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Oral vaccine of recombinant *Lactococcus lactis* expressing the VP1 protein of duck hepatitis A virus type 3 induces mucosal and systemic immune responses

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ABSTRACT

Duck hepatitis A virus (DHAV) is the major pathogen of duck viral hepatitis, which has caused great economic losses to duck breeding industry. As an effective delivery tool for protein antigens, *Lactococcus lactis* (*L. lactis*) has been successfully used to stimulate mucosal and systemic immune response. In this study, a recombinant *L. lactis* named NZ3900-VP1 was constructed, which could express VP1 protein of DHAV type 3 (DHAV-3) by using a nisin-controlled expression (NICE) system. The animal experiment in both mice and ducklings were performed to detect the immune response and protection effect of oral vaccination by the recombinant *L. lactis*. The results showed that oral vaccination with *L. lactis* NZ3900-VP1 significantly induced specific anti-VP1 IgG antibodies and mucosal secretory immunoglobulin A (sIgA) of DHAV-3 in mice and ducklings, and cytokines including interleukin-2 (IL-2), interferon gamma (IFN-γ), interleukin-10 (IL-10) and interleukin-4 (IL-4). Notably, the ducklings vaccinated with *L. lactis* NZ3900-VP1 were effectively protected when facing natural infestation of DHAV-3, which indicated that the recombinant *L. lactis* could serve as an effective vaccine to prevent DHAV-3 infection in ducklings.

1. Introduction

Duck virus hepatitis (DVH) as an acute, fatal and rapidly spreading disease, can cause a mortality rate of nearly 100% in ducklings under 3 week-age. Until now, it is one of the most pestilent diseases in waterfowl industry [1,2]. Generally, both DHAV and duck astrovirus (DAstV) could cause DVH in ducklings, while DHAV results in most cases of fatal DVH worldwide. DHAV belongs to the family *Picornaviridae* and genus *Avihepatovirus*, was first discovered and isolated in the United States in 1949 [3]. According to phylogenetic analysis and neutralization test, DHAV can be further divided into three serotypes: duck hepatitis A virus type 1 (DHAV-1), type 2 (DHAV-2) and type 3 (DHAV-3). Thereinto, DHAV-3 was first reported in Korea [4] and then became prevalent in mainland of China [2,5,6]. In China, to prevent DVH in ducklings, researchers attenuated various DHAV strains in lab and tested their protective efficiency in ducklings [1,7]. Moreover, recombinant vaccine expressing VP1 protein have been attempted in ducklings and displayed a good protection effect [8]. However, recombinant vaccines for DHAV-3 prevention are limited in lab-only, with no application of recombinant vaccine yet for a mass market offering currently.

The arrangement of DHAV-3 genome is as follows: 5′UTR-VP0-VP3-VP1-2A1-2A2-2A3-2B- 2C-3A-3B-3C-3D-3′UTR. Among them, genes VP0, VP1 and VP3 encode capsid protein, in which VP1 is the most external immunogenic protein [9]. Besides, VP1 confers specific neutralizing antibody, and thus plays an essential role in pathogenicity, evolution, and virulence [10–12]. Taken together, above advantages make VP1 gene a good candidate when constructing recombinant vaccine. Oral vaccine can strongly activate the first line immune defense in gut, which plays a vital role in

Abbreviations: DHAV-3, duck hepatitis A virus type 3; NICE, nisin-controlled expression; *L. lactis*, *Lactococcus lactis*; IgG, immunoglobulin G; sIgA, secretory immunoglobulin A; IL-2, interleukin-2; IFN-γ, interferon gamma; IL-4, interleukin-4; IL-10, interleukin-10.

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disease prevention. *Lactobacillus*, a kind of probiotics that possesses crucial influence in regulating intestinal health, has been modified as host to express bacterial and viral antigens. Previous studies have demonstrated that recombinant *lactobacillus* can elicit immune responses in animal intestine after oral administration [13–15]. *Lactococcus lactis* (*L. lactis*) as a member of lactobacillus, is well-known as a food-grade lactic acid bacteria (LAB), which has been extensively used as a delivery vehicle for oral vaccines. Among various bacterial expression modes, nisin-controlled expression (NICE) system can effectively transport the foreign protein to the bacterial cell surface [16]. In recent years, NICE system has been successfully applied in *L. lactis* to express various bacterial proteins and antigens, which greatly promoted the development of oral vaccines [17,18].

In this study, we constructed a recombinant *L. lactis* that could express recombinant protein of DHAV-3/VP1. To comprehensively evaluate the protective effect of this recombinant vaccine, in vivo experiment was performed in mice and ducklings. Moreover, the variation of cytokines was tested among different time points, to roughly estimate the immunological responses stimulated by the recombinant *L. lactis*.

2. Methods and materials

2.1. Bacterial strain and vector

DHAV-3 strain SD1101 (accession no. JQ409566) was stored in the Molecular Etiology Laboratory at Shandong Agricultural University. DHAV-3 stocks were propagated in the allantoic cavities of 10-day-old specific-pathogen-free (SPF) embryonated duck eggs. Virus titer of DHAV-3 was determined to be a 5×10^5 duckling's lethal median dose (DLD_{50})/0.2 ml. The plasmid pNZ8149 and *L. lactis* NZ3900 strain were purchased from MoBiTec GmbH (Goettingen, Germany). *L. lactis* NZ3900 was cultured in M17 medium supplemented with 0.5% glucose. The mAb 4F8 was made in our previous work [11]. *Escherichia coli (E. coli)* DH5α and pEASY-T5 Zero were purchased from Takara (TaKaRa, Dalian, China).

2.2. Construction of recombinant L. Lactis NZ3900-VP1

Of technical note, we met difficulties in directly ligating DHAV-3/VP1 gene into pNZ8149 vector by classical enzyme digestion and ligation method. Then an alternative method was demonstrated to be more efficient when ligating target genes into pNZ8149 vector. A plasmid contained full-length of DHAV-3/VP1 gene as template, was amplified with primers VP1-F/VP1+Vect-R to obtain fragment A. Then plasmid pNZ8149 as template, was amplified with primers VP1+Vector-F/Vector-R to obtain fragment B. Then the two PCR products (A and B) were fused using primers VP1-F/Vector-R and named fragment C. Fragment C was then cloned into plasmid pEASY-T5 Zero and renamed pNZ-VP1-T5 (sTable 1). Plasmid pNZ-VP1-T5 was then digested by restriction enzyme Kpn I, to obtain the pNZ-VP1 fusion fragment (3268 bp) and the pEASY-T5 Zero vector (3955 bp). The pNZ-VP1 fusion fragment was purified and ligated by itself using T4 DNA ligase. Then the connection product was transformed into *L. lactis* NZ3900 by using electroporation apparatus. The conditions were pulse of 25 μF, resistance of 200 Ω and voltage of 2000 V [19]. The recombinant *L. lactis* NZ3900 that containing plasmid pNZ-VP1 was renamed *L. lactis* NZ3900-VP1.

2.3. Protein expression and Western-blot analysis

Recombinant *L. lactis* NZ3900-VP1 were grown in M17 medium (with 0.5% glucose) and cultured at 30 °C for 8–10 h. Recombinant *L. lactis* NZ3900-VP1 cells were diluted (1:25) and grown in fresh M17 medium. When OD_{600} value was nearly 0.4, nisin was added to a final concentration of 1 ng/mL and perform a three-hour induction. After induction, cells were collected and detected by sodium dodecyl sulfate-poly-acrylamide gel electrophoesis (SDS–PAGE) analysis. Western blot analysis that described previously was performed to identify the specificity of DHAV-3/VP1 protein [11]. Monoclonal antibody 4F8 and HRP-conjugated goat anti-mouse IgG (Sigma, 1:4000) were used as primary antibody and secondary antibody respectively.

2.4. Animal experiment

The cultured recombinant *L. lactis* NZ3900-VP1 was resuspended in sterile PBS at a concentration of 5×10^{10} colony-forming unit (CFU)/ml. The corresponding control strain *L. lactis* NZ3900-pNZ (*L. lactis* NZ3900 with the pNZ8149 vector) was treated in the same way. Mice and ducklings were fed under specific pathogen-free (SPF) condition. All experiments were performed in accordance with animal ethics guidelines of the Shandong Agricultural University (Approval Number: SDAUA-2017-036).

2.4.1. Oral vaccination of mice

Totally 54 BALB/c female mice of 6-week ages were bought from Shandong Taibang biological products Co., Ltd. The BALB/c mice were then randomly divided into three groups (18 mice/group), named pNZ-VP1 group, pNZ-vector group and PBS group, respectively. The mice in group pNZ-VP1 and group pNZ-vector were orally vaccinated with recombinant *L. lactis* NZ3900-VP1 and *L. lactis* NZ3900-pNZ respectively. Each administration included three consecutive daily doses of 200 μL (5×10^{11} CFU/ml) (i.e., the animals were vaccinated at 1, 2, 3, 11, 12, 13, 21, 22 and 23 days) (Fig. 1). Three mice of each group were randomly sacrificed to collect serum and small intestine samples (3 cm length) at 10, 20, 30, 37, 44, and 51 days. The intestine samples were washed by 0.5 ml PBS to collect intestine fluids.

2.4.2. Oral vaccination of ducklings

A total of 133 one-day-age cherry valley ducklings were bought from Jingwei-husbandry company, Taian city. Five 1-day-age ducklings (without eating and drinking) were randomly slaughtered to collect serum and small intestine (3 cm length) samples. The remaining ducklings were then divided into 4 groups, named A (35 ducklings), B (35 ducklings), C (43 ducklings) and D (15 ducklings). Ducklings in group A and B were orally immunized with recombinant *L. lactis* NZ3900-VP1 and *L. lactis* NZ3900-pNZ respectively, from day 1 to day 7. Each duckling in the two groups was orally immunized with 0.5 ml recombinant *L. lactis* (5×10^{11} CFU/mL). Then five ducklings in both group A and group B were randomly sacrificed at 4, 6 and 8 days (24 h after oral immunization at 3, 5 and 7 days), to collect serum samples and small intestine samples. Group C only received food and water every day as blank control group. Five ducklings were also slaughtered each time in control group C at 4, 6 and 8 days to collect serum and small intestine samples. Ducklings in group D only received food and water at the first 6 days and then were injected with egg-yolk antibodies (anti-DHAV-3) (0.3 ml/duckling) at 7th day. At 8th day, 8 ducklings were randomly selected from group C to infect DHAV-3 (strain SD1101) by intramuscular injection (0.2 ml/duckling). Next, groups A, B, C and D were put in 2 ducklings that infected with DHAV-3 to mimic natural infection. Except corresponding experiment in four groups, all ducklings received same food and water that processed by autoclaved sterilization (Fig. 2).

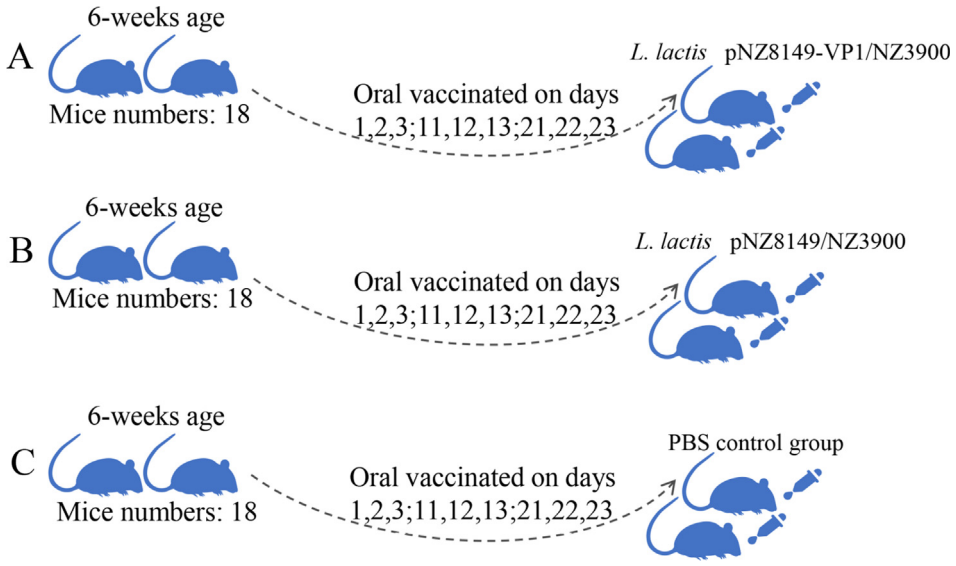

Fig. 1. Oral vaccination experiment in mice. Totally 54 BALB/c female mice were randomly divided into three groups (18 mice/group). pNZ-VP1 group (A), pNZ-vector group (B) and PBS control group (C) were orally vaccinated with recombinant *L. lactis* NZ3900-VP1, *L. lactis* NZ3900-pNZ and PBS. The animals were vaccinated at1, 2, 3; 11, 12, 13; 21, 22 and 23 days.

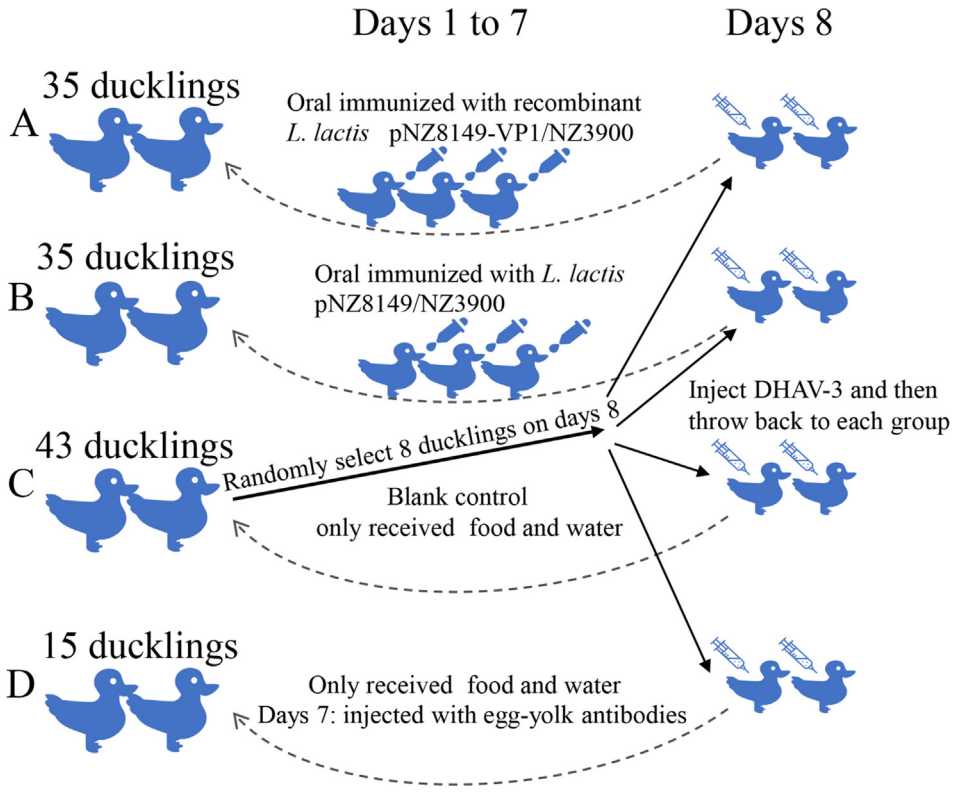

Fig. 2. Oral vaccination experiment in ducklings. A total of 133 one-day-age ducklings were randomly divided into 4 groups, named A (pNZ-VP1 group, 35 ducklings), B (pNZ-vector group, 35 ducklings), C (blank control group, 43 ducklings) and D (egg-yolk antibody group, 15 ducklings). Ducklings in group A and B were orally immunized with recombinant *L. lactis* NZ3900-VP1 and *L. lactis* NZ3900-pNZ respectively, from day 1 to day 7. Ducklings in group C and group D only received food and water at the first 6 days. Then ducklings in group D were injected with egg-yolk antibodies against DHAV-3 at the 7th day. At the 8th day, eight ducklings were randomly selected from group C to inject DHAV-3 and then each group were thrown back 2 infected ducklings to mimic natural infection.

2.5. Detection of cytokines and specific antibody in mice and ducklings

An enzyme-linked immunosorbent assay (ELISA) method described previously was used to determine the levels of specific blood-serum IgG and intestine sIgA [20]. The 96-well plate was coated with purified VP1 protein of DHAV-3 (2.0 μg/ml) and added 100 μL/well and incubated at 4 °C overnight. HRP-conjugated goat anti-mouse IgG (Sigma, 1:4000 dilution) for blood-serum or IgA (abcam, 1:50000 dilution) for intestine fluids were used as secondary antibody to detect specific antibody against VP1 in mice. HRP-conjugated goat anti-duck IgG (KPL, 1:350) was used to detect duck blood-serum. The intestine fluids of ducklings were examined

by sIgA commercial ELISA kit (Langdon Bio-technology Co., Ltd, Shanghai). Cytokines IL-2, IL-4, IL-10 and IFN-γ in blood-serum samples were detected using commercial ELISA kits (Langdon Bio-technology Co., Ltd, Shanghai) according to the manufacturer's instructions.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) and duncan's multiple-range test in GraphPad Prism5 were used to analyze data. Statistical significance of *P*-value < 0.05 was set up in data analysis.

3. Results

3.1. Construction of recombinant L. Lactis NZ3900-VP1

The recombinant plasmid containing DHAV-3/VP1 gene and pNZ8149 vector was confirmed by digestion of restriction enzyme Kpn I (sFig. 1A). The predicted fusion protein of DHAV-3/VP1 (about 37 kDa) was undetectable through SDS-PAGE analysis (sFig. 1B). Nevertheless, it could be detected in bacterial lysate by western blotting analysis (sFig. 1C).

3.2. Mucosal, humoral and cell-mediated immune responses in mice

For the effect of oral immunization in mice, sIgA antibody in the mice of pNZ-VP1 group were significantly higher ($P < 0.05$) than those in the pNZ-vector group or PBS group at 30, 37, and 44 days (Fig. 3A). Compared with the mice in pNZ-vector group and PBS group, the anti-VP1 IgG titers in vaccination group were significantly higher at 30 ($P < 0.001$) and 37 days ($P < 0.01$) (Fig. 3B). Subsequently, anti-VP1 IgA antibody levels decreased gradually over time. The cytokines IFN-γ, IL-2, IL-4 and IL-10 stimulated by the recombinant *L. lactis NZ3900-VP1* continuously increased and on its peak at 30 days but decreased subsequently. Notably, IL-2, IL-4 and IL-10 were still significantly higher than those in PBS group at 51 days (Fig. 3D, 3E; $P < 0.01$; Fig. 3F; $P < 0.05$).

3.3. Immune response and protection effect in ducklings after oral immunization

IL-2, IL-4, IL-10, IFN-γ and anti-VP1 antibody were detected at 3, 5 and 7 days after oral vaccination. The concentration of sIgA in ducklings vaccinated with NZ3900-VP1 was significantly higher than that in ducklings of pNZ-vector group and control group on days 5 and 7 (Fig. 4A), meanwhile, anti-VP1 IgG in group pNZ-VP1 presented an elevating tendency on days 3 to 7, which were all significantly higher compared to cytokine levels in blank control group (Fig. 4B). IFN-γ, IL-2 and IL-4 were all increased in ducklings vaccinated with NZ3900-VP1 and NZ3900-pNZ among days 1 to 7 (Fig. 4C–E). On days 7, IFN-γ and IL-4 in ducklings of group pNZ-VP1 were much higher than those in ducklings of group pNZ-vector, while IL-2 was finally no significant differences between ducklings in group pNZ-VP1 and group pNZ-vector. Intriguingly, IL-10 in ducklings vaccinated with recombinant *L. lactis* NZ3900-VP1 and *L. lactis* NZ3900-pNZ markedly rose on days 3 and went down on days 5–7 (Fig. 4E).

During the period of mimicking natural infection (totally 7 days), the two ducklings injected with DHAV-3 in each group all died during 24 h to 36 h while other experimental ducklings all survived in the first 36 h (Fig. 5). The ducklings administrated oral immunization of recombinant *L. lactis* NZ3900-VP1 all survived more than 60 h. In contrast, there were death events in pNZ-vector group (4/20), blank control group (5/20), and egg-yolk antibody group (1/15) within 60 h. From 60 h to 120 h, death number of ducklings was 9 in pNZ-VP1 group, 9 in pNZ-vector group, 3 in egg-yolk group and 11 in blank control group, respectively. After 120 h (5 days), left ducklings all stayed alive until the experiment over. Finally, the survival rate was 55% (11/20) for pNZ-VP1 group, 35% (7/20) for pNZ-vector group, 73.3%

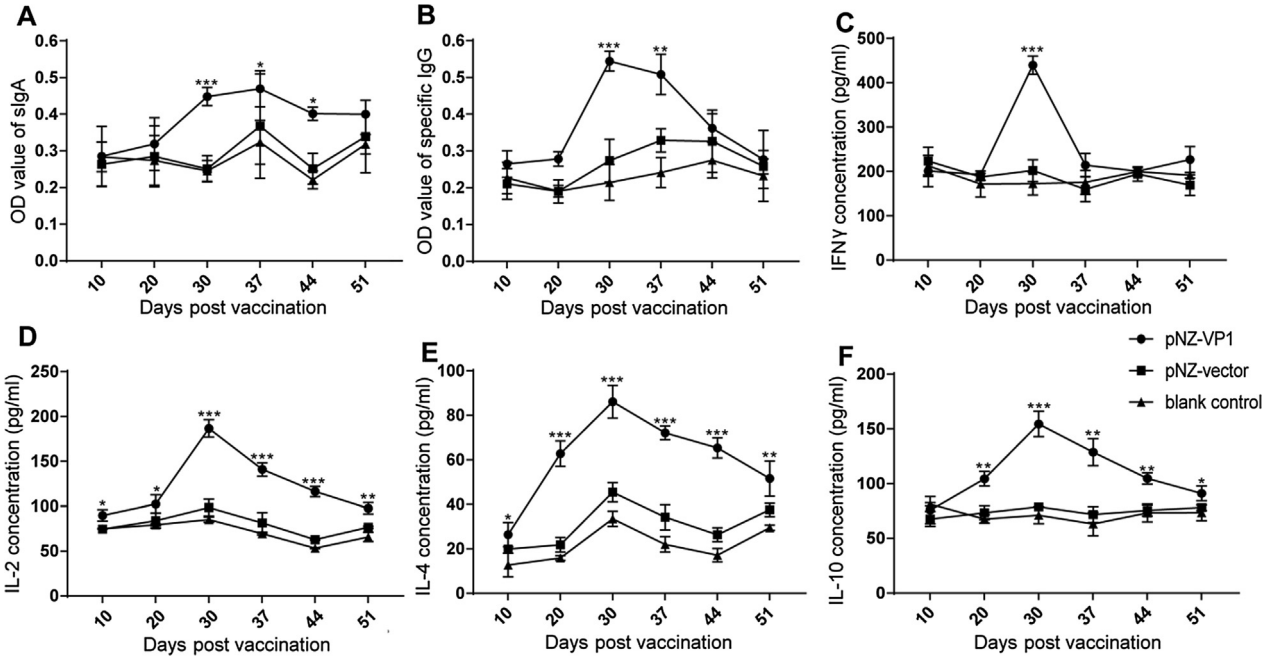

Fig. 3. Detection of immune responses in serum and intestine samples of mice. Intestinal lavage fluids and serum samples were from mice sacrificed at 10, 20, 30, 37, 44 and 51 days after oral vaccination. The sIgA (A), specific IgG (B), IFN-γ (C), IL-2 (D), IL-4 (E), and IL-10 (F) were determined by indirect ELISA. All values shown in the figures are the means ± SD of 3 independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

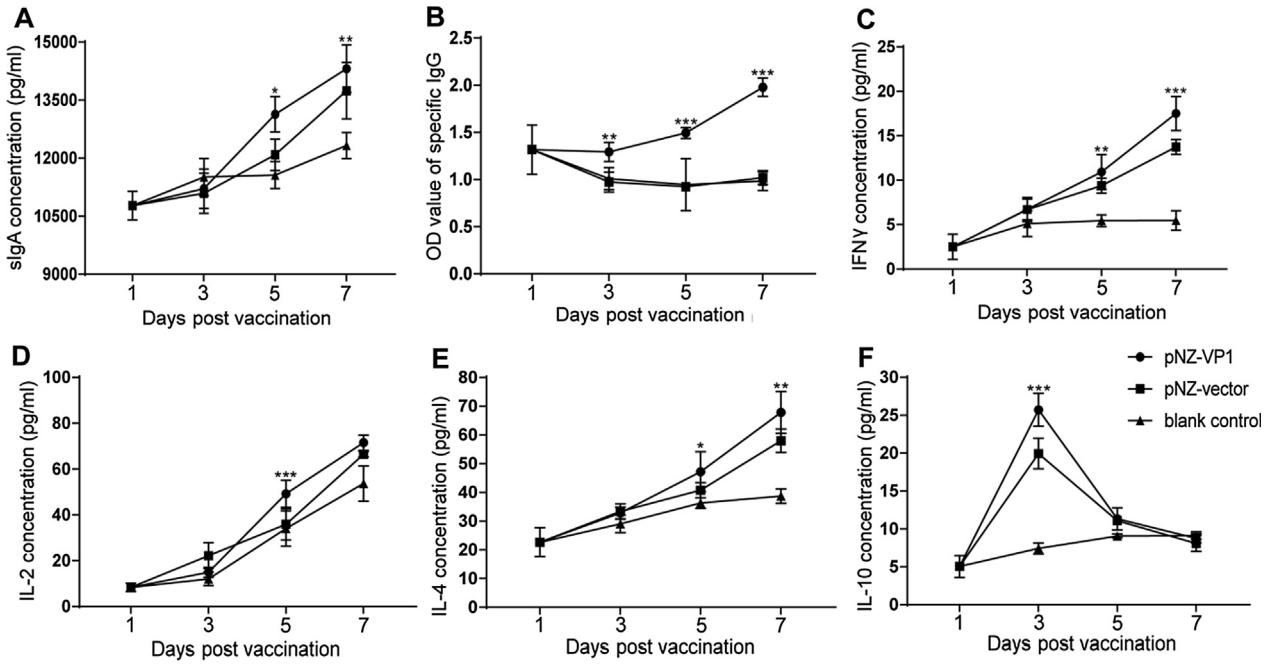

Fig. 4. Detection of immune responses in serum and intestine samples of ducklings. Serum and intestine samples were obtained from the ducklings at 1, 3, 5 and 7 days after oral vaccination. sIgA (A), specific IgG (B), IFN-γ (C), IL-2 (D), IL-4 (E) and IL-10 (F) were determined by indirect ELISA. All values shown in figure are the means ± SD of 5 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

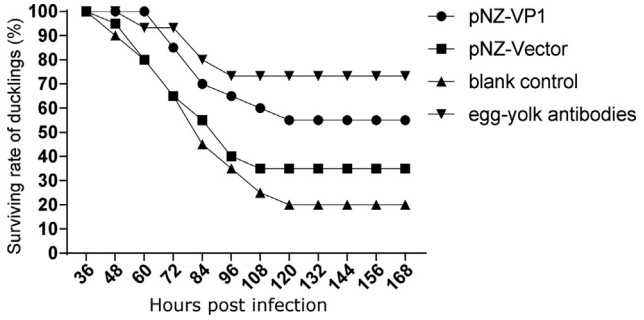

Fig. 5. Surviving rate of the ducklings after natural infection of DHAV-3.

(11/15) for egg-yolk group and 20% (4/20) for blank control group (Fig. 5).

4. Discussion

DVH, a disease with high contagious and high fatal characterization, mainly caused by DHAV, poses a severe threat to the duck industry in Southeast Asia [2,21–23]. Vaccination remains the most common method that contributes to protecting ducks against DHAV-1 and DHAV-3 infection. Intramuscular injection is a traditional immunotherapy route, which leads to a waste of time and labor force in immunizing thousands of animals. In comparison, *L. lactis* exhibits proinflammatory effects and can trigger innate inflammatory response through intestinal mucosa, which makes it efficient to work as a good oral vaccine [24]. In the present study, NICE system was used to induce the expression of DHAV-3/VP1 protein in *L. lactis*. Even though there is an innate low-copy characteristic of pNZ8149 in NICE [16], the protein expressed in *L. lactis* could be detected by western blot (sFig. 1). Besides, VP1 protein could be detected in bacterial lysate rather than culture supernatant, suggesting an intracellular expression in *L. lactis*.

The effect of oral vaccination in mice and ducklings demonstrated that the recombinant *L. lactis* NZ3900-VP1 could trigger mucosal, humoral and cell-mediated immune responses. Mucosal immune responses were normally induced by oral and nasal administration of vaccines, which could provide protection against infection in mucosal surfaces [25,26]. In the present study, the oral vaccine against DHAV-3 was constructed and experimented in ducklings and mice, which is the bold attempt to prevent DVH through mucosal immune in ducklings. After oral vaccination in ducklings, the recombinant *L. lactis* displayed the ability of eliciting strong sIgA antibodies in small intestine and specific antibody against VP1 protein in serum (Figs. 4, 5). Notably, IFN-γ, IL-2 and IL-4 in the ducklings of pNZ-VP1 group remarkably increased during vaccination period (Figs. 4, 5). These cytokines were critical in T cells immune response, B cells and anti-viruses immune response [27–30]. Furthermore, we found that the IFN-γ, IL-2 and IL-4 presented almost same increasing tendency in ducklings of pNZ-vector group, which indicated that *L. lactis* itself may confer low-grade protection. To our surprise, IL-2 and IL-4 increased in the PBS-inoculated ducklings, which might be due to the stress response in ducklings when being forced to drink PBS. Previous studies showe that IL-10 serves the role of anti-inflammatory when facing inflammatory or specific immune response [31,32]. In this study, the concentration of IL-10 in serum rose to the peak after 3-day's oral vaccination and then decreased to same level as that in blank control. We speculated that *L. lactis* as the microorganisms might cause the inflammatory response in ducklings, even though the inflammatory response faded quickly. However, we did not find any clearly pathological change after dissection.

DHAV-3 as one of the most prevalent viruses in waterfowls, has been widely vaccinated in breeder ducks and ducklings in China. In fact, it was very difficult for us to find healthy ducklings without maternal antibodies as experimental animals. In this study, we thought that the robust increase of specific IgG in the recombinant *L. lactis* group compared with control group could counteract the influence of maternal immunity, so we selected the healthy ducklings with maternal antibodies as experimental animals. The maternal antibodies of anti-DHAV-3 increased the anti-DVH ability

of ducklings at early age. In our trial, the oral drinking of recombinant *L. lactis* started at ducklings of one-day age, and the immunity was not interfered by maternal antibodies. Thus, the recombinant *L. lactis* is recommended to be used in ducklings at one-day age, and consistently oral-drunk for one week at least, which could provide a robust protection against DHAV-3 in ducklings. In this study, the good protective property and efficient immune avenue by oral drinking indicated *L. lactis* NZ3900-VP1 might be a potential vaccine against DHAV-3.

Up to now, few oral vaccines have been reported and popularized in duck industry. The recombinant *L. lactis* NZ3900-VP1 constructed in the present study demonstrates the effective protection against DHAV-3 in ducklings, which promotes the development of oral vaccine in ducks.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.06.026.

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