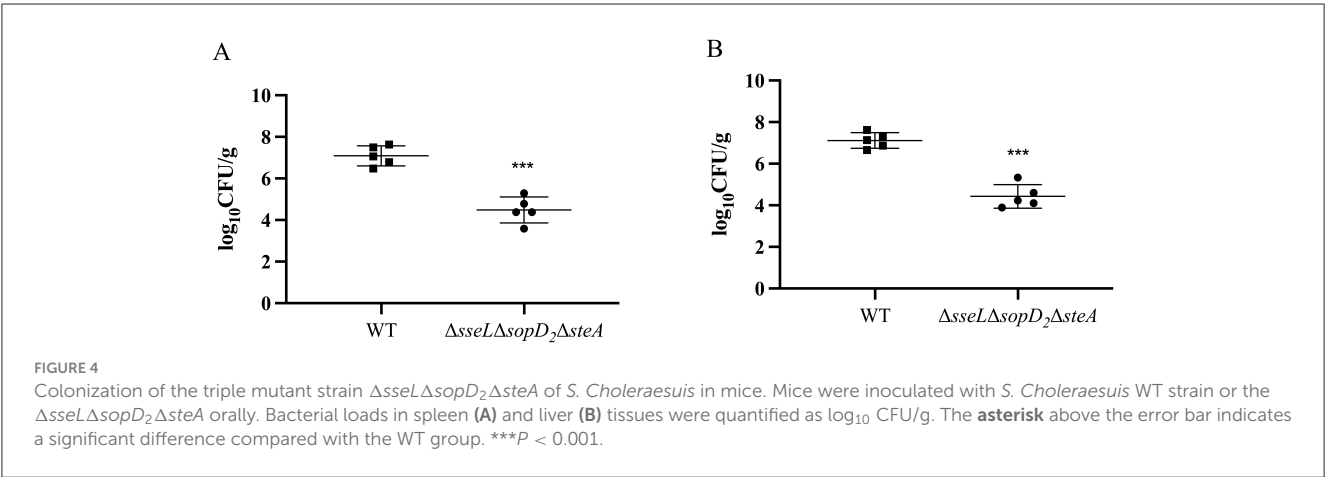
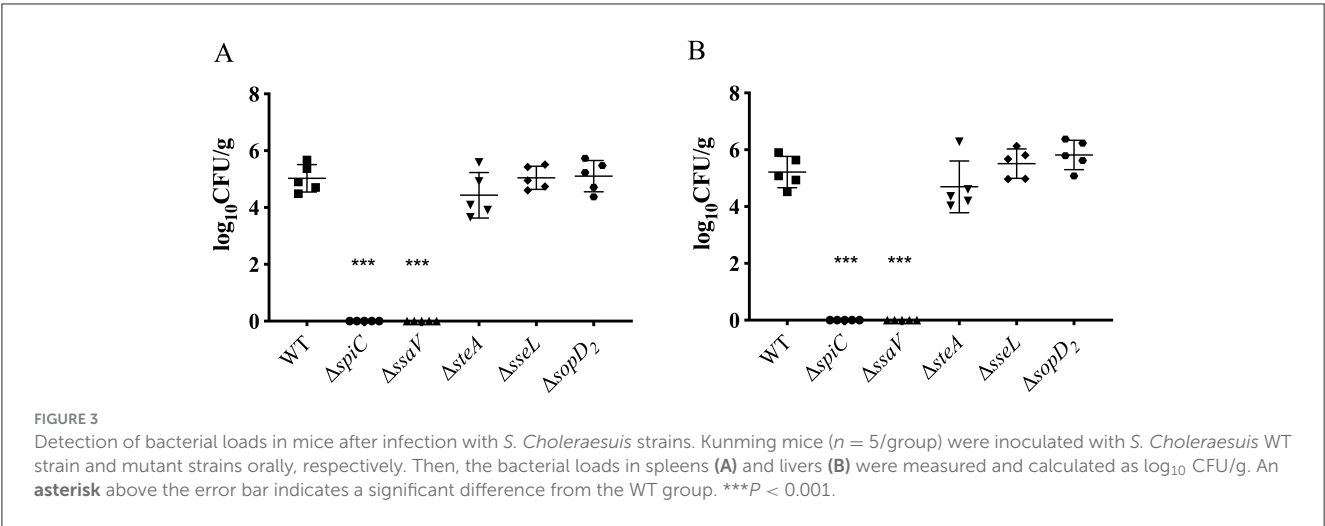


TABLE 2 The LD₅₀ of the *S. Choleraesuis* strains.

Strains	Challenge dose (CFU) and death					LD ₅₀ (CFU)
	10 ⁶	10 ⁷	10 ⁸	10 ⁹	10 ¹⁰	
WT	0/5	2/5	3/5	5/5	5/5	1.08 × 10 ⁸
$\Delta sseL\Delta sopD_2\Delta steA$	0/5	0/5	0/5	0/5	1/5	>1.65 × 10 ¹⁰
PBS					0/5	

The *spiC* mutant strain of *S. Enteritidis* exhibits stronger swimming ability (32); whereas deletion of the *spiC* of *S. Choleraesuis* did not affect swimming. These contradictions suggested that some effector molecules exhibit functional heterogeneity across different *Salmonella* serovars.

Eight effector genes *sseF*, *sseJ*, *sifB*, *sseK*, *sifA*, *sopD₂*, *steC*, and *steD* significantly enhanced the survival of *S. Choleraesuis* in macrophages, which highlights the core function of SPI-2 T3SS effector in maintaining intracellular survival and is largely consistent with previous studies. Most of these effectors are known to modulate SCV maturation, membrane dynamics, or host trafficking pathways, helping bacteria to evade lysosomal

degradation and acquire nutrients. For instance, *sifA* stabilizes the SCV membrane and recruits kinesin-1 via SKIP (33), while *sseF* and *sseG* facilitate microenvironment construction (34). The involvement of *sopD₂* and *steD* suggests a multi-effector strategy to subvert host immunity: *sopD₂* interferes with Rab GTPase-mediated trafficking (35), while *steD* downregulates MHC II surface expression through host ubiquitination machinery (36). However, *SteC* of *S. Typhimurium* barely affects bacterial proliferation in macrophages (37). Interestingly, *steA* and *pipB₂*, although previously implicated in vacuole positioning and actin remodeling (30, 38), had minimal impact on intracellular survival in *S. Choleraesuis*, hinting at possible functional redundancy or compensation by other effectors in this serovar.

All the effectors associated with intracellular survival except for *sseJ* and *steD* contribute to the virulence of *S. Choleraesuis* in mice, while three virulence determinants (*spiC*, *sseL*, and *steA*) were not related to bacterial intracellular survival. A previous study also found that protein SseL was shown to enhance the virulence of *S. Pullorum* by suppressing host NF- κ B signaling but not affect the intracellular bacterial survival (39). These findings indicated a lack of strong correlation between intracellular replicative capacity and systemic virulence.

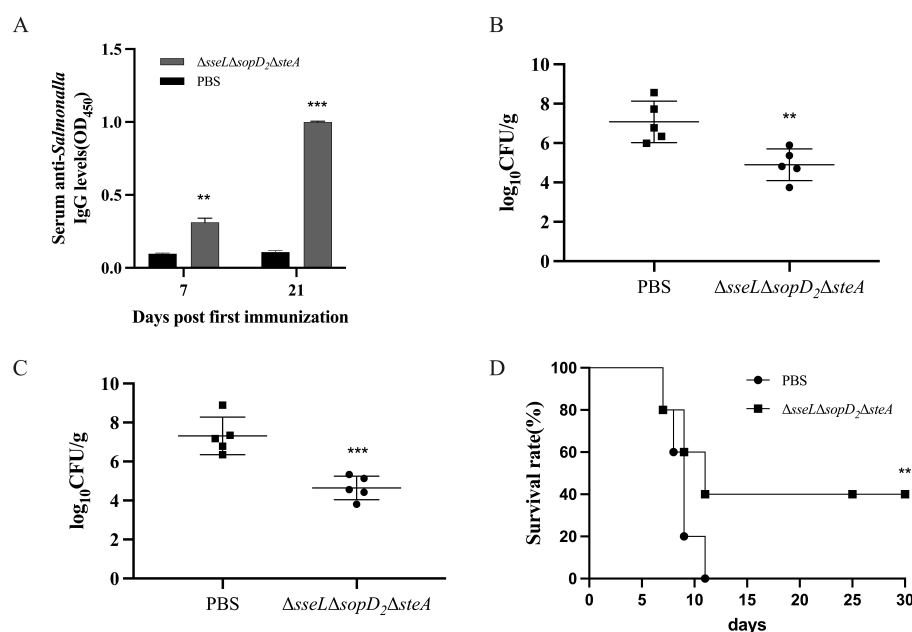


FIGURE 5

Antibody responses and protection efficacy induced by the triple mutant strain $\Delta sseL\Delta sopD_2\Delta steA$. Mice were immunized orally with the attenuated strain $\Delta sseL\Delta sopD_2\Delta steA$ twice and then challenged with the *S. Choleraesuis* WT strain 28 days post-second immunization. Indirect ELISA was used to detect serum IgG levels against whole bacterial antigens 7 days and 21 days post-immunization (A). Bacterial loads in the spleen (B) and liver (C) were measured 6 days post-challenge. (D) Survival rates of mice were monitored over a 30-day period after challenge. Asterisks above the error bars indicate significant differences compared with the PBS group. ** $P < 0.01$; *** $P < 0.001$.

Construction of live attenuated bacterial vaccines should balance the virulence and immunogenicity (40). However, we found that the $\Delta spiC$ of *S. Choleraesuis* as well as the $\Delta ssaV$ was avirulent and colonized mouse liver or spleen at levels below the threshold of detection. Too-low bacterial loads in tissues imply low immunogenicity (41). Therefore, the two strains were not considered as vaccine candidates in our study. Compared to the currently licensed live attenuated vaccine strain C500 in China, which was derived through chemical mutagenesis and has been used for over four decades, the $\Delta sseL\Delta sopD_2\Delta steA$ strain developed in this study presents both advantages and limitations. C500 has demonstrated high protection efficacy in piglets but suffers from residual virulence and has an undefined genetic background, which raise biosafety concerns and hinder its broader application, particularly in immunocompromised hosts (10). In contrast, the triple mutant strain constructed here is genetically defined and rationally attenuated by deletion of three characterized virulence genes, thereby reducing the risk of reversion and enhancing safety. However, immunization with $\Delta sseL\Delta sopD_2\Delta steA$ conferred only moderate protection (40%) against lethal challenge in mice, which is lower than the reported protection level of C500 or other strains such as the $\Delta spiC$ mutant of *S. Pullorum* that offered more than 90% protection (18). This limited efficacy may be attributed to its moderate tissue colonization and immunogenicity. Therefore, further optimization is required, such as incorporating additional immunostimulatory mutations or adjuvant delivery strategies, to enhance both antigen persistence and immune protection.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The animal studies were approved by the study protocol was approved by the Animal Ethics Committee of Sichuan Agricultural University and the Sichuan Laboratory Animal Management Committee (permit number: SYXK2019-187). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

RX: Writing – original draft, Investigation. XJ: Writing – original draft, Investigation. JL: Writing – review & editing, Investigation. DZ: Investigation, Writing – review & editing. ML: Writing – review & editing, Investigation. MW: Writing – review & editing, Investigation. RJ: Writing – review & editing, Investigation. SC: Investigation, Writing – review & editing. QY: Investigation, Writing – review & editing. YW: Validation, Writing – review & editing. SZ: Writing – review & editing, Validation. JH: Validation, Writing – review & editing. XO: Writing – review & editing, Validation. DS: Validation, Writing – review & editing. BT:

Validation, Writing – review & editing. YH: Formal analysis, Writing – review & editing. ZW: Writing – review & editing, Formal analysis. AC: Project administration, Writing – review & editing. XZ: Project administration, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This research was supported by the National Key Research and Development Program of China (2023YFD1800200), the Sichuan Veterinary Medicine and Drug Innovation Group of the China Agricultural Research System (SCCXTD-2021-18) and the Earmarked Fund for China Agriculture Research System (CARS-42-17).

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.

References

- Li H, Wu Y, Feng D, Jiang Q, Li S, Rong J, et al. Centralized industrialization of pork in Europe and America contributes to the global spread of salmonella enterica. *Nat Food*. (2024) 5:413–22. doi: 10.1038/s43016-024-00968-1
- Eddicks M, Hausleitner R, Eddicks L, Blutke A, Straubinger RK, Wolf G, et al. [detection of salmonella choleraesuis var. Kunzendorf in a fattening pig with septicemic salmonellosis A case report]. *Tierarztl Prax Ausg G Grosstiere Nutztiere*. (2016) 44:381–7. doi: 10.15653/TPG-160300
- Longo A, Losasso C, Vitulano F, Mastroianni E, Turchetto S, Petrin S, et al. Insight into an outbreak of salmonella choleraesuis var. Kunzendorf in wild boars *Vet Microbiol*. (2019) 238:108423. doi: 10.1016/j.vetmic.2019.108423
- Hajra D, Nair AV, Chakravorty D. Decoding the invasive nature of a tropical pathogen of concern: The invasive non-typhoidal salmonella strains causing host-restricted extraintestinal infections worldwide. *Microbiol Res*. (2023) 277:127488. doi: 10.1016/j.micres.2023.127488
- Chiu C-H, Su L-H, Chu C. Salmonella enterica serotype choleraesuis: Epidemiology, pathogenesis, clinical disease, and treatment. *Clin Microbiol Rev*. (2004) 17:311–22. doi: 10.1128/CMR.17.2.311-322.2004
- Luk-In S, Chatsuan T, Pulsrikarn C, Bangtrakulnonth A, Rirerm U, Kulwichit W. High prevalence of ceftriaxone resistance among invasive salmonella enterica serotype choleraesuis isolates in Thailand: The emergence and increase of CTX-M-55 in ciprofloxacin-resistant *S. Choleraesuis* isolates *Int J Med Microbiol*. (2018) 308:447–53. doi: 10.1016/j.ijmm.2018.03.003
- Jacqueline C, Samper-Cataviela C, Monzon Fernandez S, Ugarte-Ruiz M, Cuesta de la Plaza I, Alvarez J, et al. Phenotypic and genetic characterization of antimicrobial resistance in salmonella enterica serovar choleraesuis isolates from humans and animals in Spain from 2006 to 2021. *J Antimicrob Chemother*. (2024) 79:790–800. doi: 10.1093/jac/dkac022
- Alvarez DM, Barrón-Montenegro R, Conejeros J, Rivera D, Undurraga EA, Moreno-Switt AI, et al. review of the global emergence of multidrug-resistant salmonella enterica subsp. enterica serovar infantis. *Int J Food Microbiol*. (2023) 403:110297. doi: 10.1016/j.jfoodmicro.2023.110297
- Plotkin SA, Plotkin SL. The development of vaccines: How the past led to the future. *Nat Rev Microbiol*. (2011) 9:889–93. doi: 10.1038/nrmicro2668
- Zhu L, Zhao X, Yin Q, Liu X, Chen X, Huang C, et al. Mucosal IgA and IFN- γ + CD8 T cell immunity are important in the efficacy of live salmonella enterica serovar choleraesuis vaccines. *Sci Rep*. (2017) 7:46408. doi: 10.1038/srep46408
- Ji Z, Shang J, Li Y, Wang S, Shi H. Live attenuated salmonella enterica serovar choleraesuis vaccine vector displaying regulated delayed attenuation and regulated delayed antigen synthesis to confer protection against streptococcus suis in mice. *Vaccine*. (2015) 33:4858–67. doi: 10.1016/j.vaccine.2015.07.045

Generative AI statement

The author(s) declare that no Gen AI was used in the creation of this manuscript.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2025.1637327/full#supplementary-material>

- Deng W, Marshall NC, Rowland JL, McCoy JM, Worrall LJ, Santos AS, et al. Assembly, structure, function and regulation of type III secretion systems. *Nat Rev Microbiol*. (2017) 15:323–37. doi: 10.1038/nrmicro.2017.20
- Miao EA, Warren SE. Innate immune detection of bacterial virulence factors via the NLR4 inflammasome. *J Clin Immunol*. (2010) 30:502–6. doi: 10.1007/s10875-010-9395-6
- Yu X-J, Xie H, Li Y, Liu M, Hou R, Predeus AV, et al. Modulation of salmonella virulence by a novel SPI-2 injectisome effector that interacts with the dystrophin-associated protein complex. *MBio*. (2024) 15:e0112824. doi: 10.1128/mbio.01128-24
- Yu X-J, Liu M, Holden DW. Salmonella effectors SseF and SseG interact with mammalian protein ACBD3 (GCP60) to anchor salmonella-containing vacuoles at the Golgi network. *MBio*. (2016) 7:e00474–16. doi: 10.1128/mBio.00474-16
- Miao EA, Brittnacher M, Haraga A, Jeng RL, Welch MD, Miller SI. Salmonella effectors translocated across the vacuolar membrane interact with the actin cytoskeleton. *Mol Microbiol*. (2003) 48:401–15. doi: 10.1046/j.1365-2958.2003.03438.x
- Méresse S, Unsworth KE, Habermann A, Griffiths G, Fang F, Martínez-Lorenzo MJ, et al. Remodelling of the actin cytoskeleton is essential for replication of intravacuolar Salmonella. *Cell Microbiol*. (2001) 3:567–77. doi: 10.1046/j.1462-5822.2001.00141.x
- Wang Y, Huang C, Tang J, Liu G, Hu M, Kang X, et al. Salmonella pullorum spiC mutant is a desirable LASV candidate with proper virulence, high immune protection and easy-to-use oral administration. *Vaccine*. (2021) 39:1383–91. doi: 10.1016/j.vaccine.2021.01.059
- Rubirés X, Saigi F, Piqué N, Climent N, Merino S, Albertí S, et al. gene (wbbL) from Serratia marcescens N28b (O4) complements the rfb-50 mutation of Escherichia coli K-12 derivatives. *J Bacteriol*. (1997) 179:7581–6. doi: 10.1128/jb.179.23.7581-7586.1997
- Zhao X, Dai Q, Jia R, Zhu D, Liu M, Wang M, et al. Two novel salmonella bivalent vaccines confer dual protection against two salmonella serovars in mice. *Front Cell Infect Microbiol*. (2017) 7:391. doi: 10.3389/fcimb.2017.00391
- Edwards RA, Keller LH, Schifferli DM. Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression. *Gene*. (1998) 207:149–57. doi: 10.1016/S0378-1119(97)00674-5
- Zhao X, Dai Q, Zhu D, Liu M, Chen S, Sun K, et al. Recombinant attenuated salmonella typhimurium with heterologous expression of the salmonella choleraesuis O-polysaccharide: High immunogenicity and protection. *Sci Rep*. (2017) 7:7127. doi: 10.1038/s41598-017-07554-3
- Zhao X, Yang F, Shen H, Liao Y, Zhu D, Wang M, et al. Immunogenicity and protection of a pasteurized strain with a truncated lipopolysaccharide outer core in ducks. *Vet Res*. (2022) 53:17. doi: 10.1186/s13567-022-01008-w

24. Zhao X, Zeng X, Dai Q, Hou Y, Zhu D, Wang M, et al. Immunogenicity and protection efficacy of a *Salmonella enterica* serovar Typhimurium *fur*, *arcA* and *fliC* mutant. *Vaccine*. (2021) 39:588–95. doi: 10.1016/j.vaccine.2020.11.070
25. Yu X-J, Grabe GJ, Liu M, Mota LJ, Holden DW. SsaV interacts with SsaL to control the translocon-to-effector switch in the salmonella SPI-2 type three secretion system. *MBio*. (2018) 9:e01149–18. doi: 10.1128/mBio.01149-18
26. Fulde M, van Vorst K, Zhang K, Westermann AJ, Busche T, Huei YC, et al. SPI2 T3SS effectors facilitate enterocyte apical to basolateral transmigration of salmonella-containing vacuoles *in vivo*. *Gut Microbes*. (2021) 13:1973836. doi: 10.1080/19490976.2021.1973836
27. Naseer N, Egan MS, Reyes Ruiz VM, Scott WP, Hunter EN, Demissie T, et al. Human NAIP/NLRC4 and NLRP3 inflammasomes detect salmonella type III secretion system activities to restrict intracellular bacterial replication. *PLoS Pathog*. (2022) 18:e1009718. doi: 10.1371/journal.ppat.1009718
28. Zhang K, Riba A, Nietschke M, Torow N, Repnik U, Pütz A, et al. Minimal SPI1-T3SS effector requirement for salmonella enterocyte invasion and intracellular proliferation *in vivo*. *PLoS Pathog*. (2018) 14:e1006925. doi: 10.1371/journal.ppat.1006925
29. Gulati A, Shukla R, Mukhopadhyaya A. Salmonella effector SteA suppresses proinflammatory responses of the host by interfering with I κ B degradation. *Front Immunol*. (2019) 10:2822. doi: 10.3389/fimmu.2019.02822
30. Mandal K. Review of PIP2 in Cellular Signaling, Functions and Diseases. *Int J Mol Sci*. (2020) 21:8342. doi: 10.3390/ijms21218342
31. Ottemann KM, Miller JF. Roles for motility in bacterial-host interactions. *Mol Microbiol*. (1997) 24:1109–17. doi: 10.1046/j.1365-2958.1997.4281787.x
32. Wang Y, Liu G, Zhang J, Gu D, Hu M, Zhang Y, et al. WbaP is required for swarm motility and intramacrophage multiplication of salmonella enteritidis *spiC* mutant by glucose use ability. *Microbiol Res*. (2021) 245:126686. doi: 10.1016/j.micres.2021.126686
33. Dumont A, Boucrot E, Drevensek S, Daire V, Gorvel J-P, Poüs C, et al. the host target of the salmonella virulence factor SifA, promotes kinesin-1-dependent vacuolar membrane exchanges. *Traffic*. (2010) 11:899–911. doi: 10.1111/j.1600-0854.2010.01062.x
34. Feng Z-Z, Jiang A-J, Mao A-W, Feng Y, Wang W, Li J, et al. The salmonella effectors SseF and SseG inhibit Rab1A-mediated autophagy to facilitate intracellular bacterial survival and replication. *J Biol Chem*. (2018) 293:9662–73. doi: 10.1074/jbc.RA118.002708
35. D'Costa VM, Braun V, Landekic M, Shi R, Proteau A, McDonald L, et al. Salmonella disrupts host endocytic trafficking by SopD2-mediated inhibition of Rab7. *Cell reports*. (2015) 12:1508–18. doi: 10.1016/j.celrep.2015.07.063
36. Bayer-Santos E, Durkin CH, Rigano LA, Kupz A, Alix E, Cerny O, et al. The salmonella effector SteD mediates MARCH8-dependent ubiquitination of MHC II molecules and inhibits T cell activation. *Cell Host Microbe*. (2016) 20:584–95. doi: 10.1016/j.chom.2016.09.006
37. Dai Y, Zhang M, Liu X, Sun T, Qi W, Ding W, et al. Salmonella manipulates macrophage migration via SteC-mediated myosin light chain activation to penetrate the gut-vascular barrier. *EMBO J*. (2024) 43:1499–518. doi: 10.15252/embj.2023100502
38. Domingues L, Holden DW, Mota LJ. The salmonella effector SteA contributes to the control of membrane dynamics of salmonella-containing vacuoles. *Infect Immun*. (2014) 82:2923–34. doi: 10.1128/IAI.02081-14
39. Geng S, Wang Y, Xue Y, Wang H, Cai Y, Zhang J, et al. The SseL protein inhibits the intracellular NF- κ B pathway to enhance the virulence of salmonella pullorum in a chicken model. *Microb Pathog*. (2019) 129:1–6. doi: 10.1016/j.micpath.2019.03.024
40. Galen JE, Curtiss R. The delicate balance in genetically engineering live vaccines. *Vaccine*. (2014) 32:4376–85. doi: 10.1016/j.vaccine.2014.06.011
41. Pabst O, Cerovic V, Hornef M. Secretory IgA in the coordination of establishment and maintenance of the microbiota. *Trends Immunol*. (2016) 37:287–96. doi: 10.1016/j.it.2016.02.003