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Immunogenic potential and protective efficacy of a *sptP* deletion mutant of *Salmonella* Enteritidis as a live vaccine for chickens against a lethal challenge

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ABSTRACT

Salmonella Enteritidis (SE) is a highly adapted pathogen causing severe economic losses in the poultry industry worldwide. Chickens infected by SE are a major source of human food poisoning. Vaccination is an effective approach to control SE infections. This study evaluated the immunogenicity and protective efficacy of a SE *sptP* deletion mutant (C50336Δ*sptP*) as a live attenuated vaccine (LAV) candidate in chickens. 14 day-old specific pathogen-free (SPF) chickens were intramuscularly immunized with various doses of C50336Δ*sptP*. Several groups of chickens were challenged with the virulent wild-type SE strain Z-11 via the same route at 14 days post vaccination. Compared to the control group, the groups vaccinated with 1×10^6, 1×10^7 and 1×10^8 colony-forming units (CFU) of C50336Δ*sptP* exhibited no clinical symptoms after immunization. Only slight pathological changes occurred in the organs of the 1×10^9 CFU vaccinated group. C50336Δ*sptP* bacteria were cleared from the organs of immunized chickens within 14 days after vaccination. Lymphocyte proliferation and serum cytokine analyses indicated that significant cellular immune responses were induced after the vaccination of C50336Δ*sptP*. Compared to the control group, specific IgG antibody levels increased significantly in vaccinated chickens, and the levels increased markedly after the challenge. The 1×10^7, 1×10^8, and 1×10^9 CFU vaccinated chickens groups showed no clinical symptoms or pathological changes, and no death after the lethal challenge. Whereas severe clinical signs of disease and pathological changes were observed in the control group chickens after the challenge. These results suggest that a single dose of C50336Δ*sptP* could be an effective LAV candidate to against SE infection in chickens.

1. Introduction

Salmonella Enteritidis (SE) is a zoonotic pathogen, which causes sustained epidemic infections worldwide due to the consumption of raw or undercooked chicken meat or eggs since 1990s (Rodrigue et al., 1990). SE is one of the top serovars isolated causing salmonellosis in humans (Majowicz et al., 2010).

The vaccination of chickens is one of the most effective options to reduce human salmonellosis (Berghaus et al., 2011). Although several types of inactivated *Salmonella* vaccines have been evaluated in chickens (Barrow, 2007), weak protective immunity limits the development of inactivated vaccines (Chuttani et al., 1977). Deleting bacterial virulence genes to generate attenuated live vaccines is an effective solution due to its high immunogenicity and great efficacy, and both local and systemic immune responses can be elicited after immunization with LAV (Si et al., 2015; Araya et al., 2010).

Salmonella protein tyrosine phosphatase (SptP) is an effector protein encoded in *Salmonella* pathogenicity islands (SPI)-1 and secreted by the type III secretion system (Kaniga et al., 1996). The N-terminal domain of the SptP protein has a sequence similar to those of the secreted toxins *Yersinia* YopE and *Pseudomonas aeruginosa* ExoS. These toxins possess potent GTPase-activating proteins by deactivating the Rho GTPases Rac

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and Cdc42 in addition to reversing the cytoskeletal rearrangements by the SPI-1 SopE/E2/SopB effectors (Fu and Galán, 1999). The C-terminal domain was homologous to that of *Yersinia* tyrosine phosphatase YopH which exhibited the strong tyrosine phosphatase activity, and is involved in reversing the activation of mitogen-activated protein kinase (Murli et al., 2001).

Previous studies have shown that SptP was an important *Salmonella* virulence factor. The SptP mutant of Typhimurium strain ST4/74 is significantly attenuated during the intestinal colonisation (Chaudhuri et al., 2013). Our previous studies showed the *sptP* gene deletion SE mutant strain C50336Δ*sptP* possesses reduced virulence, is highly immunogenic, and provides efficacious protection for immunized mice (Lin et al., 2017). Based on above, this study further explored the bacterial persistence, host clearance, immunogenicity, safety, and protective efficacy of C50336Δ*sptP* in a chicken model. This study could pave the way for future development of a LAV against SE infection.

2. Materials and methods

2.1. Experimental animals

Healthy SPF chickens (7 day-old) were obtained from the Spirax Ferrer Poultry Science and Technology Co. Ltd. (Jinan, China). All animal experiments were approved by the Animal Welfare and Ethics Committees of Yangzhou University, and complied with the guide lines of Institutional Administrative Committee and Ethics Committee of Laboratory Animals (IACUC license number: SYXK [Su] 2016–0020).

2.2. Bacterial strains

The wild-type SE strain C50336 was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The *sptP* deletion mutant strain C50336Δ*sptP* was constructed through λ-Red-mediated recombination as described previously (Lin et al., 2017). The virulent wild-type SE strain Z-11 was a clinical isolate obtained from chickens infected with SE and was stored in our laboratory. Bacteria were cultured in lysogeny broth (LB) broth followed by overnight incubation at 37 °C at 180 rpm. XLT4 (Difco Laboratories, San Jose, CA, USA) and 1.5% LB agar were used for bacterial culture and counting CFU, respectively.

2.3. Experimental design

In total, 135 of 14 day-old SPF chickens were randomly assigned to five groups (n = 27). Each group was administered 100 μl of dilutions of suspensions of C50336Δ*sptP* containing 1×10^9, 1×10^8, 1×10^7, or 1×10^6 CFU/ml in phosphate-buffered saline (PBS) by intramuscular injection. Control chickens received 100 μl of PBS via the same route. Three chickens per group were sacrificed at 1, 3, 5, 7, and 14 days post-immunization (dpi), respectively. At 14 dpi, remaining chickens from each group (n = 12) were challenged intramuscularly with 5×10^8 CFU of the virulent wild-type SE strain Z-11 in 100 μl of PBS.

2.4. Changes in body weight and clinical symptoms after immunization

The body weight for each group of chickens was recorded at 1, 3, 5, 7, and 14 dpi, respectively. The chickens of each group were monitored from 1 to 14 dpi for clinical symptoms, including anorexia, diarrhoea, depression, and mortality.

2.5. Bacterial colonisation and persistence assay

Bacterial colonisation and persistence in the internal organs of the infected chickens were evaluated. Liver and spleen samples aseptically collected from the chickens sacrificed at 1, 3, 5, 7, and 14 dpi were weighed and homogenised in 1 ml of PBS. Serial 10-fold dilutions of tissue homogenates (100 μl each) were plated on XLT4 agar and incubated at 37 °C for 12–16 h. The bacterial counts were determined as $\log_{10}$ CFU/g.

2.6. Lymphocyte stimulation test

Peripheral blood mononuclear cells (PBMCs) were isolated from whole chicken blood at 7 and 14 dpi. The blood samples were collected into a 3.8% sodium citrate solution at a 5:1 (v:v) ratio and mixed with the same of Dulbecco's phosphate-buffered saline (DPBS; Invitrogen, Carlsbad, CA, USA). The mixture was layered onto Histopaque-1083 (Sigma, St. Louis, MO, USA) and centrifuged at 2000 rpm for 20 min at 20 °C. PBMCs were collected from the white gradient interface, washed three times with DPBS and centrifuged at 1000 rpm for 10 min at 4 °C. The PBMCs were then diluted in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) complete medium (containing 10% foetal bovine serum and 1% penicillin/streptomycin). Cell viability and number were determined using trypan blue exclusion.

Splenic lymphocytes were isolated from chicken spleens at 7 and 14 dpi. The spleens were excised in a sterile manner and washed three times with DPBS. Splenocytes were released into RPMI 1640 complete medium by grinding the tissues between frosted glass slides, and the resulting suspension was filtered through a 200-mesh grid. The filtrate was layered onto Histopaque-1083 and processed as described above for PBMCs.

The specific SEAgP antigen was prepared from the C50336 strain as described previously (Lin et al., 2017). PBMCs and splenic lymphocytes (1×10^6 cells/well) were placed in 96-well culture plates stimulated with 10 μg/ml SEAgP or 10 μg/ml Concanavalin A (Con A, a lymphocyte mitogen) as a positive control at 37 °C in a humidified atmosphere of 5% CO_2 for 48 h. Lymphocyte proliferation was measured with a BrdU kit (Roche, Basel, Switzerland). Blastogenic responses for the assay were expressed as a mean stimulation index (SI) calculated based on the optical density of stimulated cultures at 450 nm, as described previously (Rana and Kulshreshtha, 2006).

2.7. Serum interleukin (IL)-4, interferon (IFN)-γ and the IgG test

Serum samples were collected from chickens in each group at 1, 3, 5, 7, 14 dpi and 7, 14 days post-challenge (dpc). Humoral immune responses were evaluated by determining the SE-specific IgG antibody levels with enzyme-linked immunosorbent assay (ELISA), whole SE bacteria strain C50336Δ*sptP* was used as the coating antigen as described previously (Haneda et al., 2011). Serum samples were diluted 1:50 for use as the primary antibody. The secondary antibody was horseradish peroxidase (HRP)-conjugated rabbit anti-chicken IgG (Sigma; 1:10,000 dilution). HRP activity was determined by using TMB (Sigma). The reaction was stopped with 2 M H_2SO_4 and evaluated by ELISA reader at OD_{450}. Titers were reported as the highest dilution at which the optical density was 2.1-fold higher than that of the negative control (P/N ≥ 2.1).

Serum IL-4 and IFN-γ expression levels were measured with the Chicken Interleukin 4 (IL-4) ELISA Kit and the Chicken Interferon γ (IFN-γ) ELISA Kit (Cusabio, Houston, TX, USA) according to the manufacturer's protocol.

2.8. Evaluation of immune protection

The protective efficacy of C50336Δ*sptP* against a challenge with the virulent SE strain Z-11 was assessed. All chicken deaths during the 14 dpc were recorded. Body weight and clinical symptoms for each group of chickens were recorded daily.

2.9. Histological analysis

Sections of spleen, liver, ceca, and bursa of Fabricius were collected

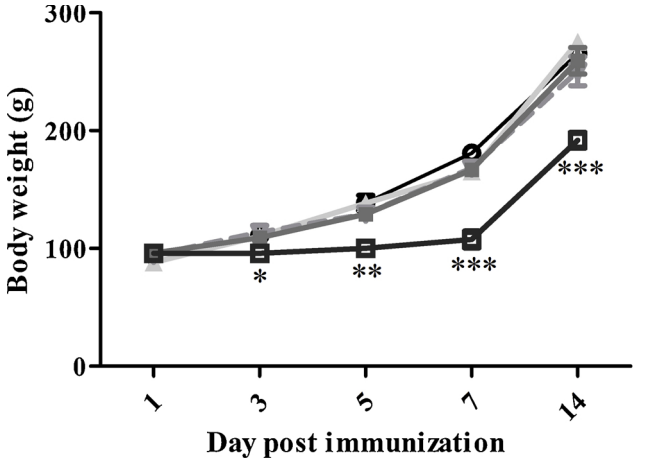

Fig. 1. The body weight of chickens after immunization. Groups of 14 day-old SPF chickens were intramuscularly immunized with 1×10^6, 1×10^7, 1×10^8 or 1×10^9 CFU of C50336Δ*sptP*, and the control group received 100 µl PBS. The body weights of the experimental chickens were determined at 1, 3, 5, 7, and 14 days post immunization. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ compared with the body weight of control group chickens by one-way ANOVA followed by Bonferroni's multiple comparison test. Data are presented as mean ± SEM.

at 14 dpi and 14 dpc, and the tissue samples were fixed in 10% (w/v) buffered formalin. Paraffin-embedded sections were stained with haematoxylin and eosin (H&E) (Wuhan Goodbio Technology Co. Ltd., Hubei, China).

2.10. Statistical analysis

All data were presented as mean ± standard error (SEM) using GraphPad Prism 5 software (La Jolla, USA). To detect significant

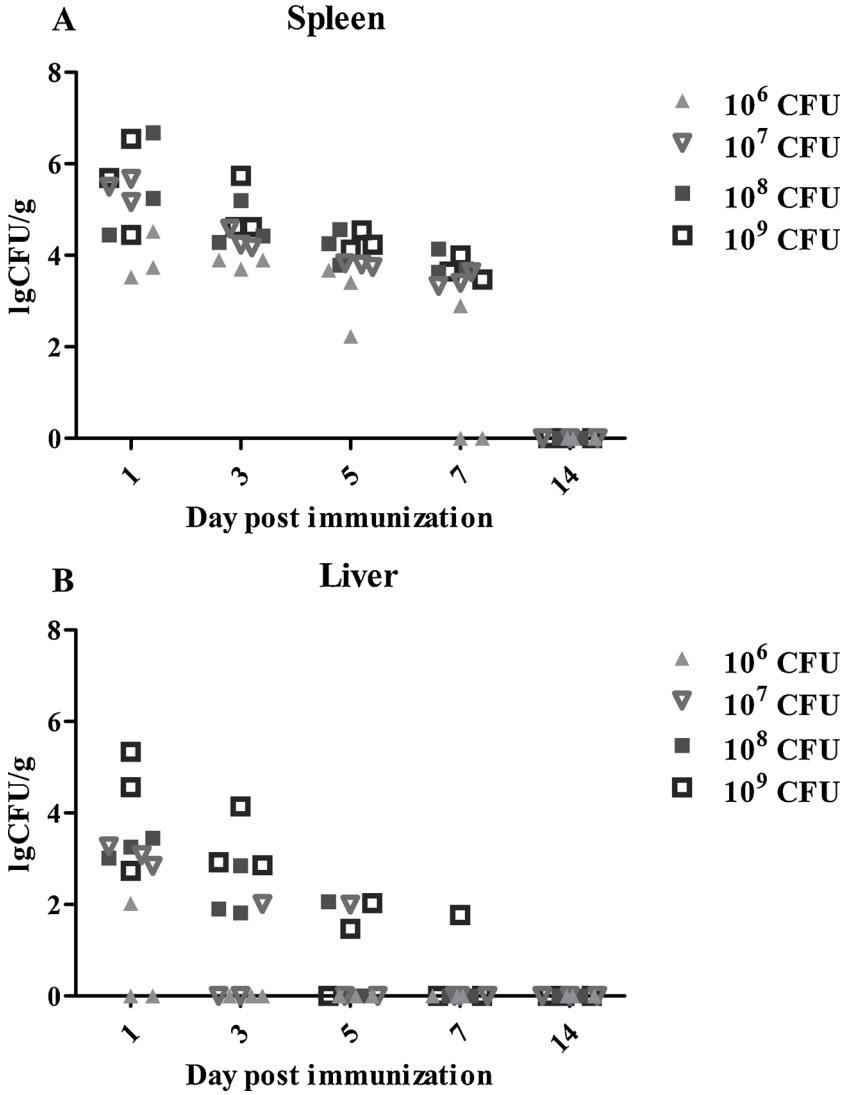

Fig. 2. *Salmonella* colonisation in chicken organs post immunization. The bacterial colonisation and persistence in the spleen (A) and liver (B) of chickens inoculated with different doses of C50336Δ*sptP*. Control chickens received 100 µl of PBS, and all spleen and liver samples from control group chickens were negative for *Salmonella*. Data are presented as mean ± SEM of $\log_{10}$ CFU/g.

differences between experimental groups, one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test were conducted. Log-rank (Mantel-Cox) Test were conducted for the survival curve. Statistical significance was determined at p values of < 0.05 (*), < 0.01 (**), or 0.001 (***).

3. Results

3.1. Body weight and clinical symptoms after vaccination

The changes in body weight and clinical symptoms were monitored in chickens inoculated with C50336Δ*sptP*. No significant difference in body weight was observed and no obvious clinical signs occurred in the 1×10^6, 1×10^7, and 1×10^8 CFU immunized groups after vaccination (Fig. 1). In contrast, weight gain was suppressed in the 1×10^9 CFU vaccinated chickens. Which was significantly lower than that in the other three groups at 5, 7, and 14 dpi. Clinical signs of disease, including slight and temporary lethargy, anorexia, and diarrhoea were observed in the 1×10^9 CFU immunized group. No clinical changes were observed in the other three immunized groups.

3.2. Colonisation and persistence of C50336ΔsptP in spleen and liver

After inoculation with C50336Δ*sptP*, bacteriological analysis of the chicken organs showed that all liver and spleen samples obtained from the non-vaccinated group were negative for bacterial recovery. As shown in Fig. 2, C50336Δ*sptP* colonisation reached the highest level at 1 dpi and decreased gradually in the liver and spleen thereafter. *Salmonella* in the liver was cleared more rapidly than that in the spleen of hosts without a functional SptP. The viable counts in the spleens and livers from the low-dose immunization group were lower than those from chickens inoculated with high-dose C50336Δ*sptP*. Only one chicken from the 1×10^6 CFU immunized group was positive for *Salmonella* in the liver at 1 dpi. Of the three livers from each vaccinated group examined at 7 dpi, only one sample from the 1×10^9 CFU immunized group was positive, and no colonies were observed at 14 dpi. In addition, only two spleen samples from the 1×10^6 CFU immunized group were negative at 7 dpi, and no C50336Δ*sptP* was detected in the spleens of immunized chickens at 14 dpi.

3.3. Histological analysis after immunization with C50336ΔsptP

H&E stained spleens revealed slight infiltration of heterophilic granulocytes and an increased number of nucleated oval shaped red blood cells (RBCs) in the 1×10^9 CFU vaccinated group compared to the other groups at 14 dpi. As shown in Fig. 3, liver cells from the 1×10^6, 1×10^7, and 1×10^8 CFU vaccinated groups showed no difference compared with those from the control group after immunization, whereas liver cells from the 1×10^9 CFU immunized group were obviously swollen. No definite lesions were detected in the ceca of all inoculated chickens compared with the control group, though there was a slight increase in gland secretions. The lymphocytes in the medulla of the bursal follicle decreased slightly after vaccination with high-dose C50336Δ*sptP* compared to those of the control group.

3.4. Humoral immune responses after immunization

To evaluate the humoral immune response in SPF chickens following inoculation with C50336Δ*sptP*, serum SE antigen-specific IgG levels were determined by indirect ELISA. As shown in Fig. 4, chickens immunized with C50336Δ*sptP* had significantly higher levels of serum IgG than the control group after vaccination. The specific IgG levels of the high-dose vaccinated group of chickens were higher than those of chickens immunized with low-dose C50336Δ*sptP*.

3.5. Cellular immune responses after immunization

The cellular immune responses in the chickens after immunization were evaluated using splenic and peripheral lymphocyte proliferation assays. In Fig. 5, the SI values of the splenic lymphocytes from all vaccinated groups of chicken increased significantly at 7 and 14 dpi after ConA or SEAgP stimulation. The splenic lymphocytes of the

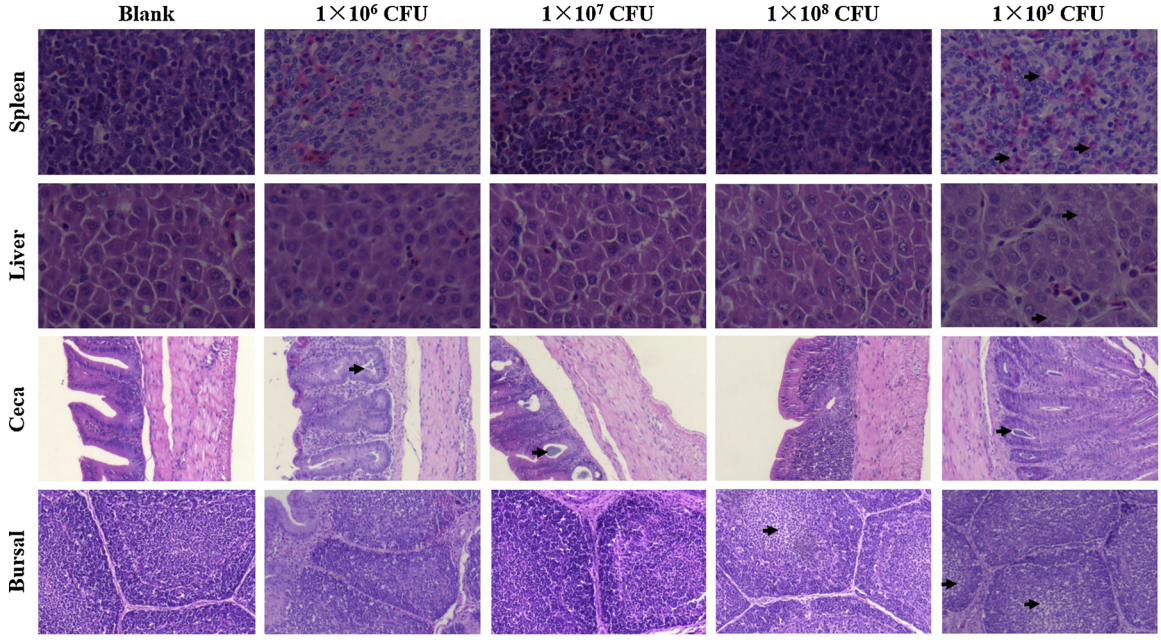

Fig. 3. Histological analysis after C50336Δ*sptP* immunization. Representative images of haematoxylin and eosin-stained spleen, liver, cecal, and bursal tissue sections at 14 days post PBS (control) or C50336Δ*sptP* intramuscular injection. Arrows in spleen sections represent the accumulation of red blood cells or inflammatory cells. Arrows in liver sections indicate swelling. Arrows in cecal sections represent increased gland secretion. Arrows in bursal sections indicate a reduction of lymphocytes in the medulla of the bursal follicle. Magnification for spleen and liver sections is 400×, and magnification for the cecal and bursal sections is 100 × (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

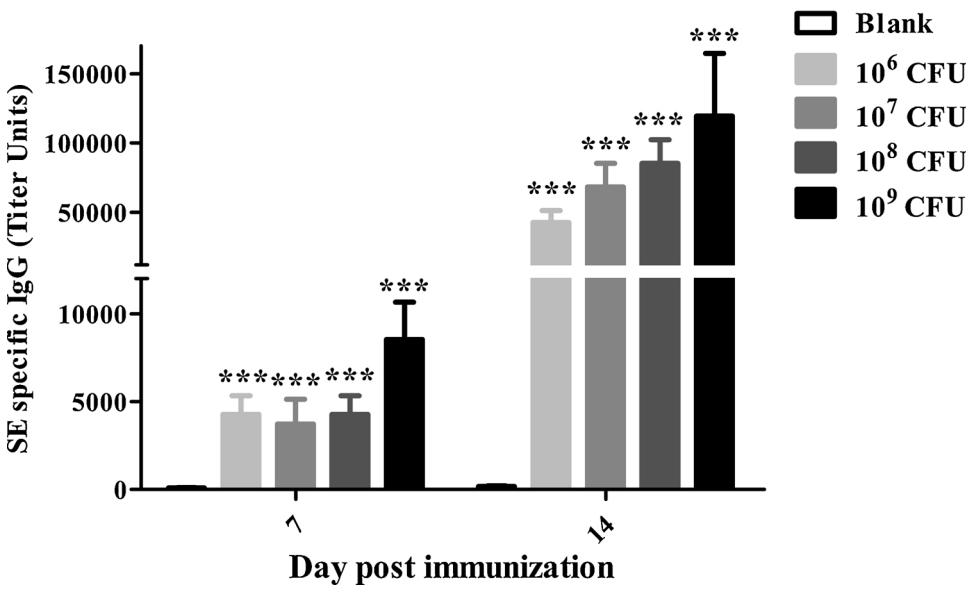

Fig. 4. Determination of serum IgG levels. *Salmonella* Enteritidis (SE)-specific IgG antibody titer in the serum of chickens from each group at 7 and 14 days post immunization were detected by enzyme-linked immunosorbent assay. ***, $p < 0.001$ compared with control group by one-way ANOVA followed by Bonferroni's multiple comparison test. Data are presented as mean ± SEM.

1×10^9 CFU vaccinated group proliferated more following ConA or SEAgP stimulation at 7 and 14 dpi. ConA-stimulated peripheral blood lymphocytes proliferated significantly in all vaccinated groups compared to those of the control group at 7 and 14 dpi. SEAgP induced dramatic proliferation of the peripheral blood lymphocytes from the inoculated groups in a dose-dependent manner. In contrast, only stimulation with ConA led to lymphocyte proliferation in the control group at 7 and 14 dpi.

In addition, the secretion of IL-4 and IFN-γ in serum after immunization was examined with ELISA (Fig. 6). The expression of the Th2 cytokine IL-4 increased significantly in the serum of the 1×10^8 and 1×10^9 CFU vaccinated groups compared to the unvaccinated chickens at 1 and 3 dpi. Chickens from the vaccinated groups exhibited significantly higher expression of the Th1 cytokine IFN-γ in serum than the unvaccinated control chickens at all tested time points after immunization. There was no significant difference in the levels of IFN-γ induced by high-dose or low-dose immunizations, suggesting that a single low-dose vaccination was able to induce strong cellular immune response. Furthermore, the expression of IFN-γ was significantly higher than that of IL-4 in the serum of all immunized groups after inoculation, demonstrating that the SE strain C50336Δ*sptP* induced a Th1 immune response in chickens.

3.6. Immune protective efficacy of C50336ΔsptP after challenge

The chickens from all groups were challenged with the virulent wild-type SE strain Z-11 at 14 dpi. Results showed that the protective efficacy of immunization groups was significantly higher than that of control group (Fig. 7). None of the chickens in the 1×10^7, 1×10^8, or 1×10^9 CFU inoculated groups died. Only one chicken died at 5 dpc in the 1×10^6 CFU vaccinated group, whereas 11 of 12 chickens in the control group died within 5 dpc. Rapid decreases in overall health and weight were observed in the challenged chickens from the control group at 3 and 5 dpc (Fig. 8). Slight and temporary anorexia, diarrhoea,

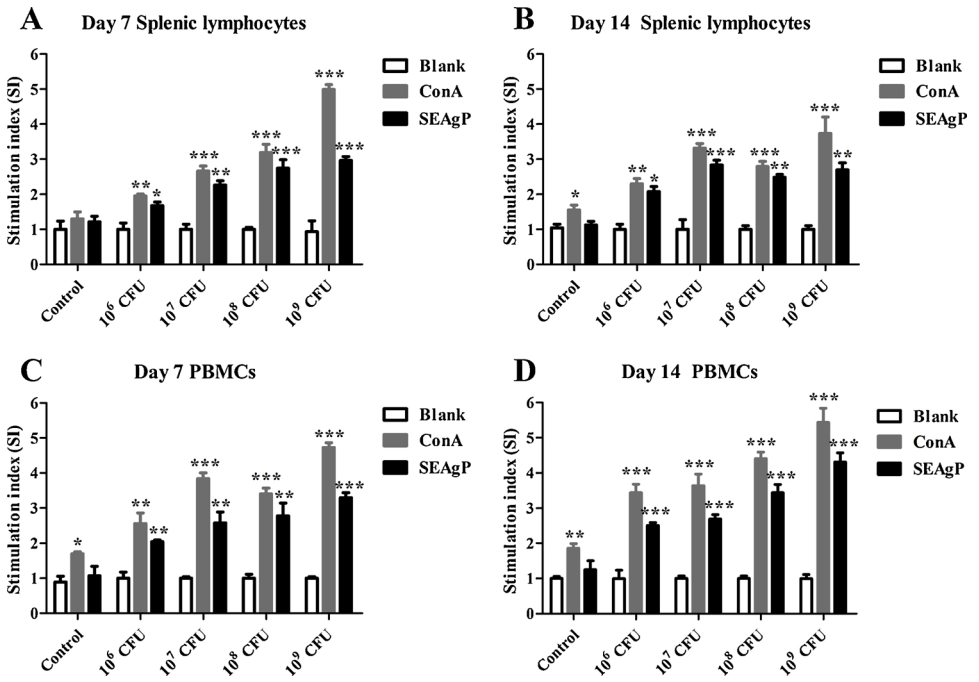

Fig. 5. Stimulation index (SI) values from the lymphocyte proliferation assay. SI of chicken lymphocyte samples determined by splenic (A, B) and peripheral (C, D) lymphocyte proliferation assay using SEAgP or Concanavalin A (Con A) for the chickens inoculated with C50336Δ*sptP* or PBS (Blank) at 7 and 14 days post immunization. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ compared with control group by one-way ANOVA followed by Bonferroni's multiple comparison test. Data are presented as mean ± SEM.

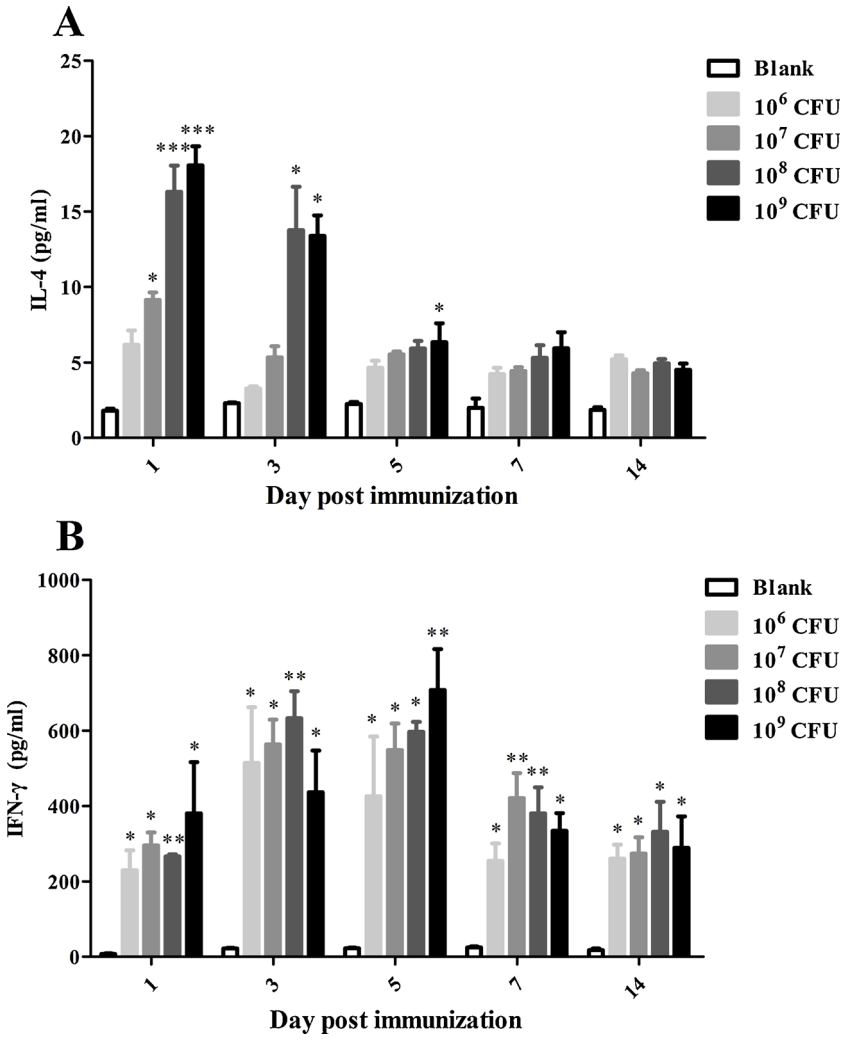

Fig. 6. Serum cytokine assay after immunization. Expression of key Th1 [interferon (IFN)-γ, B] and Th2 [interleukin (IL)-4, A] associated cytokines in serum after immunization with C50336Δ*sptP* or PBS (Blank) measured by enzyme-linked immunosorbent assay. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ compared with control group by one-way ANOVA followed by Bonferroni's multiple comparison test. Data are presented as mean ± SEM.

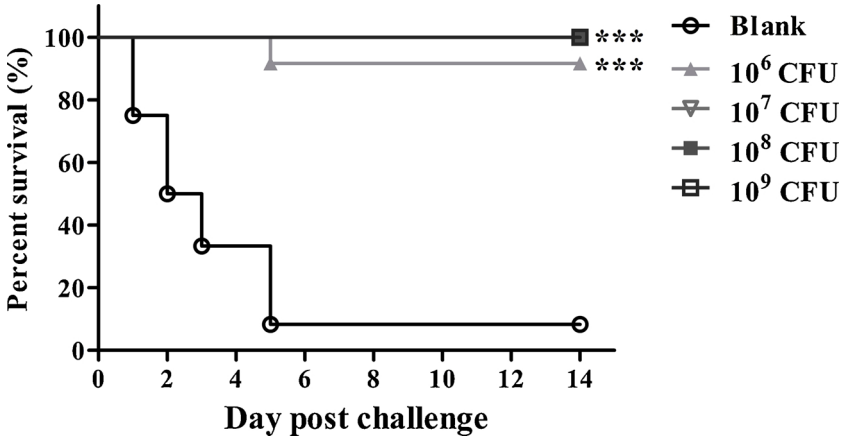

Fig. 7. The protective efficacy of C50336Δ*sptP*. Chickens immunized with different doses of C50336Δ*sptP* or PBS were intramuscularly challenged at 2 weeks post immunization with virulent *Salmonella* Enteritidis (SE) strain Z-11, and mortality was recorded. ***, $p < 0.001$ compared with control group by Log-rank (Mantel-Cox) Test for the survival curve.

and depression were observed following challenge in the 1×10^6 CFU immunized group compared with the blank control group. No clinical symptoms were detected after challenge in the other three high-dose immunized chickens.

Following challenge with the virulent SE strain Z-11, stronger specific serum IgG levels were detected in all immunized groups at 7 dpc compared to serum IgG levels at 14 dpi, and the specific IgG antibody response was further elevated at 14 dpc (Fig. 9). The high-dose immunized chickens showed higher titers than the low-dose immunized chickens, especially at 14 dpc.

3.7. Histopathological examination after challenge

As shown in Fig. 10, more heterophilic granulocyte infiltration and

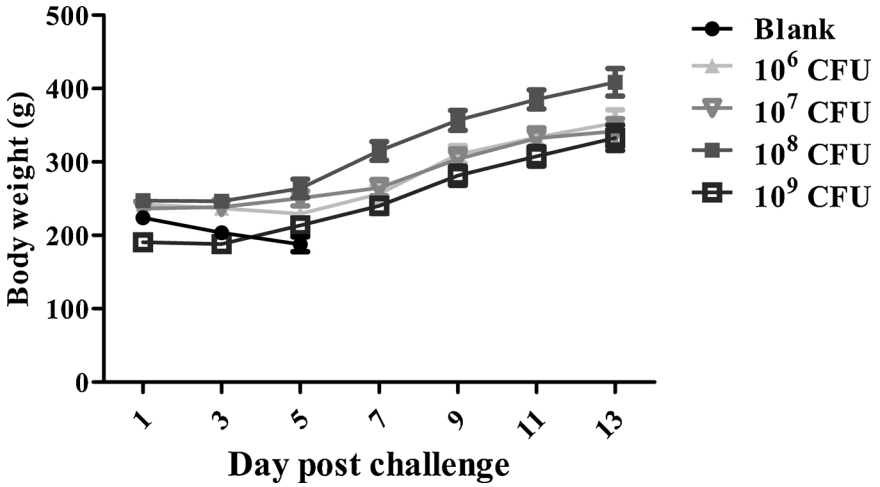

Fig. 8. The body weights of chickens after challenge. Changes in the body weights of chickens vaccinated with different doses of C50336Δ*sptP* or PBS (Blank) and then intramuscularly challenged at 14 days post immunization with the virulent *Salmonella* Enteritidis (SE) strain Z-11. Data are presented as mean ± SEM.

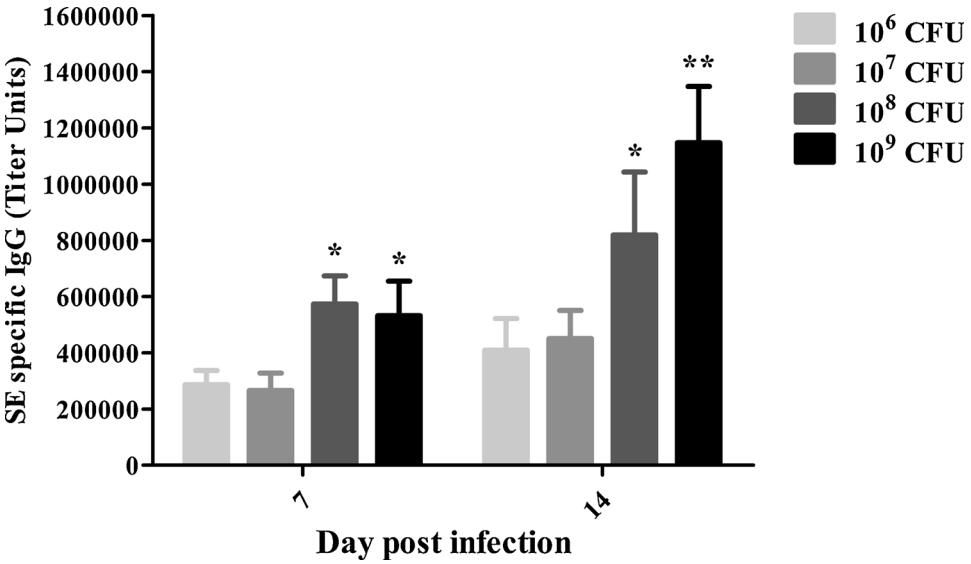

Fig. 9. Serum IgG antibody titers after challenge. Vaccinated and control group chickens were intramuscularly challenged at 14 days post infection. Then, *Salmonella* Enteritidis (SE)-specific IgG antibody titers in the serum of each group of chickens were detected by ELISA at 7 and 14 days post challenge. *, $p < 0.05$ and **, $p < 0.01$ compared with 1×10^6 CFU vaccinated group by one-way ANOVA followed by Bonferroni's multiple comparison test. Data are presented as mean ± SEM.

nucleated oval shaped RBCs were found in the spleens of the control and 1×10^6 CFU inoculated groups after challenge than in the 1×10^7, 1×10^8, and 1×10^9 CFU vaccinated groups. The liver cells from the 1×10^7, 1×10^8, and 1×10^9 CFU inoculated groups showed slight swelling at 14 dpc, whereas liver cells from the control group showed obvious swelling and the infiltration of heterophilic granulocytes after challenge. The 1×10^6 CFU immunized group showed slight heterophilic granulocyte infiltration at 14 dpc. Furthermore, the ceca of the control group displayed severe desquamation of intestine villi, epithelial damage with loss of glands, and thickening of the mucosa. The inoculated groups displayed slight intestine villi desquamation and epithelial damage. Severe lesions appeared in the medulla of the lymph follicles from the control group of chickens after challenge. In addition, massive lymphocytic necrosis, severe follicular atrophy, destruction of the follicular architecture and heterophilic granulocyte infiltration were observed in the control group. In contrast, the bursa of Fabricius from all vaccinated chickens showed only slight lymphocytic necrosis at 14 dpc.

4. Discussion

SE is a major public health problem, causing millions of foodborne salmonellosis each year (Matheson et al., 2010). Vaccination is one of the most effective strategies to control SE infection in chickens (Beal et al., 2006a). An ideal vaccine for efficient control of pathogenic *Salmonella* infections should be safe and induce both humoral and cellular immunity (Jones and Falkow, 1996), which requires a vaccine with improved immunogenicity. LAVs are more appropriate and have been commonly developed, which are more effective than killed or subunit vaccines for inducing the cross-protection against different *Salmonella* serovars in animals (Gantois et al., 2006; Barrow, 2007).

Previous studies showed that some live *Salmonella* vaccines could induce transient diarrhoea after inoculation, and abscesses formed at the inoculation site (Cooper et al., 1992). In our previous studies, C50336Δ*sptP* displayed no side effects in terms of growth performance in mice (Lin et al., 2017). Similarly, this study showed only the 1×10^9 CFU immunized chickens presented slight clinical signs indicating that live vaccines were safe, and the adverse reactions was most likely induced by the huge amount of endotoxin. In contrast, a *nuoG* mutated *Salmonella* vaccine shown reduce the mortality of chickens from 75% to less than 8%, but necrotic lesions were observed on the spleen and liver after immunization (Zhang-Barber et al., 1998). Chickens immunized with the SE mutant JOL919 had significantly lower lesion scores than the control group, while pathological changes were still found in the organs of the immunized chickens (Nandre et al., 2014).

The survived live *Salmonella* vaccine strains could colonise in the

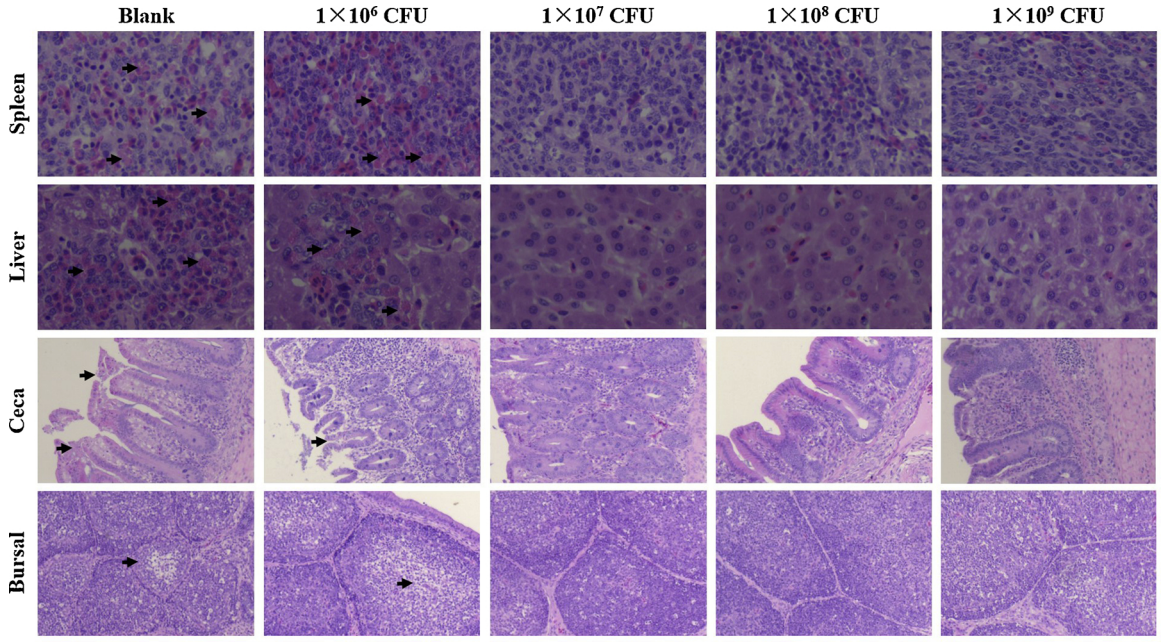

Fig. 10. Histopathological analysis after challenge. Representative images of haematoxylin and eosin-stained spleen, liver, cecal, and bursal tissue sections at 14 days post challenge after intramuscular injection with the virulent *Salmonella* Enteritidis (SE) strain Z-11. Arrows in spleen sections represent the accumulation of red blood cells or inflammatory cells. Arrows in liver sections indicate infiltration of heterophilic granulocytes. Arrows in cecal sections represent intestine villi desquamation and epithelial damage. Arrows in bursal sections indicate follicular emptiness with lymphocytic necrosis. Magnification for spleen and liver sections is 400×, and magnification for cecal and bursal sections is 100 × (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

chicken for slaughtering, which increase the possibility of *Salmonella* entering the slaughterhouse and contaminating chicken meat (Zhang-Barber et al., 1998; Bohez et al., 2008, 2007). These bacteria can also be vertically transmitted among birds by faecal shedding, contributed to systemic disease with pathology (Silva et al., 1981). Chickens immunized with a vaccine consisting of attenuated strains of *Salmonella* Enteritidis, Typhimurium, and Infantis were still positive for *Salmonella* in cloacal swabs 10 weeks after immunization (Varmuzova et al., 2016). The live attenuated SE strain constructed in study could be cleared from liver and spleen within 14 days after inoculation in chicken, which was a great advantage for a vaccine candidate. Similar results were also reported in other studies, the double-gene mutant JOL1587 mostly cleared by day 7 post inoculation (Kamble and Lee, 2016). Another *Salmonella* vaccine strains were not detected in the spleen at 14 dpi (Pei et al., 2014).

The humoral immune response plays an important role in preventing *Salmonella* infections (Peng et al., 2011). It has been demonstrated that lower prevalence of *Salmonella* in vaccinated flocks is associated with high IgG antibody titers in field experiments (Berghaus et al., 2011). At the early stage of infection, the induction of SE-specific systemic IgG antibody could contribute to enhance bacterial killing before SE penetrated the cell membrane by restoring the dendritic cells capacity for SE taking up (Riquelme et al., 2012). Previous studies reported that the novel live SE vaccine strain JOL919 (Nandre et al., 2012), as well as LAV strain LVR02 (Betancor et al., 2005) could induce significant systemic IgG response in chickens after vaccination. A *Salmonella* attenuated *spiC* mutant induced higher levels of IgG antibody in chickens inoculated intramuscularly compared to chickens vaccinated orally (Geng et al., 2014). Similarly, the SE-specific serum IgG levels in all chickens immunized with C50336Δ*sptP* were significantly higher than those of the non-vaccinated group. Additionally, enhanced induction of the specific IgG antibody response was observed in a dose-dependent manner at 14 dpc. In contrast, a commercial live vaccine containing the SE mutant strain induced a very low level of IgG antibody in young turkeys after a second immunization (Hesse et al., 2018b). Another study reported that chickens immunized with an attenuated *Salmonella* mutant had slightly higher IgG levels compared to the control group (Penha Filho et al., 2012).

Live vaccines conferred better protection against intracellular pathogens than inactivated vaccines, by inducing a stronger cellular immune response (Mastroeni et al., 2001). Cell-mediated immune mechanisms were important for resolving *Salmonella* infections with the help of T cell proliferation and the expression of the Th1-type cytokine IFN-γ because *Salmonella* could survive and replicate within macrophages (Beal et al., 2004, 2006b). Previous studies demonstrated that LAVs are effective for increasing lymphocyte proliferation in response to SE antigens and ConA in chickens (Babu et al., 2003; Cheng et al., 2016), which consistented with this study. In contrast to the immunopotentiating effect of a live vaccine, Babu et al. (2004) observed a strong negative effect from a killed *Salmonella* vaccine on splenic proliferation in response to ConA.

The activation of cell-mediated immune responses could induce Th1/Th2 T lymphocytes to secrete cytokines, such as IFN-γ and IL-4 (Mayr et al., 2005), which promote protective effects. All immunized groups exhibited high serum IFN-γ expression levels in this study, suggesting that the strong cellular response induced by C50336Δ*sptP* should be of the Thl type. This finding was consistent with a previous study which a live attenuated *Salmonella* vaccine can induce a Th1 type immune response after immunization (Kamble et al., 2016). Chappell et al. (2009) indicated that Th1-mediated cellular response with high IFN-γ expression is key to clearing *Salmonella* infections. This may provide an important explanation for the rapid clearance of C50336Δ*sptP* from chicken spleens and livers in this study. Lehmann et al. (2006) reported that the cellular and humoral immune responses activated by an SE live vaccine were regulated by Th1 cells via the secretion of IFN-γ, whereas no substantial contribution to SE live vaccine-induced immunity was made by Th2 cells.

Immune protection efficacy is the most important index to evaluate the effectiveness of a vaccine. Vaccines that induce a strong immune response without high protection efficacy are substandard. Previous

study proved that the protective effect of a live vaccine is higher than that of an inactivated vaccine (Peng et al., 2011). And intramuscular immunization could provide higher immune protection efficacy than the oral immunity in chickens after a *Salmonella* challenge (Geng et al., 2014). A LAV strain 1009Δ*spiC*Δ*crp* conferred 100% protection against a lethal *Salmonella* challenge in chickens after two intramuscular injections (Cheng et al., 2016). In the current study, the survival rate of the 1×10^7, 1×10^8, and 1×10^9 CFU vaccinated groups was 100% after a single dose, whereas 91.7% mortality was observed in the control group. In contrast, a live attenuated SE vaccine only provided 80% protection in chickens immunized twice against challenge (Si et al., 2015). Slight pathological changes were observed in the organs of low-dose vaccinated chickens, while more serious heterophilic granulocyte infiltration, epithelial damage and lymphocytic necrosis were detected in unvaccinated chickens after challenge. Our results were similar to a previous study, which the SE vaccine elicited less severe pathological changes compared to unimmunized SPF chickens after a lethal challenge, particularly in the mucous epithelium of the small intestine (Peng et al., 2011).

In conclusion, this study demonstrated that a live attenuated SE strain C50336Δ*sptP* could strongly elicit both humoral and cellular immune responses with minimal clinical symptoms in SPF chickens. The SE strain was cleared rapidly from the organs of immunized chickens. A single dose of C50336Δ*sptP* could provid an efficient protection against the lethal challenge with highly virulent SE, and few clinical symptoms and pathological changes were observed in vaccinated chickens. These data indicated the strain C50336Δ*sptP* as a safe, highly immunogenic and effective candidate vaccine to against SE infection, and revealed a novel alternative for vaccination which could be applied in the poultry industry.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

Acknowledgments

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