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A live attenuated strain of HY9901Δ*dctP* provides protection against *Vibrio alginolyticus* to the pearl gentian grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatus*)

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In recent decades, vibriosis caused by *Vibrio alginolyticus* has become a severe threat to the global mariculture industry. There is an urgent need for an effective vaccine to alleviate this unoptimistic situation. In this study, we evaluated the safety, immunoprotection, and specific and non-specific immune response effect of Δ*dctP* strain as a live-attenuated vaccine to pearl gentian grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatu*). The results demonstrate that the safe dose of Δ*dctP* was ≤1.0 ×10^6 CFU in pearl gentian grouper. The relative percent survival of the pearl gentian grouper challenged with the Δ*dctP* mutant strain by intraperitoneal injection reached 74.4%, which was significantly higher than that of the control group. Meanwhile, the expression level of immune-relative genes, including *IgM*, *IL-1β*, *IL-8*, *IL-10*, *MHC-Iα*, *MHC2*, *TNF-α*, *TLR3*, and *CD4*, were upregulated in liver, spleen, and head kidney within 28 d post-vaccination. Moreover, specific antibody IgM, total serum protein as well as activities of catalase, superoxide dismutase, and lysozyme in serum were significantly up-regulated in vaccinated groupers compared with those in control. Collectively, Δ*dctP* could be used as a live-attenuated vaccine candidate against *V. alginolyticus* infection in pearl gentian grouper.

KEYWORDS

***vibrio alginolyticus*, *dctP* gene, pearl gentian grouper, live-attenuated vaccine, immune response**

Introduction

Vibrio alginolyticus, a gram-negative opportunistic pathogen, mainly distributed in the coastal and estuarine environment of coastal countries, has caused serious economic losses to the aquaculture industry in recent decades (Balcázar et al., 2010; Jacobs Slifka et al., 2017). As one of the important pathogens, *V. alginolyticus* extremely caused diseases to many aquatic animals in China, such as *Litopenaeus vannamei* (Liu and Chen, 2004), *Epinephelus coioides* (Wang et al., 2014), *E. bruneus* (Harikrishnan et al., 2012), *Rachycentron canadum* (Rameshkumar et al., 2017), and especially pearl gentian grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatus*), which caused severe threat to the development of grouper farming industry (Chen et al., 2019; Pang et al., 2022).

Pearl gentian grouper, a new hybrid species, is hybridized between ♀*E. fuscoguttatus* × ♂*E. lanceolatus*. Pearl gentian grouper is widely cultured because of its high economic value. However, the development of the grouper farming industry was severely restricted by the outbreak of vibriosis in recent years. Vibriosis is a common bacterial disease that affects all stages of grouper growth and accounts for about 66.7% of all grouper diseases, and can result in up to 50% mortality in pearl gentian grouper. Several species, including *V. alginolyticus*, *V. harveyi*, and *V. vulnificus* are pathogenic to pearl gentian grouper (Wei et al., 2020). After being infected with *V. alginolyticus*, pearl gentian groupers have symptoms of pelvic and pectoral fin hyperemia, anal redness, and even death in serious cases (Zhang et al., 2021). Therefore, it was urgent to find effective ways to solve the current unfavorable situation of pearl gentian grouper farming.

In order to mitigate this situation, antibiotics are commonly used to control vibriosis, which leads to antibiotic-resistant bacteria and drug residue problems emerging (Xiong et al., 2010). Therefore, healthy, effective, and sustainable treatments to combat the risk of vibriosis were urgently needed, such as vaccinatopm. Vaccines could induce immune responses in the host, keeping the host sensitive enough to respond to pathogen infection, such as live attenuated vaccines, inactivated vaccines, subunit vaccines, and DNA vaccines (Mohd-Aris et al., 2019). Among them, live attenuated vaccines have greater and longer adaptive immune protection in fish: they could not only carry natural antigenic structures expressed by pathogens and mimic real infection after natural contact but also provides long-term and unaltered antigen presentation to better stimulate humoral and cell-mediated immune responses (J. Wang et al., 2014; Mohd-Aris et al., 2019; Ji et al., 2020). Therefore, live attenuated vaccines had attracted more and more attention in aquatic animal disease prevention and control (Liu et al., 2018; Chen et al., 2019a; Zhang et al., 2021; Pang et al., 2022).

Some studies had shown that *V. alginolyticus* HY9901 inframe-deletion strains as live attenuated vaccines surely could improve specific and non-specific immunity and reduce mortality of pearl gentian groupers, such as Δ*vscB*, Δ*acfA*, and Δ*clpP* (Chen et al., 2019a; Chen et al., 2020; Pang et al., 2022). VscB as a T3SS chaperone protein, could assist the secretion or transport of the T3SS effector proteins and transported into host cells and participate in host intracellular functions (Pang et al., 2022). ClpP is an ATP-dependent serine protease, it can degrade abnormal proteins in cells, participate in protein quality control and affect the physiological and biochemical metabolism of cells (Chen et al., 2020). AcfA is one of the accessory colonization factors and plays a significant role in the efficient colonization of *Vibrio* in the intestine (Chen et al., 2019a). DctP as C_4-dicarboxylate binding protein, is part of the TRAP transporter system that is capable of collaborating with DctQ and DctM to transport 4-carboxylate dicarboxylates into the cytoplasm and be used as carbon and energy in bacteria (Kelly and Thomas, 2001; Rhie et al., 2018). *dctP* was firstly proved to be one of virulence factors in *Vibrio cholerae* (Sharma et al., 2011). In *V. aginolyticus*, *vscB*, *acfA*, *clpP*, and *dctP* have performed different functions, and play different roles in stabilizing the internal environment of the bacterium as well as improving the invasive ability of specific species. Although some live attenuated vaccines such as Δ*vscB*, Δ*acfA*, and Δ*clpP* were constructed in *V. aginolyticus*, more live attenuated vaccine candidates should be studied and developed to finally screen the most suitable vaccine through clinical application evaluation.

In our previous study, a HY9901Δ*dctP* strain was also constructed and confirmed that the virulence against pearl gentian grouper was significantly reduced (Zhang et al., 2021). However, until now, there have been no studies of a vaccine that is related to *dctP*. Therefore, we wondered whether Δ*dctP* also had potential as a live attenuated vaccine candidate.

Based on the former studies, in this study we analyzed and evaluated the immune protection effect of Δ*dctP* strain as live attenuated vaccines against pearl gentian groupers. Firstly, we evaluated the safety of Δ*dctP* strain. Moreover, we not only detected the expression pattern of immune-related genes in the liver, spleen, head kidney, and thymus but also detected enzyme activity catalase (CAT), superoxide dismutase (SOD), lysozyme (LZM), total serum protein and specific antibody IgM in serum. Finally, mortality rates were measured. The results provided a more comprehensive understanding of Δ*dctP* strain as an attenuated vaccine protecting pearl gentian grouper against Vibriosis caused by *V. alginolyticus*.

Materials and methods

Bacterial strains and fish

V. alginolyticus HY9901 was isolated from disease groupers and stored in our laboratory. *V. alginolyticus* HY9901 Δ*dctP* was constructed from our former study (Zhang et al., 2021) and stored in our laboratory. *V. alginolyticus* HY9901, Δ*dctP* was

cultured in Tryptic Soy Broth (TSB, Huankai, Guangzhou, China) containing 2% NaCl at 28°C for the following study.

Healthy pearl gentian groupers with a body weight 40.0 ± 4.0 g were purchased from East-island fish farm (Zhanjiang, China). Groupers were cultured in an aerated tank containing seawater (filtered by a sea water filter bag) at 26.0 ± 1.0°C, and fed with commercial grouper feed twice a day for 2 weeks, to adapt them to a different environment. Before the experiment, six fish were randomly chosen for bacterial recovery from the spleen, liver, and head kidney on TSA plates (Tryptic soy broth with 1% agar) to ensure the fish were not infected with *V. alginolyticus* or other bacterium. Fish were anesthetized with 100 ng/ml tricaine methanesulfonate (MS-222) before vaccination and sample collection. All animal experiments were strictly executed according to ethical standards and approved by Guangdong Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Economic Animals Ethics Committee.

Preparation of live-attenuated vaccine

Δ*dctP* strain was cultured in TSB with 2% NaCl at 28°C for 12h, then collected and centrifugated at 5000 rpm for 15 min. Bacterium were washed twice with phosphate-buffered saline (PBS, pH 7.2). After that, the concentration of Δ*dctP* was adjusted with PBS to 1.0×10^9, 1.0×10^8, 1.0×10^7, 1.0×10^6, and 1.0×10^5 CFU/ml using UV-Visible Spectrophotometer Evolution 220 (Thermo Fisher Scientific, USA).

Vaccine safety evaluation

A total of 150 fish were randomly divided into 5 groups (30 fish/group), each group was intraperitoneally injected with 0.1 ml 10^9, 10^8, 10^7, 10^6, and 10^5 CFU/ml *V. alginolyticus* HY9901 Δ*dctP*, respectively. *V. alginolyticus* were re-isolated from the liver, spleen, and head kidney of dead fish. All dead fish were counted in 14 days in order to calculate the safety concentration. The experiment was performed three times.

Pearl gentian grouper vaccination

360 fish were randomly divided into PBS and Δ*dctP* group (180 fish/group), each group were intraperitoneally injected with 0.1 ml PBS (control group) and 1×10^5 CFU/ml *V. alginolyticus* HY9901 Δ*dctP* (experimented group), respectively. Liver, spleen, head kidney and thymus (n=3) were collected from 7 d to 28 d and blood (n=3) from 7 d to 56 d post-vaccination, once a week. The serum was collected by centrifugated the blood at 3500 rpm for 20 min at 4°C. Serum, liver, spleen, head kidney and thymus were stored at -80°C until used.

Pathogen load of the live-attenuated vaccine in tissues

Pathogen load of Δ*dctP* in tissues (liver, spleen, and head kidney) from three randomly selected fish of the Δ*dctP* group was calculated, from 1 d to 9 d post-vaccination, once a day. Liver, spleen, and head kidney were obtained and weighed 1.0 ± 0.05 g, respectively. All 3 tissues were added in 3 ml PBS and homogenized together using a motor-driven tissue grinder (Sangon Biotech, Guangzhou, China) at 8000 rpm. Homogenates were 10-fold gradient diluted, with 100 µl on a TCBS plate (Sangon Biotech, Guangzhou, China) at 28°C for 24 h. Colonies with yellow centers were counted. This experiment was performed three times.

Relative percent survival (RPS) of pearl gentian grouper after *V. alginolyticus* HY9901 challenge

After 28 d post-vaccination, 180 fish of PBS and Δ*dctP* groups (90 fish/group) were intraperitoneally injected with 0.1 ml *V. alginolyticus* HY9901 with a concentration of 7.42×10^8 CFU/ml (Zhang et al, 2021) The mortality of each group was monitored for 14 d after bacterium challenge. RPS was calculated using the formula: RPS (%) = [1-(Mortality of vaccinated fish/Mortality of control fish)] × 100%. Bacterium were re-isolated from the liver, spleen, and head kidney of dead fish and incubated on TSA plates. The 16s rDNA (Table 1) was used to identify whether the fish died of *V. alginolyticus* HY9901 infection. The experiment was performed three times.

Quantitative real-time quantitative PCR (qRT-qPCR) analysis

The qRT-qPCR was used to analyze the gene expression of *IgM* (immunoglobulinM), *IL-1β* (interleukin 1β), *IL-8* (interleukin 8), *IL-10* (interleukin 10), *MHC-Iα* (major histocompatibility complex Iα), *MHC2* (major histocompatibility complex II), *TNF-α* (tumor necrosis factor α), *TLR3* (Toll-like receptors 3), and *CD4* (cluster of differentiation 4), with β-actin as internal reference gene. Total RNA was extracted from all samples (liver, spleen, head kidney, and thymus) using TransZol Up Plus RNA Kit (TransGen Biotech, Beijing, China). cDNA was amplified using EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (Takara, Beijing, China), with 1000ng RNA as a template. The qRT-PCR amplification was performed using PerfectStart® Green qPCR SuperMix (TransGen Biotech, Beijing, China) for gene expression analysis. The primers are listed in Table 1. qRT-PCR reaction system (total volume of 10 µl) was as follows: Template (cDNA) 1 µl, Forward primer (10

μM) 1 μl, Reverse primer (10 μM) 1 μl, 2×perfectStart® green qPCR superMix 5 μl and Nuclease-free water 2 μl. The reaction condition was as follows: preincubation at 94°C for 300s, 3-step amplification including 40 cycles of 94°C for 10s, 60°C for 15s, and 72°C for 20s, with melting and cooling performed finally. All data were analyzed by the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001).

Indirect ELISA for detection of antigen-specific serum antibody IgM

Antigen-specific serum antibody IgM was detected using a slightly modified ELISA method (Wei et al., 2020). *V. alginolyticus* HY9901 was incubated at 28°C overnight, then added to 0.5% methanol and shook at 28°C for 48 h. *V. alginolyticus* was centrifuged and washed using PBS, following adjusting the concentration to 1.0×10^8 CFU/ml using 0.05 M pH 9.6 carbonate buffer solution. 100 μl *V. alginolyticus* was added to each well of the 96-well microplate and incubated at 4°C overnight. Wells were washed with PBS added with 0.05% Tween-20 (PBST) three times, then blocked in 100 μl 5% non-fat powdered milk (Sangon Biotech, Guangzhou, China) for 2 h. After that, wells were washed with PBST three times, then added to diluted serum (2-fold continuous gradient dilution, from 2^1 to 2^{12} fold dilution) and incubated at 37°C for 1 h. Then, wells were washed with PBST three times, following adding 100 μl 1:1000 rabbit-anti-pearl gentian grouper IgM polyclonal antibody and incubated at 37°C for 1 h. After that, wells were washed with PBST three times, wells were incubated 100 μl 1:2000 mouse-anti-rabbit IgG conjugated to HRP (Boster, Wuhan, China) at 37°C for 1 h. Wells were washed with PBST three times and added to 100 μl TMB substrate solution (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) for 15 min at room temperature. Finally, 50 μl 2 M H_2SO_4 was quickly added to wells to stop the reaction, then the absorbance of OD_{450} of wells was detected using a microplate reader (Thermo Fisher scientific, USA). The formula for antibody titer: Antibody titer (experimental group T/control group C) = (OD_{450} of serum in experimental group - OD_{450} of serum in blank control). The final antibody titer was the dilution ratio of antiserum when T/C > 2.1. The experiment was performed three times.

Enzyme activity and total serum protein in fish serum

The activities of CAT, SOD, LZM, and total serum protein of fish serum samples were detected using a catalase assay kit (Visible light), superoxide Dismutase assay kit (WST-1 method), lysozyme assay kit and total protein quantitative assay kit, respectively. All kits were purchased from Nanjing Jiancheng Bioengineering Institute in China.

Histopathological analysis post-vaccination

To assess the safety of live-attenuated vaccine Δ*dctP*, livers and spleens of PBS and Δ*dctP* group 28 days after vaccination were collected for histological examination. The tissues of dying fish (*V. alginolyticus* HY9901 group, fish challenged by *V. alginolyticus* in experiment 2.6) were used as positive pathogen control. All tissues were fixed using 10% paraformaldehyde fixative for 48h, then dehydrated in ethanol with increasing concentrations (50%→70%→80%→90%→95%→100%). After that, tissues were equilibrated with xylene, following embedding in paraffin. Finally, paraffin blocks were cut to 4 μm thickness, stained by Haematoxylin–Eosine (Sangon Biotech, Shanghai, China), and the pathological alterations were observed using an optical microscope.

Statistical analysis

Data were statistics by one-way analysis of variance (ANOVA), followed by using SPSS 26.0 software with Duncan's new multiple range test. Data were shown as mean ± SE, and the difference between data were marked as * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

Results

Safety situation of the live-attenuated vaccine

The safety situation of live attenuated vaccine Δ*dctP* was shown in Table 2. Simply, there were no fish dead after injection with 0.1 ml 10^7, 10^6, and 10^5 CFU/ml Δ*dctP*. Therefore, ≤1.0×10^6 CFU Δ*dctP* was the safe vaccine dose for these experimented groupers.

Pathogen load of the live-attenuated vaccine in tissues

Δ*dctP* rapidly spread but only temporally survived in tissues and gradually vanished from the host's body (Figure 1). The results indicated that the highest bacterial number was detected in the liver, spleen, and head kidney (tissues mixture) at 24 h post-vaccination, and the bacteria had totally disappeared from all tissues at 9 d post-vaccination.

Vaccine efficacy

The efficacy of the live-attenuated vaccine was evaluated by fish challenged with HY9901 28 d post-vaccination. Grouper

TABLE 1 Primers used in this study.

Primers	Primer sequence (5′–3′)	Purpose	Product size (bp)	Theoretical Tm (°C)
16s rDNA-F	TTGCGAGAGTGAGCGAATCC	Identification of bacteria species	150	61.1
16s rDNA-R	ATGGTGTGACGGGCGGTGTG			66.7
IgM-F	TACAGCCTCTGGATTAGACATTAG	Expression of IgM	116	58.6
IgM-R	CTGCTGTCTGCTGTTGTCTGTGGAG			66.1
IL-1β-F	TCTGGGCATCAAGGGCACACA	Expression of IL-1β	137	66.6
IL-1β-R	CCATGTCGCTGTTCGGATCGA			66.3
IL-8-F	AGTCATTGTCATCTCCATTGCG	Expression of IL-8	129	60.0
IL-8-R	AAACTTCTTGGCCTGTCCTTTT			59.3
IL-10-F	TTCGACGAGCTCAAGAGTGAG	Expression of IL-10	150	58.0
IL-10-R	TGCCGTTTAGAAGCCAGATACA			60.4
MHC-Iα-F	GCCGCCACGCTACAGGTTTCTA	Expression of MHC-Iα	138	66.3
MHC-Iα-R	TCCATCGTGGTTGGGGATGATC			66.2
MHC2-F	CCACCCGAACAAACAGACC	Expression of MHC2	140	58.1
MHC2-R	TGATGCCCCCTCCAACACT			60.5
TNF-α-F	GCCACAGGATCTGGCGCTACTC	Expression of TNF-α	136	65.8
TNF-α-R	CTTCCGTCGCTGTCCTCATGTG			65.1
TLR3-F	TCTCCATTCCGTCACCTTCC	Expression of TLR3	112	59.5
TLR3-R	TCATCCAGCCCGTTACTATCC			59.2
CD4-F	TTGCGGTGCAAAATCCACTG	Expression of CD4	160	62.6
CD4-R	TGCCATCAGTCCAGGACAAC			58.6
β-actin-F	GGACTCTGAGCGCCGTC	Expression of β-actin	196	56.3
β-actin-R	GTGCGGCGATTTCATCTTCC			62.7

began to die starting on the second day and ending by the ninth. The RPS of the Δ*dctP* group was 74.4%, which was a significant increase compared with the PBS group, which was 23.3% (Figure 2). The results showed that Δ*dctP* only had negligible virulence and significant immunoprotection to groupers, suggesting it is a better candidate as a live-attenuated vaccine.

Immune gene expression of grouper during immune protection of the vaccine

To evaluate the immune response of the pearl gentian grouper immunized with Δ*dctP* live-attenuated vaccine, the transcriptional levels of nine immune-related genes in the liver, spleen, head kidney, and thymus of groupers were measured over 7 d, 14 d, 21 d, and 28 d post-vaccination (Figures 3–6). The expression level of *IgM* was highly expressed in 4 tissues at all time points, except for the liver at 28 d, with the maximum expression levels (10.5-folds) appearing in the thymus at 14 d (Figures 3A, 4A, 5A, 6A). The expression level of *MHC-Iα* (Figures 3E, 4E, 5E, 6E) and *TLR3* (Figures 3H, 4H, 5H, 6H) up-regulated to the highest in 4 tissues at 14 d, with the highest expression levels of 7.1-folds appeared in liver and 11.3-folds in the thymus. *IL-8* (Figures 3C, 4C, 5C, 6C) expressed to the highest level in 4 tissues at 21 d, with 10.2-folds in the thymus expressed to the highest compared with the other three tissues. The expression level of *MHC2* rose to the top in the spleen, head kidney, and thymus at 28 d (Figures 4F, 5F, 6F) and in the liver at 21 d (Figure 3F), with the results of 9.2-folds, 4.6-folds, 17.8-folds, and 6.8-folds, respectively. The expression level of *CD4* rose to the peak in the spleen, head kidney, and thymus at 21 d (Figures 4I, 5I, 6I), and in the liver at 7 d (Figure 3I), with

TABLE 2 Safety evaluation of live attenuated vaccine Δ*dctP*.

Vaccination dose (CFU/fish)	Experimental fish	Dead fish	Mortality (%)
1.0×10^8	10×3	6 ± 0.0	20 ± 0.0
1.0×10^7	10×3	2 ± 0.3	6.67 ± 3.3
1.0×10^6	10×3	0	0
1.0×10^5	10×3	0	0
1.0×10^4	10×3	0	0

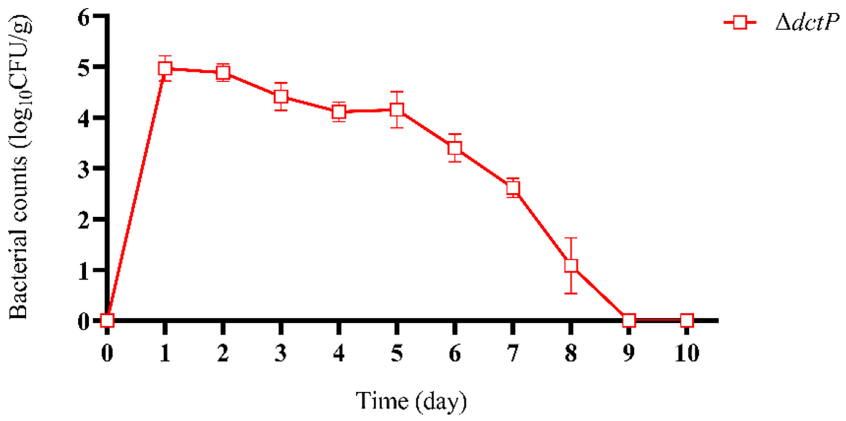

FIGURE 1
Pathogen load of liver, spleen, and head kidney mixture by Δ*dctP* strain. Values are means ± SEM (n = 3).

the results of 4.7-folds, 5.4-folds, 3.0-folds, and 3.7-folds, respectively. Moreover, the expression levels of *IL-1β* (Figures 3B, 4B, 5B, 6B), *IL-10* (Figures 3D, 4D, 5D, 6D), and *TNF-α* (Figures 3G, 4G, 5G, 6G) have different expression patterns in different tissues, but significantly increased compared to those in PBS group at most of sampling time points. The highest expression levels of *IL-1β*, *IL-10*, and *TNF-α* were 8.8-folds in the spleen at 21 d, 6.0-folds in the spleen at 21 d, and 9.8-folds in the liver at 7 d, respectively.

Antibody titers in serum of vaccinated fish

The antibody level of IgM was evaluated by ELISA from 1 to 8 weeks post-vaccination (Figure 7). Antibody titers of Δ*dctP* group displayed the tendency of increasing from 1 to 4 weeks and reached the highest level, then it began to drop from 5 weeks. The specific antibody titers in the Δ*dctP* group were significantly increased ($p < 0.05$) than those in the PBS group at all time points.

Enzyme activity of (CAT, SOD, and LZM) and total serum protein

The enzyme activity and total serum protein in fish serum from 7 d to 42 d were thoroughly detected. CAT activity rose to the highest level at 14 d post-vaccination and gradually dropped to a normal level at 28 d in the Δ*dctP* group (Figure 8A). SOD activity significantly increased from 7 d to 28 d (rising to the highest levels). After that, it decreased to the normal level at 35 d

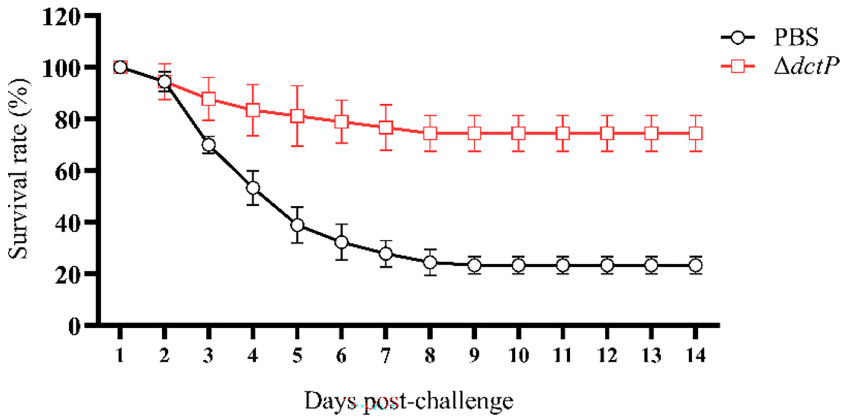

FIGURE 2
Survival rate of vaccinated groupers challenged with *V. alginolyticus* for 14 days. Values are means ± SD (n = 3).

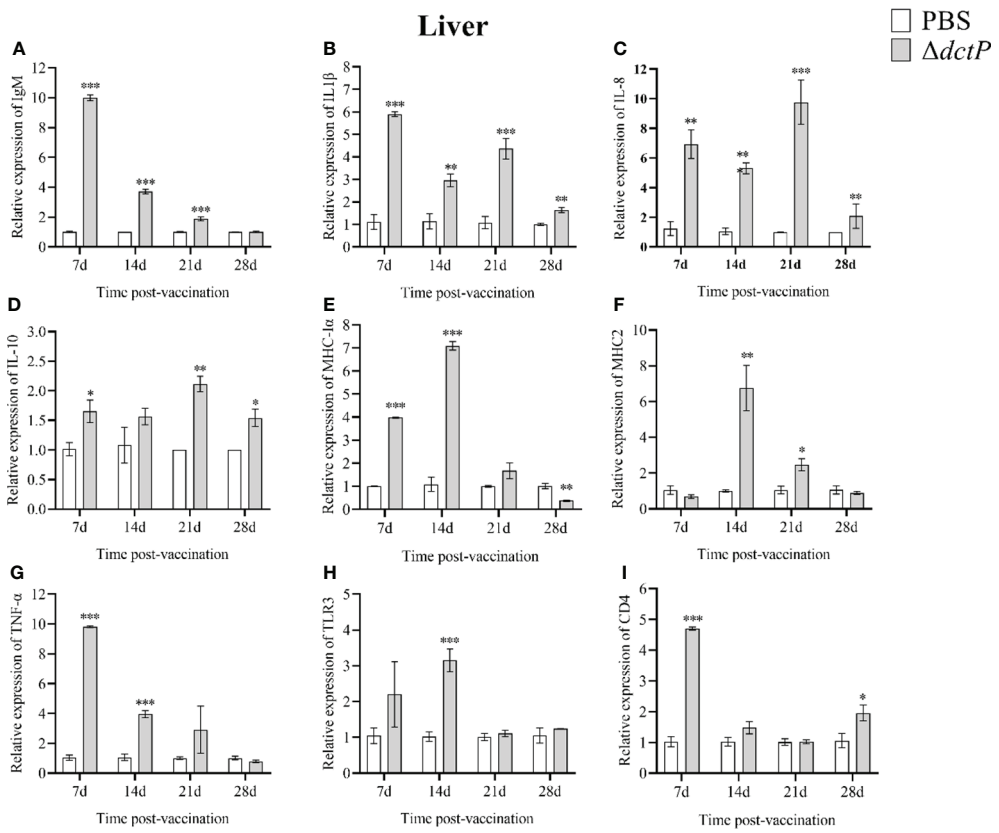

FIGURE 3
The expression levels of immune-related genes in the liver of pearl gentian groupers by qRT-PCR post-immunization. The mRNA level of each immune-related gene was normalized to that of β-actin and relative expression was calculated as the values of the vaccinated tissues by those of the controls. **(A)** *IgM*, **(B)** *IL-1β*, **(C)** *IL-8*, **(D)** *IL-10*, **(E)** *MHC-Iα*, **(F)** *MHC2*, **(G)** *TNF-α*, **(H)** *TLR3*, and **(I)** *CD4*. Bars represented the mean relative expression (n=3) and error bars represent standard errors. The asterisks indicated significant differences: *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$), between PBS group and Δ*dctP* group.

(Figure 8B). LZM activity began to rise at 7 d and reached its peak at 14 d, then gradually decreased until it returned to normal levels at 42 d (Figure 8C). Total serum protein gradually increased and reached the peak at 28 d, then decreased to the normal level at 42 d (Figure 8D). The activities of CAT and LZM reached the top at 14 d, and the SOD activity and total serum protein reached the peak at 28 d.

Histological analysis of livers and spleens post-immunization

There were no tissue lesions in the Δ*dctP* group (Figures 9C, F) compared with the control group (Figures 9A, D). However, histopathological changes were seen in the liver and spleen of fish that were infected with *V. alginolyticus*. Hemorrhage, enlargement, and the cytoplasmic ratio were reduced in the liver (Figure 9B). Necrotic foci and splenic cord were unclear in the spleen (Figure 9E).

Discussion

In recent decades, the outbreak of vibriosis hindered the development of aquatic industry, which also stimulated the development of aquatic vaccines. Currently, many live-attenuated vaccines have been applied and achieved positive effects in preventing vibriosis infection in experimental aquatic animals (Zhang et al., 2012; Gao et al., 2014; Liu et al., 2018; Chen et al., 2019a; Pang et al., 2022). To prevent vibriosis caused by *V. alginolyticus*, our study firstly used *dctP* mutant strain as a live-attenuated vaccine to immunize pearl gentian groupers, notwithstanding Δ*dctP* has not been applied to any aquatic animals as a live-attenuated vaccine so far. The results indicated that Δ*dctP* injection could guarantee safety, prolong immune protection effect, improve specific and non-specific immunity and reduce mortality of pearl gentian groupers, which opened up the possibility of Δ*dctP* to be an effective live-attenuated vaccine in the future.

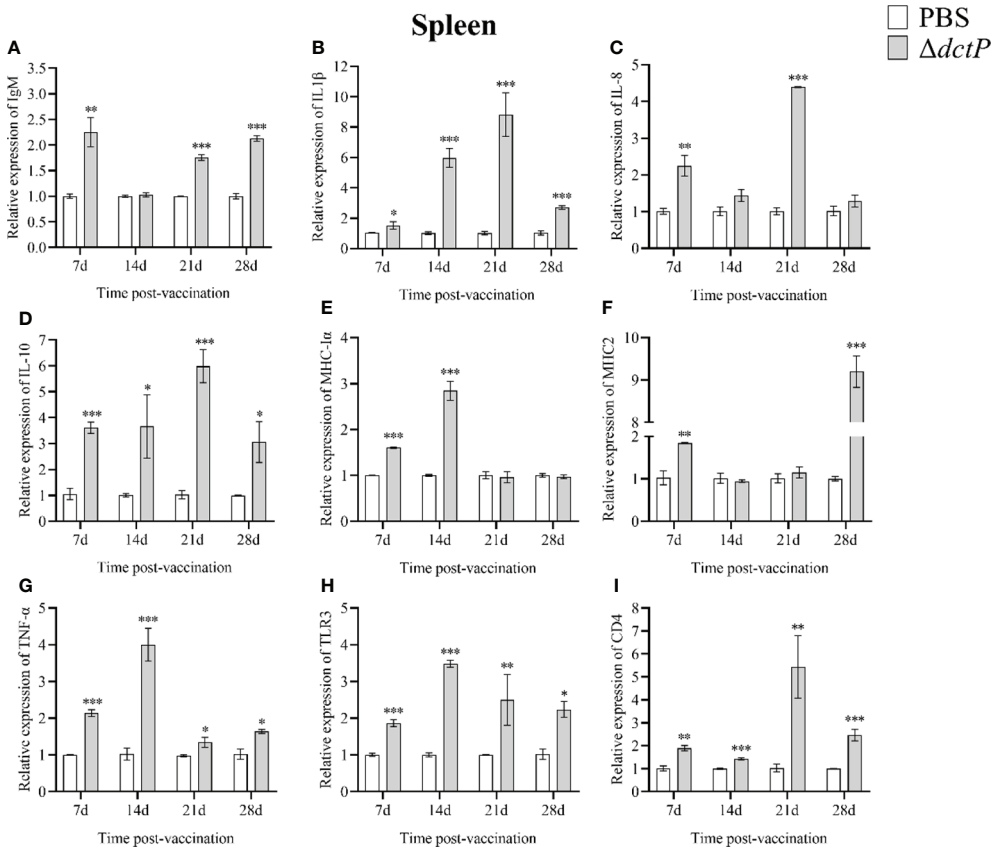

FIGURE 4
The expression levels of immune-related genes in the spleen of pearl gentian groupers by qRT-PCR post-immunization. The mRNA level of each immune-related gene was normalized to that of β-actin and relative expression was calculated as the values of the vaccinated tissues by those of the controls. **(A)** *IgM*, **(B)** *IL-1β*, **(C)** *IL-8*, **(D)** *IL-10*, **(E)** *MHC-Iα*, **(F)** *MHC2*, **(G)** *TNF-α*, **(H)** *TLR3*, and **(I)** *CD4*. Bars represented the mean relative expression (n=3) and error bars represent standard errors. The asterisks indicated significant differences: *($p < 0.05$), **($p < 0.01$) and *** ($p < 0.001$), between PBS group and Δ*dctP* group.

Live attenuated vaccines not only can proliferate in the body for a few days but also do not cause diseases. Therefore, it more effectively activates the immune response and provides longer-lasting protection in animals (Baxter, 2007). Nevertheless, for fish, live-attenuated vaccines have side effects of the possibility of virulence recovery or retention and persistence in the organism (Tlaxca et al., 2015). In this study, we tested Δ*dctP* to ensure its safety before being used as a live attenuated vaccine candidate. In our previous study, the genetic stability and LD_{50} of Δ*dctP* has tested, and the result showed that Δ*dctP* was stable to continue 30 generations and the virulence was almost 40-folds lower than that of the HY9901 (Zhang et al., 2021). In this study, we tested the safety dose situation of Δ*dctP* in fish and concluded that $\leq 1.0 \times 10^6$ CFU was safe injection dose for our experimental groupers (40.0 ± 4.0g). Moreover, we evaluated the pathogen persistence in the spleen and head kidney (tissues mixture) and indicated that Δ*dctP* could be disappeared totally at 9 d post-vaccination. The above results indicated that Δ*dctP* was safe enough to be applied as a live-attenuated vaccine candidate.

RPS is an important and relatively straightforward indicator of vaccine effectiveness. Several studies have developed *V. alginolyticus* mutant strains as live-attenuated vaccines to protect groupers and *Danio rerio*, with RPS ranging from 71% to 87% (Pang et al., 2018; Chen et al., 2019a; Chen et al., 2019; Zhou et al., 2020). In our study, the RPS of groupers injected with Δ*dctP* was 74.4%, indicating that Δ*dctP* could provide better effective protection against challenge by *V. alginolyticus*.

Multiple studies have concluded that live-attenuated vaccines induce stronger humoral and cellular immune responses (Killeen and DiRita, 2001). As immune organs, the liver, spleen, head kidney, and thymus play an important role in humoral and cellular immune processes. The liver plays an important role in innate immunity, metabolism, and growth, supporting host defense and promoting metabolic regulation of

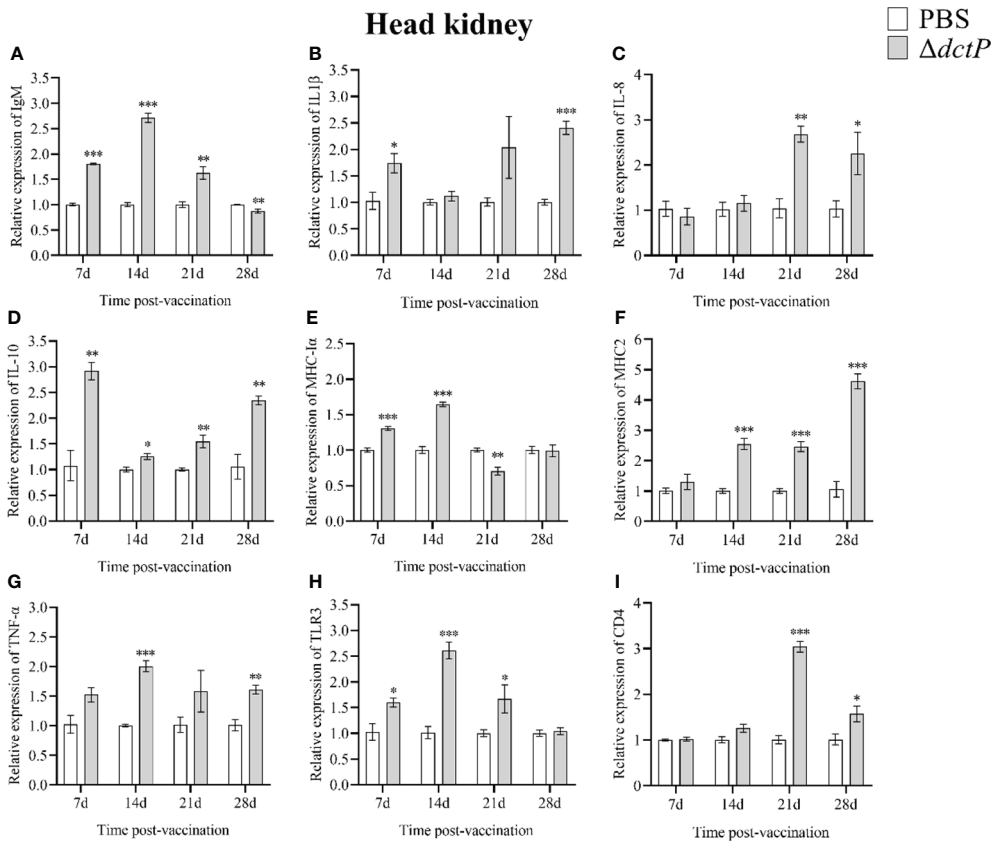

FIGURE 5
The expression levels of immune-related genes in the head kidney of pearl gentian groupers by qRT-PCR post-immunization. The mRNA level of each immune-related gene was normalized to that of β-actin and relative expression was calculated as the values of the vaccinated tissues by those of the controls. **(A)** *IgM*, **(B)** *IL-1β*, **(C)** *IL-8*, **(D)** *IL-10*, **(E)** *MHC-Iα*, **(F)** *MHC2*, **(G)** *TNF-α*, **(H)** *TLR3*, and **(i)** *CD4*. Bars represented the mean relative expression (n=3) and error bars represent standard errors. The asterisks indicated significant differences: *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$), between PBS group and Δ*dctP* group.

effective immune function (Causey et al., 2018). The spleen is the hematopoietic organ of fish with the function of purifying blood (macrophages in the spleen can capture and phagocytose foreign substances in the blood) (Fänge and Nilsson, 1985). The head kidney is the hematopoietic organ of fish and the origin of immune cells, especially B lymphocytes, which play an important role in humoral immune response (Zapata et al., 2006). The thymus is the central immune organ as well as the site of proliferation and differentiation of lymphocytes (Burnet, 1962). Therefore, the expression levels of some immune-related genes in these immune organs were analyzed in this study. IL-1 and IL-8 could activate and regulate immune cells, mediate T and B cell activation, proliferation, and differentiation, or play an important role in inflammatory response (Dinarello, 1997; Remick, 2005). IL-10 is an anti-inflammatory cytokine that plays a role in limiting the immune response to pathogens and therefore preventing damage to the host (Saraiva and O'Garra, 2010). TNF mainly participates in the body's defense response and is also an important pro-inflammatory cytokine and immunoregulatory molecule (Nascimento et al., 2007). TLR3 plays an important role in non-specific immunity (innate immunity) in fish that involves in the defense process against bacteria invasion (Wang et al., 2018). *IL-1β*, *IL-8*, *IL-10*, *TNF-α*, and *TLR3* were upregulated in tissues at different time points post-vaccination, suggesting that the innate immune response of pearl gentian groupers could be activated by Δ*dctP*. IgM is the earliest immunoglobulin in the initial humoral immune response of the animal body that can kill bacteria and activate complements to promote phagocytosis (Castro et al., 2013). CD4 is the co-receptor of TCR on helper T cell (T_H cell) that could bind with MHC class II and MHC class I on antigen-presenting cells (APC) to participate in the TCR recognition antigen process and the transduction of T cell activation signal, respectively (Chaplin, 2010). In the present study, specific immune-associated genes: *IgM*, *MHC-Iα*, *MHC2*, and *CD4* were elevated to varying degrees in tissues at different time points post-vaccination, suggesting that specific immunity including

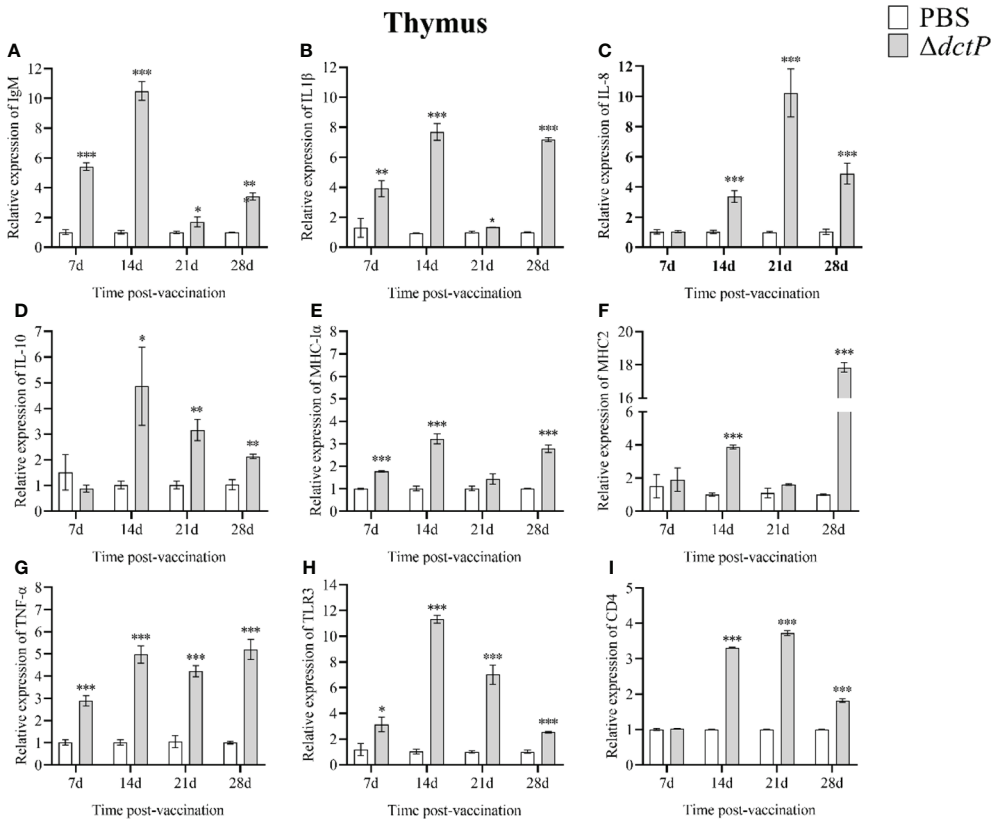

FIGURE 6
The expression levels of immune-related genes in the thymus of pearl gentian groupers by qRT-PCR post-immunization. The mRNA level of each immune-related gene was normalized to that of *β-actin* and relative expression was calculated as the values of the vaccinated tissues by those of the controls. **(A)** *IgM*, **(B)** *IL-1β*, **(C)** *IL-8*, **(D)** *IL-10*, **(E)** *MHC-Iα*, **(F)** *MHC2*, **(G)** *TNF-α*, **(H)** *TLR3*, and **(I)** *CD4*. Bars represented the mean relative expression (n=3) and error bars represent standard errors. The asterisks indicated significant differences: *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$), between PBS group and Δ*dctP* group.

the cellular and humoral immune response of pearl gentian groupers could be also induced by Δ*dctP*.

The primary response is the process in which the antigen first enters the body, stimulates B cells to activate, proliferate and differentiate into plasma cells, and finally secrete antibodies (LeFor and Bauer, 1970). IgM is the earliest antibody produced in serum in the primary response. In our study, the expression level of specific antibody IgM reached the peak at 28 d post-immunization, and it was still upregulated even 56 d post-vaccination. The result was consistent with previous studies (Pang et al., 2018; Chen et al., 2019a; Chen et al., 2019), indicating that the effective and long-lasting humoral immune response was induced by Δ*dctP*.

Antioxidants in serum could defend against free radicals produced in the body and maintain internal stability. According to their response to general free radical invasion, they are classified as first, second, third, and fourth line defense antioxidants (Ighodaro and Akinloye, 2019). SOD and CAT, as antioxidants in the first line of defense, are important and indispensable in the whole defense strategy of antioxidants. SOD catalyzes the decomposition of superoxide anions ($^*O_2$) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), thereby reducing potentially harmful superoxide anions (Ighodaro and Akinloye, 2019). CAT catalyzes the degradation or reduction of H_2O_2 to H_2O and O_2, thus completing the detoxification process simulated by SOD (Marklund, 1984; Chelikani et al., 2004). Lysozyme could cleave the β-1,4-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in cell walls, dissolving bacteria by rupturing the cell wall and releasing the contents (Bera et al., 2005). Blood lysozyme concentration is also an indicator of monocyte and granulocyte proliferation (Bera et al., 2005). Total serum proteins contain albumin and total globulin, which is the indicator of enhanced immunity (Peters, 1985; Busher, 1990). In our study, CAT, SOD, LZM and total serum protein were significantly up-regulated post-vaccination, which demonstrated non-specific immunity of grouper could be widely induced by Δ*dctP*.

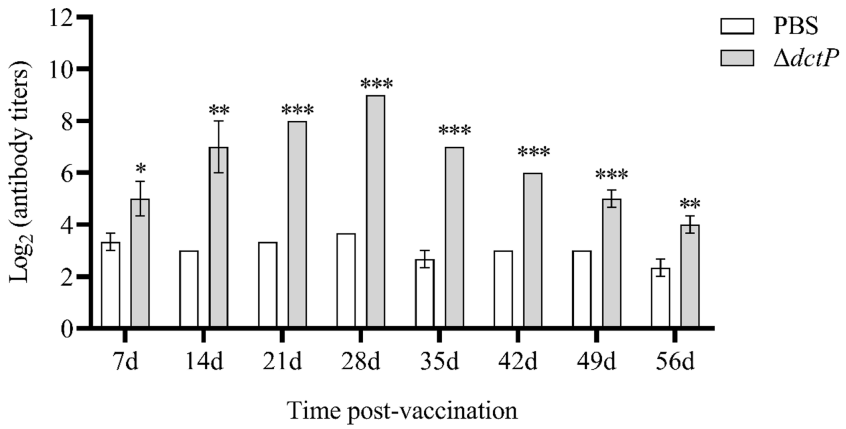

FIGURE 7
Analysis of *V. alginolyticus*-specific serum antibody titers of IgM at 7d, 14d, 21d, 28d, 35d, 42d, 49d, and 56d post-vaccination. Specific antibody titers were detected by ELISA. Bars represented the mean relative expression (n = 3) and error bars represent standard errors. The asterisks indicated significant differences: *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$), between PBS group and Δ*dctP* group.

In addition to injection vaccination, immersion and oral vaccination are more convenient for clinical application. Immersion vaccination has several advantages including reduced handling and stress to fish, lower cost, avoidance of damage to fillets, and the potential to effectively stimulate the teleost immune response by delivery through a route utilized by aquatic pathogens. After immersion live-attenuated vaccination, antigens are taken up by the skin, gills, or gut, and thus adherence and invasion of epithelial cells stimulates the immune system, resulting in a response and the protection of the fish (Locke et al., 2010; Bøgwald and Dalmo, 2019). Live-attenuated oral vaccination has the advantage of intestinal colonization and rapid immune responses, which could result in improved immunological protection against specific bacterial infections

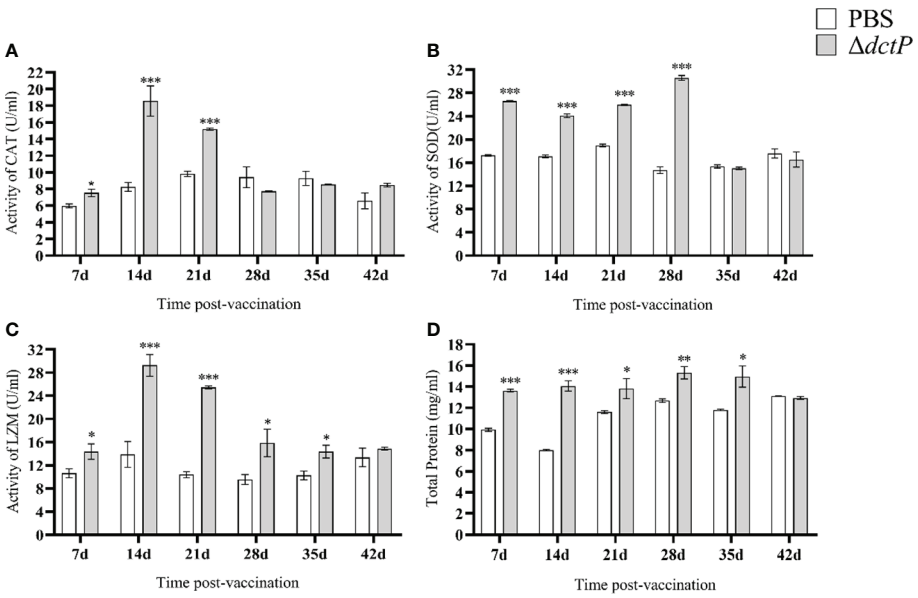

FIGURE 8
CAT, SOD, LZM activity and total serum protein in serum of pearl gentian groupers at 7d, 14d, 21d, 28d, 35d and 42d post-vaccination. **(A)** CAT activity; **(B)** SOD activity; **(C)** LZM activity; **(D)** total serum protein. Bars represented the mean relative expression (n = 3) and error bars represented standard errors. The asterisks indicated significant differences: *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$), between PBS group and Δ*dctP* group.

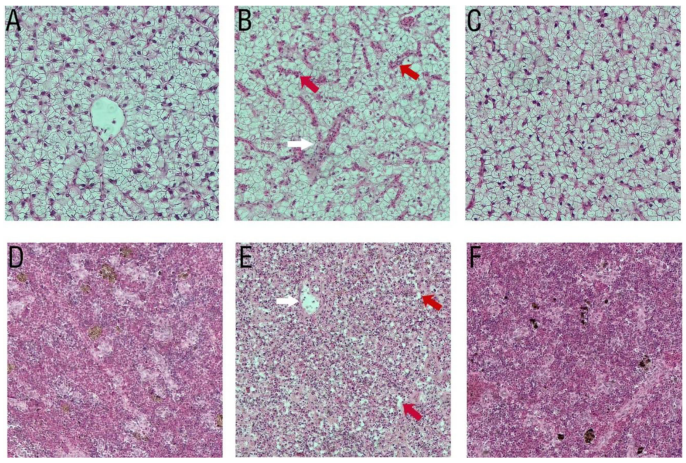

FIGURE 9
Histopathological changes in liver (100 × magnification) and spleen (100 × magnification) of pearl gentian grouper after infection with *V. alginolyticus*. **(A, D)** PBS group, **(B, E)** PBS group infected with V. alginolyticus HY9901, **(C, F)** ΔdctP group infected with V. alginolyticus HY9901. **(A, C)** Liver of group and Δ*dctP* group show normal hepatocytes structure and organization. **(B)** Liver of *V. alginolyticus* HY9901 group show pronounced liver sinus congestion (red arrow) and hemorrhage (white arrow). **(D, F)** Spleen of PBS group and Δ*dctP* group show normal hepatocytes structure and organization. **(E)** Spleen of *V. alginolyticus* HY9901 group shows splenic cord-unclear (red arrow) and necrotic foci (white arrow).

(Ryan et al., 2006). Therefore, Δ*dctP* as a live-attenuated vaccine may be also suitable for immersion and oral vaccination.

In conclusion, Δ*dctP* as a live-attenuated vaccine is not only safe for grouper, but effective in protecting fish from vibriosis caused by *V. alginolyticus* by enhancing specific and non-specific immune responses. Therefore, the current study provides an insight into the protection of grouper with live attenuated vaccines in the future.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Guangdong Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Economic Animals Ethics Committee.

Author contributions

Experimental design, SC, JJ, and YH. Sampling, YZ, ZZ, and JZ. Analyses, YZ, ZZ, and JZ. Supervision, SC and JJ. Writing, original draft, YZ. Writing, review, and editing, SC, JJ, and YH. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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