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Oral delivery of *Bacillus subtilis* spores expressing grass carp reovirus VP4 protein produces protection against grass carp reovirus infection

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ABSTRACT

Grass carp (*Ctenopharyngodon idellus*) hemorrhagic disease (GCHD), caused by grass carp reovirus (GCRV), has given rise to an enormous loss in grass carp industry during the past years. Up to date, vaccination remained to be the most effective way to protect grass carp from GCHD. Oral vaccination is of major interest due to its advantages of noninvasive, time-saving, and easily-operated. The introduction of oral vaccination has profound impact on aquaculture industry because of its feasibility of extensive application for fish in various size and age. However, the main challenge in developing oral vaccine is that antigens are easily degraded and are easy to induce tolerance. *Bacillus subtilis* (*B. subtilis*) spores would be an ideal oral vaccine delivery system for their robust specialty, gene operability, safety and adjuvant property. VP4 protein is the major outer capsid protein encoded by GCRV segment 6 (S6), which plays an important role in viral invasion and replication. In this study, we used *B. subtilis* spores as the oral delivery system and successfully constructed the *B. subtilis* CotC-VP4 recombinant spores (CotC-VP4 spores) to evaluate its protective efficacy in grass carp. Grass carp orally immunized with CotC-VP4 spores showed a survival rate of 57% and the relative percent survival (RPS) of 47% after the viral challenge. Further, the specific IgM levels in serum and the specific IgZ levels in intestinal mucus were significantly higher in the CotC-VP4 group than those in the Naive group. The immune-related genes including three innate immune-related genes (IL-4/13A, IL-4/13B, CSF1R), four adaptive immune-related genes (BAFF, CD4L, MHC-II, CD8), three inflammation-related genes (IL-1β, TNF-α, TGF-β) and interferon type I (IFN-I) related signaling pathway genes were significantly up-regulated in the CotC-VP4 group. The study demonstrated that the CotC-VP4 spores produced protection in grass carp against GCRV infection, and triggered both innate and adaptive immunity post oral immunization. This work highlighted that *Bacillus subtilis* spores were powerful platforms for oral vaccine delivery, and the combination of *Bacillus subtilis* spores with GCRV VP4 protein was a promising oral vaccine.

1. Introduction

Grass carp (*Ctenopharyngodon idellus*) is an essential pisciculture species with an annual production of 6.1 million tons worldwide [1,2]. The aquaculture industry has faced enormous challenges of the grass carp hemorrhagic disease (GCHD) that, during the past years, economic impacts addressed the urgency to tackle this aquacultural illness [3]. GCHD, is a hemorrhagic disease caused by grass carp reovirus (GCRV). The epidemic of GCHD has yielded a mortality rate of up to 81.4% [4]. However, no effective antiviral therapeutics, to date, has been developed to treat the disconcerting disease, becoming an unsolved burden. To conquer GCRV and control the infection, vaccination prevails to be

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the constructive practice that could induce immunity in protecting against GCRV infection [5–7]. Three immunization routes are used to deliver the vaccine in the fish culture industry, which include injection, immersion and oral administration. To our notice, one attenuated vaccine is commercially licensed which requires physical injection to realize the vaccination. However, the injective vaccine was not a success and was impractical nor achievable due to the large sample size of the fish culture industry [3,8]. Given previous experience, developing an obtainable and operation-friendly vaccine may be the essential missing ingredient. Accordingly, oral vaccination may be a more reasonable and convenient approach for mass vaccine implementation [9–11]. Nevertheless, the major obstacle in developing oral vaccine is that during the delivery, process, storage and transportation, protein antigens are susceptible to degradation [3]. New methods must address the concern that antigen degradation will impede the potency of the oral vaccine. Achieving a stable oral vaccine will be highly facilitated by engagement of *Bacillus subtilis* (*B. subtilis*) spores which could attest vaccine effectiveness and potency. *Bacillus subtilis* spores have features to weather extreme conditions such acidity, heat and desiccation that could serves as an ideal vaccine vector. Antigens are harnessed on the spore surface with the outer coating proteins (e.g., CotB, CotC and CotG) as the fusion partners [12]. Moreover, *B. subtilis* spores display safety and strong adjuvant property that could assist in activation of the protective immunity and reducing tolerance [13,14].

Four types of vaccines, to date, are developed to prevent GCHD—inactivated vaccine, attenuated vaccine, recombinant subunit vaccine, and DNA vaccine [3,8]. A major disadvantage of inactivated vaccine, attenuated vaccine and DNA vaccine is their poor safety under natural conditions, as they may be detrimental to the animals and the environments [3]. Recombinant subunit vaccine such as protein vaccine, on the other hand, demonstrated to be safe and with good application prospects.

The pathogen of GCHD—GCRV, is the most virulent type among *Aquareovirus* species recognized so far [15]. There are three genotypes of GCRV (GCRV I, II, III). Among them, GCRV-II is the major genotype causing GCHD [16]. GCRV is a double-stranded RNA virus composed of 11 segments (S1-S11). In each segment, one protein is encoded [17]. VP4 protein is the major outer capsid protein encoded by segment 6 (S6) [15]. It is conservative and plays an important role in viral invasion and replication [18,19], which can be used as a candidate protein vaccine [6]. However, little studies focus on formulating VP4 vaccines in oral form [6,20].

In the present study, we developed an oral vaccine by combining *Bacillus subtilis* spores with GCRV VP4 protein and evaluated its protection in grass carp model. HuNan1307, a virulent strain of GCRV-II, was used for the subunit vaccine preparation, and the coat protein C (CotC) of *B. subtilis* spores was used as the fusion partner. The aim of this work was: (i) to construct pEB03-CotC-S6 recombinant plasmid, *B.s*-CotC-S6 recombinant strain and recombinant spores expressing CotC-VP4 fusion protein (CotC-VP4 spores); (ii) to confirm the VP4 expression on the spore surface; (iii) to verify the immune protection of *B.s*-CotC-VP4 recombinant spores against GCRV; and (iv) to evaluate the immune response and preliminary mechanism.

2. Methods

2.1. Construction of pEB03-CotC-S6 recombinant plasmid and B.s-CotC-S6 recombinant strain

The pEB03-CotC-S6 recombinant plasmid was constructed in accordance with the steps as the schematic representation shown in Fig. 1. In brief, the full-length coding sequence (CDS) of GCRV segment 6 (S6) (1953bp) (GenBank: KU254571.1) was amplified by PCR using pVAX1-S6 (kindly provided by Pearl River Fisheries Research Institute (PRFRI) of Chinese Academy of Fishery Sciences, Guangzhou, Guangdong, China) as template with specific primers. The sequence of the forward

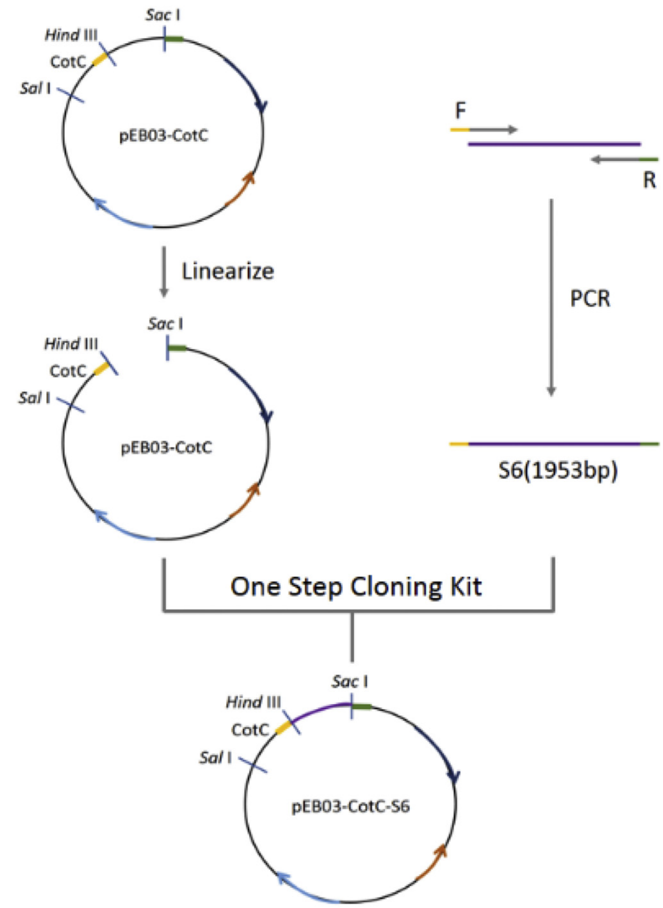

Fig. 1. Schematic representation of constructing pEB03-CotC-S6 recombinant plasmid. The pEB03-CotC plasmid was linearized by using *Hind* III and *Sac* I as restriction sites. The full-length coding sequence of GCRV S6 (1953bp) was amplified by PCR using pVAX1-S6 as template with specific primers (introducing homologous sequences of linearized vector ends (highlighted as yellow and green), aiming to make the ends of amplified inserts and linearized vectors identical to each other). Mixed the linearized vectors and inserts for recombination reaction by using a ClonExpress One Step Cloning Kit. Then the pEB03-CotC-S6 recombinant plasmid was constructed. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

primer was 5′- AAA AGC ATA AAA AAC ACT ACA AGC TTA TGG GAA ACG TCC AGA CGA AC-3' (underlined were homologous sequences of pEB03-CotC), the sequence of the reverse primer was 5′- AAA TGA GTG GCA AAG TGC TAG AGC TCC TAA GAC GGA GGA GGC CAG T-3' (underlined were homologous sequences of pEB03-CotC). The pEB03-CotC plasmid was previously constructed and stored in our laboratory and was used as a vector. It was linearized by using *Hind* III and *Sac* I as restriction sites. The amplified products and enzyme-digested products were determinated by DNA gel electrophoresis and then recovered by a high-quality gel recovery kit (TIANGEN, Beijing, China). After acquisition of the S6 insert and the linearized pEB03-CotC vector, the recombination reaction was conducted by using a ClonExpress One Step Cloning Kit (Vazyme, Nanjing, China) in strict accordance with the manufacturer's instructions. Then the recombination products were transformed into DH5α competent *E.coli* strain and the bacterial solution was plated on a agar plate and mono-colonies were picked after overnight culture for colony PCR to select the positive colonies. Remaining bacterial solution of positive colonies was used to extract the pEB03-CotC-S6 recombinant plasmid by TIANprep Rapid Mini Plasmid Kit (TIANGEN, Beijing, China). S6 gene fragments were verified by PCR amplification using the pEB03-CotC-S6 recombinant plasmid as template and further confirmed by DNA sequencing. In the end, the recombinant plasmid was chemically transformed into *B. subtilis* WB600 strain (routinely preserved in our laboratory) to harvest the *B.s*-CotC-S6 recombinant strain. PCR amplification and DNA sequencing were also done to confirm the recombinant strain was successfully constructed *B. subtilis* WB600 strain with the pEB03-CotC plasmid (*B.s*-CotC), which was routinely preserved in our laboratory, was used as the control in the subsequent experiments.

2.2. Preparation of recombinant spores

The method of harvesting the recombinant spores was as described previously [21,22]. Briefly, *B.s*-CotC-S6 and *B.s*-CotC were cultured in Difco Sporulation Medium (DSM, BD, Franklin Lakes, USA) to induce sporulation by the exhaustion method [23]. 1 ml of the spore liquid induced at different time points (0 h, 6 h, 12 h, 24 h) was centrifuged and the precipitate was collected for further analysis. After induction for 24 h, the spores were harvested by centrifugation and were treated with 4 mg/ml lysozyme for 30 min at room temperature (RT) to break residual sporangial cells, then were washed with 1 M NaCl and 1 M KCl with 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, St. Louis, USA) to inhibit proteolysis, followed by washing three times with deionized water. After the final wash, the spores were resuspended in deionized water and treated in a 65 °C water bath for 1 h to kill the residual WB600 propagules. Spore samples were used immediately or stored at 4 °C until use.

2.3. SDS-PAGE and Western blotting

VP4 expression on the spore surface was analyzed by SDS-PAGE and Western blotting. Coat protein of the spores was first extracted as follows: the induced 24 h spores were treated with sodium dodecyl sulfate (SDS)-dithiothreitol (DTT) extraction buffer (0.5% SDS, 0.1 M DTT, 0.1 M NaCl) at 37 °C for 2 h, followed by washing with 1 M Tris-HCl buffer (pH 8.0) for six times, then were suspended in 5 ml broken buffer (50 mM Tris-HCl, 0.5 mM EDTA, 1 mM PMSF) and were ultrasonicated for 5 min. The coat protein was collected from the precipitate after centrifugation. The spores induced at different time points (0 h, 6 h, 12 h and 24 h) as well as the supernatant and precipitate of the ultrasonic lysates were analyzed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and were visualized by Coomassie brilliant blue G-250 staining. rVP4 prokaryotic protein (kindly provided by PRFRI) and CotC spores were used as the controls.

The separated supernatant and precipitate of the ultrasonic lysates were also transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, USA) after SDS-PAGE for Western blotting analysis. After blocking with 5% skim milk in phosphate buffered saline (PBS)-Tween (PBST), the membrane was incubated with rabbit anti-rVP4 serum (kindly provided by PRFRI, 1:1000 diluted with PBST) for 2 h at RT. Followed by washing 5 times with PBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5000 diluted with PBST, Sigma-Aldrich, St. Louis, USA) and finally visualized by the enhanced chemiluminescence (ECL) method.

2.4. Immunofluorescence

Immunofluorescence was done to verify whether VP4 was displayed on the spore surface according to improved methods previously described [24,25]. Briefly, 200 μl of the CotC-VP4 or CotC (used as control) spore suspension was fixed onto slides. The slides were blocked with normal goat serum overnight at 4 °C followed by incubation with rabbit anti-rVP4 serum (1:200 diluted with PBST) used as the primary antibody for 2 h at RT, naive rabbit serum at the same dilution was used as a control. After washing thoroughly, cyanine3 (Cy3)-labeled goat anti-rabbit IgG (1:500 diluted with PBST, Invitrogen, Carlsbad, USA) was used as the secondary antibody for incubation for 1 h at RT (in dark). The samples were incubated for 3–5 min with a DNA staining solution-4′, 6-diamidino-2-phenylindole (DAPI). Finally, the images of the samples were captured under a fluorescent microscope (Leica DFC500 Digital Camera, Barnack, German) in dark.

2.5. Flow cytometry

To detect the expression rate of VP4 expressed on the spore surface, flow cytometry was applied as previous reports [24,26]. Briefly, about 10^6 CotC-VP4 or CotC (used as control) spores were resuspended in 30 mM $NaPO_4$ buffer (pH = 7.4, containing 2.4% paraformaldehyde and 0.04% glutaraldehyde), and were placed at RT for 10 min, then on ice for 50 min. After washing with PBS for 3 times, 200 μl GTE buffer was added to resuspend the precipitate, followed by placing at 37 °C for 30 min. After wash, the tubes were incubated with rabbit anti-rVP4serum (1:200 diluted with 1% BSA-PBS) as the primary antibody for 1 h at 37 °C, naive rabbit serum at the same dilution was used as a control. Then the tubes were washed with PBS for 4 times. Next, fluorescein (FITC)-conjugated goat anti-rabbit IgG (1:50 dilution, AB-clonal, USA) was added as the secondary antibody for incubation for 1 h at RT. After final wash, the spores were resuspended in PBS and transferred into the flow tube and counted with a flow cytometer (BD FACS Verse). Data were analyzed with the software FlowJo version 7.6.1 (Tree Star, Inc., USA).

2.6. Ethics statement

The animal study was carried out in accordance with National Institutes of Health on animal care and the ethical guidelines and protocols approved by the Animal Ethics and Welfare Committee of SYSU (Permit Numbers: SCXK (Guangdong) 2016-105XS).

2.7. Preparation of experimental fish and forages

Healthy and lively grass carp (23 ± 2 g mean weight and 11 ± 1 cm body length) were kindly obtained from PRFRI. Fish were acclimatized to the laboratory conditions for two weeks prior to any administration. After two weeks, grass carps (n = 160) were randomly distributed into four 50 L tanks (40 fish per tank). The temperature of each tank maintained at 25–28 °C and sufficient oxygen was supplied to all fish. The water was replaced every two days.

The CotC-VP4 spores and CotC spores were harvested according to the above method. The number of spores was calculated as 1.0×10^{12} spores/ml by using the gradient dilution method. Commercial forages were purchased from Foshan Shunde Jinfeng Feed Plant (Guangdong, China). Spores diluted with sterile water were sprayed to the surface of forages in accordance with 1.0×10^{12} spores/g with a sprinkling can, then cod-liver oil were wrapped onto the mixed forages to prevent the loss of the spores in water. After thorough drying, forages were stored at −20 °C until use.

2.8. Oral immunization and sample collection

There were four groups in total. Grass carp orally treated with forages containing CotC-VP4 spores were the experimental group (CotC-VP4 for short). Grass carp orally treated with forages containing CotC spores were the negative control group (CotC group for short), and grass carp orally treated with normal forages without spores were the blank control group (Naive group for short). At the same time, grass carp which were intraperitoneally injected only once at week 0 with a dose of 1×10^3 TCID50 (200 μl) of the commercial attenuated GCRV vaccine (PuLin Biological Products Co., Ltd., Guangzhou, China) were the positive control group (Injection group for short). Grass carp were fed with prepared forages continuously in accordance with 1% of the fish weight (in a final concentration equivalent to 2.3×10^{11} spores/

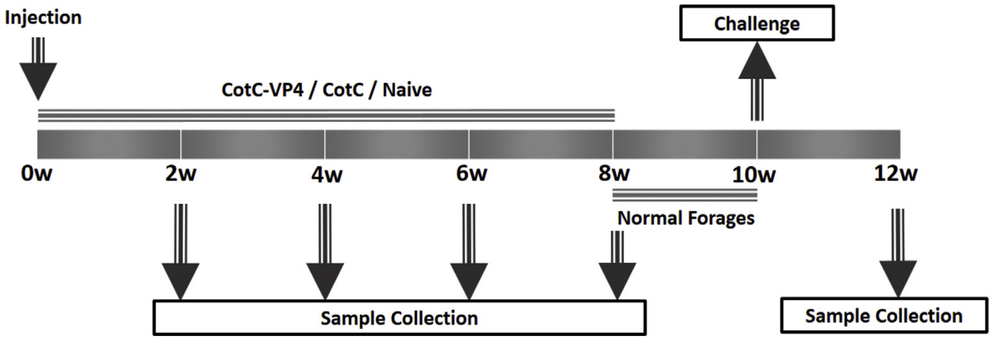

Fig. 2. Strategy of oral administration of grass carp. CotC-VP4 group, the experimental group, which was orally treated with forages containing CotC-VP4 spores. CotC group, the negative control group, which was orally treated with forages containing CotC spores. Naive group, the blank control group, which was orally treated with normal forages without spores. Injection group, the positive control group, which was injected with commercial attenuated vaccine. Samples collection meant samples were collected at week 2, week 4, week 6, week 8 and week 12 after the first oral administration. The samples included blood, intestine mucus, and liver, spleen, head kidney, kidney, intestine and muscle tissues.

fish/day, amount to 1×10^{-3} μg/g (protein/fish)) for 8 weeks in the CotC-VP4 group and CotC group. Meanwhile, grass carp were treated with normal forages in Naïve group and Injection group. And grass carp in all groups were treated with normal forages from week 8 to week 12 (Fig. 2).

Samples were collected at week 2, week 4, week 6, week 8 after the first oral administration (Fig. 2). Blood, intestine, spleen, head kidney, kidney and hind gut tissues were obtained from 5 fish per group at each time point. Clove oil was added into water to anesthetize fish by immersion before dissection. Sample collection was performed according to previous methods [27]. Briefly, blood was extracted from the caudal vein of fish with 1 ml sterile syringe. The blood was put at RT for 1–2 h to clot before stored at 4 °C overnight, followed by centrifuging at 4 °C, 4000 rpm for 20 min to collect serum. About the same length of intestine was aseptically isolated from each fish, then was lavaged with 1 ml sterile PBS and scraped with tweezers to collect lavage fluid. The lavage fluid was centrifuged at 5000 rpm for 20 min at 4 °C before supernatant collection as intestinal mucus. Serum and intestinal mucus samples were stored at −20 °C until use. The spleen, head kidney, kidney and hind gut tissues were carefully separated with sterile scissors and tweezers, then each sample was added with 200 μl sample protector (TaKaRa Bio, Otsu, Japan) before stored at −80 °C.

2.9. Detection of the spores' colonization in intestine by PCR

At the time point of 10 weeks post the first oral administration before challenge, the intestine (2 cm length) was taken from the CotC-VP4 spores immunized grass carp, and the intestinal contents were scraped under aseptic conditions, followed by thoroughly mixed with 500 μl of sterile physiological saline, and the mixture was placed in a water bath at 68 °C for 15 min to kill the bacteria except spores. After the mixture was serially diluted to 10^{-5}, 100 μl was uniformly coated on the spectinomycin-resistant LB agar plates. Then the plates were placed in a 37 °C incubator for 14–18 h. Several colonies were picked for PCR amplification to detect the colonization of spores in intestine. The specific primers were as follows: Forward: 5′- TAC AAG CTT ATG GGA AAC GTC CAG ACG AAC-3'; Reverse: 5′- CTA GAG CTC CTA AGA CGG AGG AGG CCA GT -3'. Finally, the amplified products were identified by 1% agarose gel electrophoresis.

2.10. Challenge and determination of viral load

At the time point of 10 weeks post the first oral administration, grass carp (n = 20 for each group) were challenged with 1×10^3 TCID50 GCRV strain HuNan1307 by intraperitoneal injection, with a heating rod in each tank to keep the water temperature constant at 28 °C. Any clinical sign or mortality was monitored. The target tissues of GCRV including spleen, kidney, intestine, liver and muscle tissues were collected from dead fish daily until 14 days post-challenge when no fish died in all groups. All fish were euthanized and the five tissues were collected at day 15 post-challenge. The cumulative survival rate was recorded to assess the protection efficacy of CotC-VP4 spores and the relative percent survival (RPS) was calculated according to Amend's method [28]. The formula was as follows: RPS = [1- (% mortality of immunized group/% mortality of control group)] × 100. Further, virus-specific Taqman probe was used to detect the viral load by qRT-PCR in the five tissues of 5 dead fish in each group except for 5 euthanized fish in Injection group to confirm that the fish died of GCRV infection and to determine the antiviral effect of the oral vaccine.

2.11. Detection of specific antibody levels by ELISA

The specific IgM antibody levels in serum and the specific IgZ antibody levels in intestinal mucus against VP4 in immunized fish or control fish were measured by ELISA as described in previous study [29]. Briefly, rVP4 was used as antigen, the serum samples were used as primary antibody, and HRP-conjugated rabbit anti-grass carp IgM (Zoonbio Biotech Corp., Nanjing, China) was used as secondary antibody to determine the specific IgM antibody levels in serum. In addition, the intestinal mucus samples were used as primary antibody and HRP-conjugated mouse anti-grass carp IgZ (Zoonbio Biotech Corp., Nanjing, China) was used as secondary antibody to determine the specific IgZ antibody levels in intestinal mucus. 100 μl of rVP4 (5 μg/ml) was coated in each well of plates and incubated at 4 °C overnight. After blocking with 5% skim milk at 37 °C for 2 h, fish samples were added to the plates and were incubated for 1 h at 37 °C (serum was 1:50 dilutions, intestinal mucus was 1:5 dilutions). Then the plates were washed and incubated with HRP-conjugated rabbit anti-grass carp IgM (1:5000 dilutions) for serum samples or HRP-conjugated mouse anti-grass carp IgZ (1:300 dilutions) for intestinal mucus samples for 1 h at 37 °C, followed by incubation with tetramethylbenzidine (TMB) substrate (BD, Biosciences Pharmigen) for 10–15 min at RT after washing. Finally, 2 M H2SO4 was used to stop the reaction and the OD value of each well at 450 nm was read with a precision microplate reader (Bio-Rad iMark, USA).

2.12. Determination of immune-related genes expression by qRT-PCR

Four tissues including spleen, head kidney, kidney and hind gut were collected to analyze immune-related genes expression after oral administration by qRT-PCR according to the manufacturer's protocol (TransGen Biotech, Beijing, China). In brief, total RNA of the tissue samples was extracted with the TRIzol reagent, then total RNAs were reversely transcribed into cDNA with TransScript All-in-One First-Strand cDNA Synthesis SuperMix (One-Step gDNA Removal) kit (TransGen Biotech, Beijing, China), and finally, the expression of a set of immune-related genes was determined using quantitative real-time polymerase chain reaction (qRT-PCR). The immune-related genes included three innate immune-related genes, five adaptive immune-related genes, three inflammation-related genes and IFN-I related signaling pathway genes. The innate immune-related genes were interleukin-4/13A (IL-4/13A), interleukin-4/13B (IL-4/13B) and

Table 1
Genes and primer sequences used in the qRT-PCR assays.

Genes	Forward (5′-3′)	Reverse (5′-3′)
18S	ATTTCCGACACGGAGAGG	CATGGGTTTAGGATACGCTC
IL-4/13A	ATGATGAAGACTATACTACTGC	CTCTAAGAGTGTTTTCGGTG
IL-4/13B	CAAGCAGCAAAGGTCCTGAATG	TCACTGGATGTTCCTCTGAAGC
CSF1R	TCTTACGGCATTCTGCTGTG	TGAATGTTGGTCTCTCAGCG
Foxp3	GCAGGGAAGTGTTCAAAGAA	TAGCTGCATGGCCTGCAGTT
BAFF	GTTGTCGCTGCCATCACCTC	CCCTCAAACCCTCCACCTCA
CD8	GAGTCTCTGCACGGATCTAT	GTGTAGTGTTCCGAATTTAAGT
CD4L	ATGTGTCCAGGTGTCATAGT	GGAATTTTGACTGTATAGGAT
MHC-II	TACTACCAGATTCACTCGG	CGGGTTCCAGTCAAAGAT
IL-1β	GATTCGAAAGTTCGATTCAATCT	TTCAGTGACCTCCTTCAAGAC
TNF-α	TGTGCCGCCGCTGTCTGCTTCACGCT	GATGAGGAAAGACACCTGGCTGTAGA
TGF-β	TGGACTGGAAGTGGATGCAT	TCTAGCACTTGGGGTACACG
IFN-I	GGTGAAGTTTCTTGCCCTGACCTTAG	CCTTATGTGATGGCTGGTATCGGG
RIG-I	ACTACACTGAACACCTGCGGAA	GCATCTTTAGTGCGGGCG
MDA5	CAGGAGCGACTCTTGGACTATG	AAAGACGGTTTATTTGAATGGAAG
LGP2	CGTCTACTCGGTGGTGGCT	AAACTCCCTGGGACTCATACTCT
TLR3	GAGAACAATCGTGACTCCCTGA	CCAGTAGAGAACACAGCGAGGT
TLR7	GAGCATACAGTTGAGTAAACGCAC	TCTCCAAGAATATCAGGACGATAA
TLR8	TCACATCGCTTCCAGGTCTC	ACGGTGAAATAATGGGGGTT
TLR9	CAGTTGCGTTATCTCGGGGT	CTTCAGGAGGGGAATGATGGT
TLR21	ACAATCGTATTCTACAGGTCGGAG	GATCTTTGAGTCCATGTAAGGCTTT
TLR22	TATACAGCAGCCGCAAAACC	CCAGGAACACCAGGATCAGC
IPS-1	GACCGTAAGAAGTCAGCCTCC	CCTGAATAACTCTTGATAGCCCTC
IRF3	TGTGGACACTGACGGACCCTTC	CGGCTGCTGATATGCTGGAGAA
IRF7	GAAGAGACCTTGGGGACGAG	TTGAGGACGGATAATGCGAT
NF-kB	AGGCACTTCCTCAGCACTACGAT	AAAACCTCCTCCCATTCCACC
Mx1	CTGGGGAGGAAGTAAAGTGTTCT	CAGCATGGATTCTGCCTGG
Mx2	ACATTGACATCGCCACCACT	TTCTGACCACCGTCTCCTCC
Mx3	CCTTAAAGACGCTGAAGACCA	GCAACCTCATCTCACGCAA

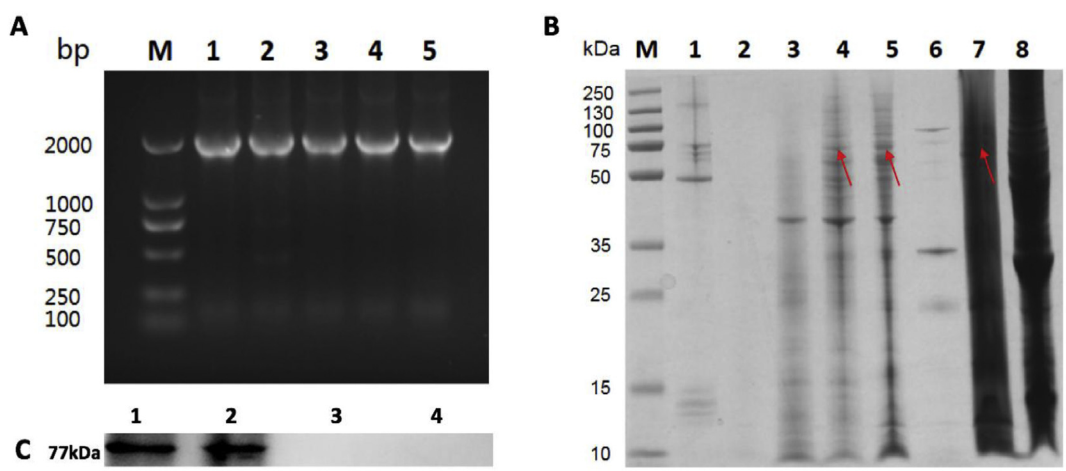

Fig. 3. Successful construction of pEB03-CotC-S6 recombinant plasmid and CotC-VP4 recombinant spores. (A) The full-length coding sequence of S6 was cloned into a pEB03-CotC plasmid and was confirmed by PCR with pEB03-CotC-S6 recombinant plasmid as template. Lane M: DNA marker, Lane 1–5: S6 gene (1953 bp). **(B)** The expression of CotC-VP4 fusion protein at different incubation time by SDS-PAGE. Lane M: protein marker, Lane 1: VP4 prokaryotic protein, Lane 2: 0 h induction, Lane 3: 6 h induction, Lane 4: 12 h induction, Lane 5: 24 h induction, Lane 6: supernatant of CotC-VP4, Lane 7: precipitation of CotC-VP4, Lane 8: CotC spores. The arrows indicated the possible expression band. **(C)** Expression identification of CotC-VP4 fusion protein by Western blotting. Lane 1, 2: precipitation of CotC-VP4, Lane 3,4: supernatant of CotC-VP4.

colony-stimulating factor 1 receptor (CSF1R). The adaptive immune-related genes were B-cell activating factor (BAFF), cluster of differentiation 4-like (CD4L), major histocompatibility complex class II (MHC-II), cluster of differentiation 8 (CD8) and forkhead box P3 (Foxp3). The inflammation-related genes were interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), transforming growth factor beta (TGF-β). The interferon type I (IFN-I) related signaling pathway genes were IFN-I, retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), laboratory of genetic and physiology 2 (LGP2), Toll-like receptor (TLR) 3, TLR7, TLR8, TLR9, TLR21, TLR22, IFN-β promoter stimulator-1 (IPS-1), IFN regulatory factor (IRF) 3, IRF7, nuclear factor-Kb (NF-kB), Myxovirus resistance (Mx) 1, Mx2 and Mx3. The primers for each gene were listed in Table 1 qRT-PCR reactions were performed in a 20 μl final volume and were carried out with CFX96 Real-Time PCR Detection System (Bio-Rad). CFX Manager software was used to analyze the threshold cycle (Ct) value [30]. Each assay was performed in triplicate with 18S RNA as an internal control to calculate the relative folds of expression by using the ΔΔCt method [31,32]. More details could be found in the literature [33].

2.13. Statistical analysis

Data from three independent experiments were presented as means ± standard deviation (SD). Multiple pairwise comparisons were performed by using one-way analysis of variance (ANOVA) with Bonferroni tests using GraphPad Prism vision (version 6.01 for Windows, San Diego). The survival rate of fish was analyzed using the Kaplan-Meier survival curve with log-rank test. In all analyses, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Successful construction of pEB03-CotC-S6 recombinant plasmid and CotC-VP4 recombinant spores

The full-length coding sequence of S6 (1953 bp) was ligated to the 3′ end of CotC of pEB03-CotC vector. CotC-S6 gene fragment was successfully amplified by PCR with the recombinant plasmid as a template (Fig. 3A). The pEB03-CotC-S6 plasmid was then transferred into *B. subtilis* WB600 strain and induced for sporulation in DSM medium. The recombinant spores expressing CotC-VP4 fusion protein (CotC-VP4 spores) were harvested. CotC spores were harvested by the same method which used as the control. The molecular weight of the CotC-VP4 fusion protein was approximately 77.1 kDa, corresponding to the molecular weight of VP4 (68.3 kDa) plus CotC (8.8 kDa). As shown in Fig. 3B, the CotC-VP4 fusion protein was expressed in CotC-VP4 spores, while no matching band was observed in CotC spores, and the expression of the CotC-VP4 fusion protein gradually increased with incubation time (from 0 h to 24 h). In addition, large amount of CotC-VP4 fusion protein was in the extract precipitation which contained the coat protein of the spores, but almost no corresponding band was present in the supernatant (Fig. 3B). Western blotting analysis also showed specific band at 77 kDa in the precipitation while no band in the supernatant (Fig. 3C). Taken together, these results indicated that the pEB03-CotC-S6 recombinant plasmid and the CotC-VP4 spores expressing CotC-VP4 fusion protein were successfully constructed.

3.2. Immunofluorescence and flow cytometry analysis of VP4 expressed on the spore surface

After the pEB03-CotC-S6 recombinant plasmid and the CotC-VP4 spores expressing CotC-VP4 fusion protein were successfully constructed, we next used immunofluorescence and flow cytometry to further confirm VP4 expression on the spore surface. Immunofluorescence results showed that red fluorescence (VP4) was abundantly distributed on the CotC-VP4 spore surface incubated with rabbit anti-rVP4 serum (Fig. 4A). Almost no red fluorescence was visualized on the CotC spore surface incubated with rabbit anti-rVP4 serum (Fig. 4B), or the CotC-VP4 spore surface incubated with naive rabbit serum (Fig. 4C). Flow cytometry results showed that VP4 was abundantly distributed on the CotC-VP4 spore surface with a high positive rate (44.7%) (Fig. 4D), while a low positive rate of VP4 was detected on the CotC spore surface incubated with rabbit anti-rVP4 serum (3.60%) (Fig. 4E), or the CotC-VP4 spore surface incubated with naive rabbit serum (2.45%) (Fig. 4F). Our results showed that VP4 was expressed on the spore surface.

3.3. Colonization of spores in intestine

The intestinal contents in the CotC-VP4 spores immunized group were collected and diluted for plates and PCR at week 10. As shown in Fig. 5A, there were several colonies grown on the plates. And the specific PCR products (about 2000 bp in agarose gel electrophoresis) could be amplified by using specific primers from colonies on plates (Fig. 5B), suggesting that the CotC-VP4 spores could colonize in the intestine of immunized grass carp.

3.4. Assessment of protective efficacy of CotC-VP4 spores

After 8 weeks of continuous oral administration with the prepared forages to grass carp, we evaluated the protective efficacy of CotC-VP4 spores. All groups of grass carp were challenged with 1×10^3 TCID50 GCRV strain HuNan1307 at week 10. The mortality and clinical signs of the challenged fish were recorded daily for two weeks post-challenge. In the challenge trials, the dead or moribund fish showed typical clinical symptoms of GCRV infection, including dark body color, different degrees of congestion, hemorrhage in operculum, gill, fin base, muscle and intestinal wall tissues and around the eyes. About 57% of the grass carp were protected in the CotC-VP4 spores immunized group; the attenuated vaccine group showed a higher protection rate reaching 100%. The protection rate of the CotC spores immunized group was 36% and the protection rate of the Naive group was less than 19% (Fig. 6). The CotC-VP4 spores immunized group (CotC-VP4 group) showed a higher protection rate in comparison with that of the blank control group (Naive group) (57% vs 19%, $p = 0.0043$), demonstrating that the CotC-VP4 spores could protect the fish from hemorrhagic disease in certain extent. The RPS of each group were shown in Table 2. Grass carp orally immunized with CotC-VP4 spores showed the RPS of 47% compared to those in the blank control group post-challenge.

3.5. Determination of GCRV viral load in different tissues after challenged with GCRV

After we assessed the protective efficacy of CotC-VP4 spores, we tested whether the protective effect of the oral administration of CotC-VP4 spores was achieved by reducing the viral load in the immunized fish. The transcriptional levels of GCRV were quantified by qRT-PCR in spleen, kidney, intestine, liver and muscle tissues of the immunized and unimmunized fish after challenge with GCRV (Fig. 7). The levels of GCRV gene expression in the immunized fish were lower than that in unimmunized fish, regardless of the assessed organs. Furthermore, the viral load was the highest in the spleen among all tissues in unimmunized fish, and was also high in intestine, liver and kidney. Conversely, the viral load greatly reduced or even almost undetectable in the liver, kidney, and spleen of the orally immunized fish, suggesting that the oral vaccine greatly reduced the amount of virus in fish internal organs.

3.6. Detection of specific antibody levels in serum and intestinal mucus

We next explored the potential antiviral mechanism involved. Specific antibodies in serum and intestinal mucus were measured by ELISA. The result indicated that specific IgM levels in the serum of the CotC-VP4 group increased from week 2 and reached its peak at week 4. The IgM levels were significantly higher in the CotC-VP4 group than those in the Naive group until week 6 (Fig. 8A). In intestinal mucus, specific IgZ levels in the CotC-VP4 group were significantly higher than those in the Naive group at week 2 and week 4, and reached its peak at week 2 (Fig. 8B). The results demonstrated that the CotC-VP4 spores triggered both systemic and local mucosal humoral immunity in grass carp. Taken together, our findings suggested the elevated levels of specific IgM and IgZ may protect the grass carp of CotC-VP4 spores immunized group against GCRV.

3.7. Expression of immune-related genes in different tissues

In week 6 post the first oral administration, immune-related genes expression was examined by qRT-PCR in the tissues of spleen, head kidney, kidney, and hind gut. Three innate immune-related genes, IL-4/13A, IL-4/13B and CSF1R, were detected. IL-4/13A and IL-4/13B showed a more than four-fold higher trend in the CotC-VP4 group versus the Naive group (Fig. 9A and B). No significant differences were found among the four groups in the remaining three tissues. CSF1R was around two-fold higher in the CotC-VP4 group than that in the CotC group and Naive group in all the tissues (Fig. 9C). The results suggested that the CotC-VP4 spores could induce the innate immunity in grass

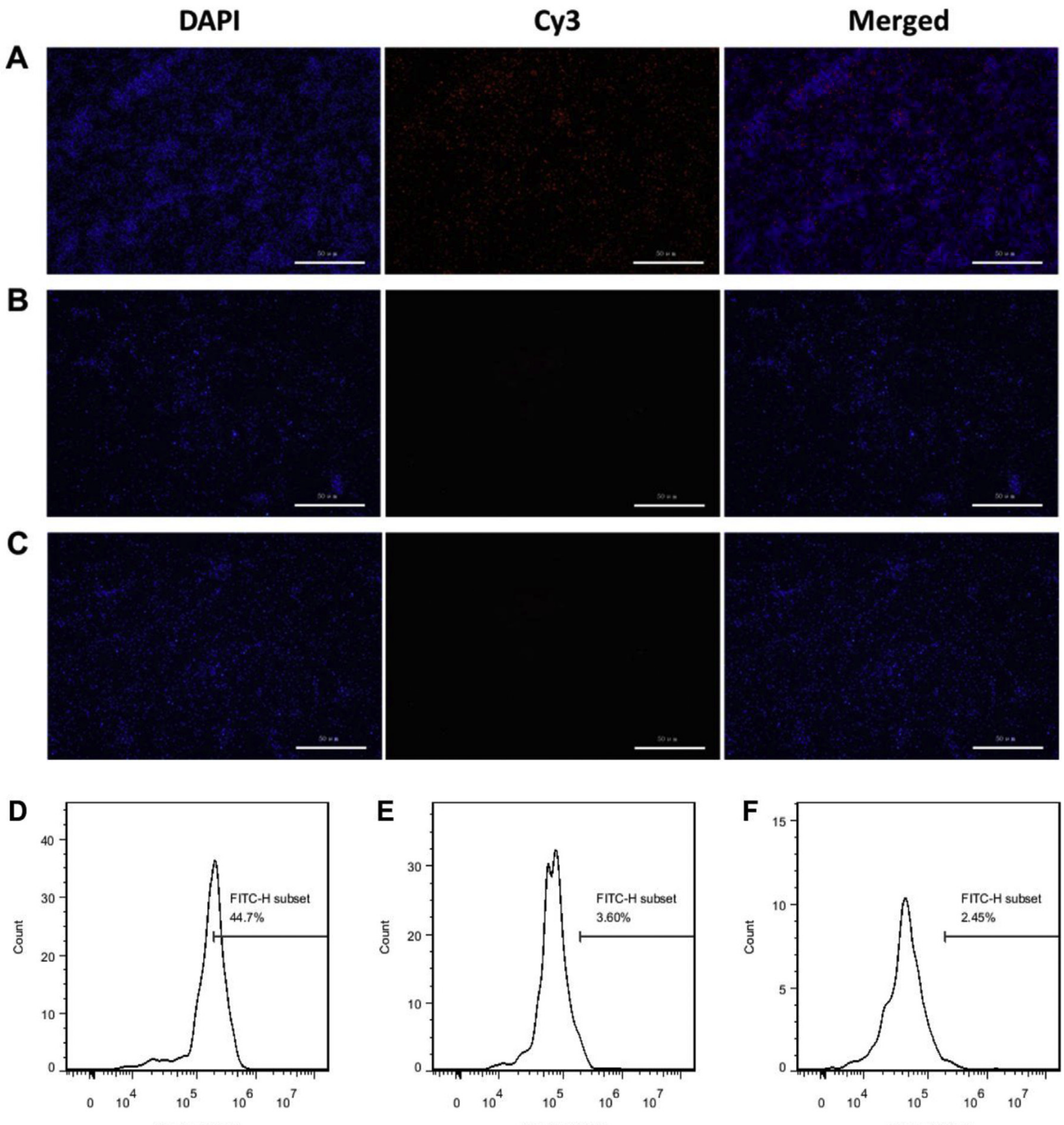

Fig. 4. Immunofluorescence and flow cytometry analysis of VP4 expressed on the spore surface. **(A), (B)** and **(C)** The CotC-VP4 spores were observed by immunofluorescence. The CotC-VP4 spores and CotC spores were incubated with rabbit anti-VP4 serum **(A** and **B)**, and CotC-VP4 spores were incubated with naive rabbit serum **(C)**, respectively, followed by Cy3-labeled goat anti-rabbit IgG. The CotC-VP4 spores were stained as red and the nucleus was stained as blue. All images were magnified at 400 × . *Scale-bar*: 50μm. **(D), (E)** and **(F)** The positive expression rate of VP4 on the coat of recombinant spores was detected by flow cytometry. The CotC-VP4 spores and CotC spores were incubated with rabbit anti-VP4 serum **(D** and **E)**, and CotC-VP4 spores were incubated with naive rabbit serum **(F)**, respectively, followed by FITC-conjugated goat anti-rabbit IgG. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

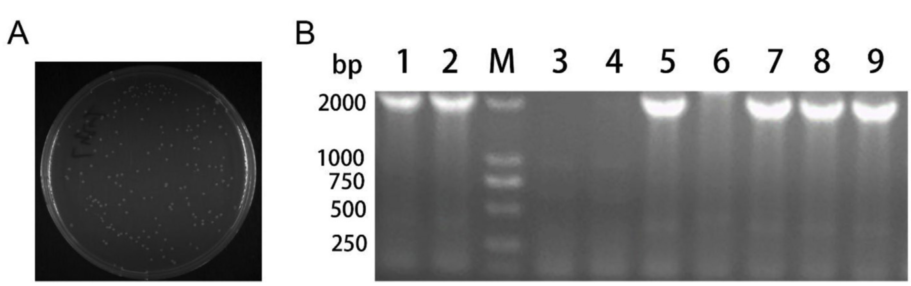

Fig. 5. Detection of the spores' colonization in intestine of fish. (**A**) *B. subtilis* colonies of intestinal contents in CotC-VP4 group were grew on the spectinomycin-resistant LB agar plates. (**B**) PCR specific amplification the fragment of CotC-VP4 in colonies from CotC-VP4 group intestinal dilution contents. Lane M: DNA marker, Lane 1–9: colonies picked up from contents dilution plates.

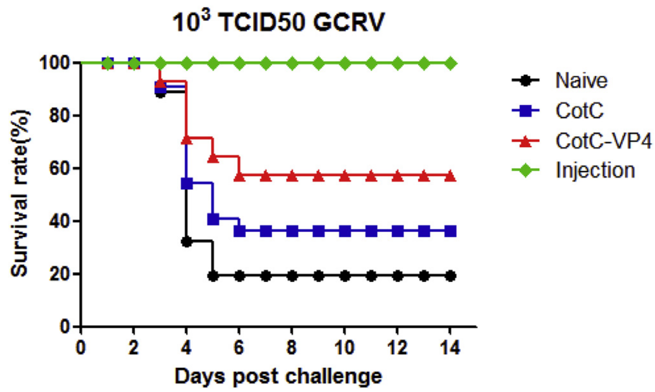

Fig. 6. Cumulative survival rate of grass carp after challenged with 10^3 TCID50 GCRV. The survival rate of fish was analyzed using the Kaplan-Meier survival curve with log-rank test. One representative of three similar independent experiments was shown (n = 20).

carp.

Next, we detected five adaptive immune-related genes—BAFF, CD4L, MHC-II, CD8 and Foxp3. BAFF was induced to a certain extent in the CotC-VP4 group versus the Naive group and was significantly higher in head kidney and kidney (Fig. 9D). The adaptive cellular immune markers—CD4L, MHC-II and CD8 were higher in almost all tissues in the orally immunized fish (Fig. 9E, F and G). However, the transcription levels of CD4L showed no significant differences between the CotC-VP4 group and the Naive group (Fig. 9E). MHC-II and CD8 expression levels were three to six times significantly higher in the immunized fish than those in the Naive group (Fig. 9F and G). Foxp3 was around two-fold higher in the CotC-VP4 group than that in other groups in spleen, but there were no significant differences in head kidney, kidney, and hind gut (Fig. 9H). The results suggested that the CotC-VP4 spores could induce the adaptive immunity in grass carp.

We also examined the expression of the inflammation-related genes of IL-1β, TNF-α and TGF-β. The up-regulation of IL-1β mRNA expression was only found in spleen and hind gut of the CotC-VP4 group (Fig. 9I). Meanwhile, the up-regulation of TNF-α and TGF-β mRNA expression was found in spleen, head kidney and kidney of the CotC-VP4 group (Fig. 9J and K), suggesting that an inflammatory response was triggered. As IFN-I was critical in antiviral immunity, the expression level of IFN-I was examined. IFN-I elevated nine times in spleen and four times in head kidney in the CotC-VP4 group compared to that in the Naive group (Fig. 9L).

Further, we checked whether IFN-I related signaling pathways were activated in immunized fish. We detected the expression levels of RIG-I, MDA5, LGP2, TLR3, TLR7, TLR8, TLR9, TLR21, TLR22, IPS-1, IRF3, IRF7, NF-kB, Mx1, Mx2 and Mx3 in spleen and kidney. In spleen, all the expression levels of the signaling pathway genes except for IPS-1 (RIG-I, MDA5, LGP2, TLR3, TLR7, TLR8, TLR9, TLR21, TLR22, IRF3, IRF7, NF-kB, Mx1, Mx2 and Mx3) were up-regulated (Fig. 10A and C). Moreover, the expression levels of MDA5, IRF7, Mx1, Mx2 and Mx3 were up-regulated for more than ten times. Nevertheless, only the expression levels of RIG-I, LGP2, TLR9, IRF7, Mx1, Mx2, and Mx3 were up-regulated in the kidney (Fig. 10B and D). Specifically, TLR9 was nearly ten times fold up-regulated, and Mx-2 was 13 times fold up-regulated in the CotC-VP4 group compared to those in the Naive group.

Taken together, we could conclude that the CotC-VP4 spores were the cause of the comprehensive activation of the innate and adaptive cellular immunity in grass carp.

4. Discussion

GCHD is a severe viral infectious disease in freshwater aquaculture in China. Although there is an attenuated vaccine in the market to prevent GCHD, the disease has not been effectively alleviated. Rough estimation indicates the annual economic loss due to GCHD is at least 1 billion, seriously affecting grass carp farmers' enthusiasm and the healthy development of freshwater aquaculture [3]. As a result, research on the prevention of GCHD is becoming pivotal of great significance.

This is the first study to use *Bacillus subtilis* (*B. subtilis*) spores as the oral delivery system in presenting GCRV VP4 protein for the prevention of GCHD. We successfully constructed the recombinant *B. subtilis* spores with VP4 protein displayed on the surface. Then, we evaluated the protective efficacy against GCRV infection and immune response as well as preliminary mechanism caused by CotC-VP4 spores in grass carp after oral administration.

To explore the protective efficacy of the oral vaccine against GCRV, we assessed the survival rate of vaccinated fish challenged with GCRV. The survival rate was 57% and the relative percent survival was about 47% compared to the Naive group. Previous study by Tian YY et al. used the same protein (GCRV VP4), but the immunization route was intraperitoneal injection [6]. The relative percent survival ranged from 47% to 82% as grass carp were immunized with 1 μg/g, 3 μg/g and 5 μg/g (recombinant protein/fish) of rVP4. Although our study appeared to have a similar or lower protective efficacy compared with theirs, the amount of protein (1×10^{-3} μg/g (protein/fish)) in our study was much fewer than theirs, suggesting *B. subtilis* spores were powerful platforms for oral vaccine delivery. Moreover, this oral vaccine delivery system also came with the advantage of antigen-saving, efficient and cost-effective. Despite the relative low percent survival in the study, we found the effect would be still influential to the current agriculture industry. Our study affirmed the feasibility of *B. subtilis* spores as a delivery system and VP4 as one of the vital proteins suitable for developing vaccines. Considering the single protein vaccine was already effective without co-immunization with other proteins, adding co-immunization proteins could be more effective, based on our results. Another factor should be considered is the amount of virus in natural infection would be less than that in our experiment. We could expect a better protective effect when the vaccine is applied in real world.

According to previous studies, the virus was widely distributed in the organs and tissues of grass carp after intraperitoneal injection with GCRV, with a high RNA copy number in liver, spleen, kidney, intestine and muscle tissues [34,35]. In our study, we also evaluated the viral load by qRT-PCR in all the five tissues mentioned above after challenge with GCRV, in order to confirm the cause of infection, infection extent and protection effect of the CotC-VP4 spores. We found that causality of the grass carp death is due to GCRV infection, while interestingly, the viral loads were decreased in all the tissues, including the

Table 2
Mortality rate and relative percentage survival (RPS) of grass carp challenged with 10^3 TCID50 GCRV.

Group	Mortality rate (%)	Survival rate (%)	Relative percent survival (RPS) (%)	*p* value	Significance
Naive	80.65	19.35	–	–	–
CotC	63.64	36.36	21.09	0.011	*
CotC-VP4	42.86	57.14	46.86	0.0043	**
Injection	10.53	89.47	86.94	< 0.001	***

Log-rank test was used for the statistical analysis by comparison with the Naive group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

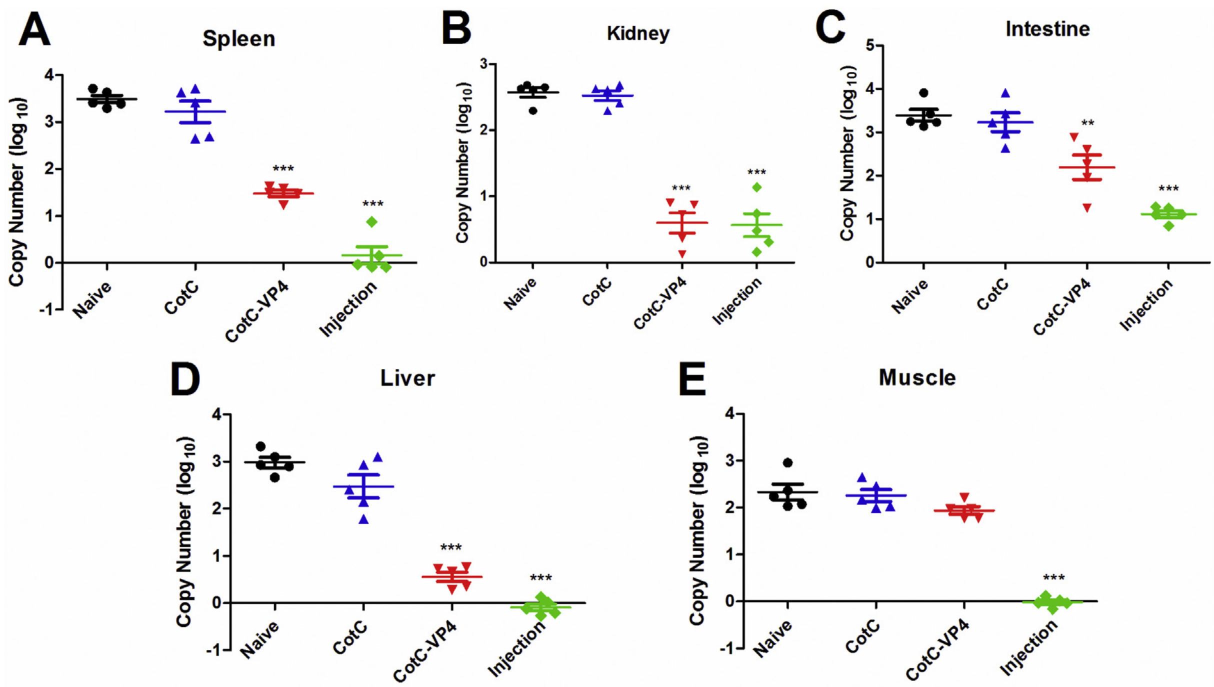

Fig. 7. Determination of GCRV viral load in different tissues after challenged with GCRV. (A) GCRV viral load in spleen. **(B)** GCRV viral load in kidney. **(C)** GCRV viral load in intestine. **(D)** GCRV viral load in liver. **(E)** GCRV viral load in muscle. Data were presented as the means ± SD. Comparison between the CotC-VP4 group with the Naive group or the Injection group was shown. Values that were significantly different from the control were indicated by asterisks (one-way ANOVA, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

administrative organ (intestine), peripheral immune organ (spleen) and the target organs (spleen, kidney, intestine, liver and muscle). Combined with the results of the detection of the spores' colonization in intestine by PCR, we could speculate that fish assigned to CotC-VP4 group may intake the CotC-VP4 spores through the intestinal mucosal barrier. The CotC-VP4 invasion may help to remove GCRV and result in the low viral load. However, further studies are needed to investigate the mechanism of CotC-VP4 uptake and immunization. Our results indirectly suggested that the CotC-VP4 spores could not be degraded and induced local mucosal immunity and systemic immunity in grass carper.

IgM is the most abundant immunoglobulin in blood and in mucosal-associated lymphoid tissue (MALT). It is also considered to be the most important immunoglobulin in systemic immunity for disease resistance in teleost fish [36–38]. IgZ/IgT is ubiquitous in intestine, skin, and gill, which plays a vital role in mucosal immunity [36]. We evaluated the immune response by detecting the specific antibody levels with ELISA. In intestinal mucus, specific IgZ levels increased only in the CotC-VP4 group but not in other groups, which indicated local mucosal immune response was induced by CotC-VP4 spores. Specific IgZ levels in intestinal mucus reached the peak at week 2 and specific IgM levels in serum reached the peak at week 4 in the CotC-VP4 group. It hinted that local mucosal immune response in intestine was earlier than systemic humoral immune response. Previous studies suggested the concentration of antibodies against antigens usually corresponded with the protection/survival rate after vaccination challenge [3,39]. In our study, oral immunization seemed to trigger higher antibodies than injection immunization. However, the maintaining duration of antibodies in the CotC-VP4 group was different to that of the Injection group, which could be an explanation for the variation of the protective rate. The results of ELISA were in line with the results of challenge trials, indicating that the elevated specific antibody levels might protect the fish from GCRV infection.

It is known that immunization with an antigen would stimulate the

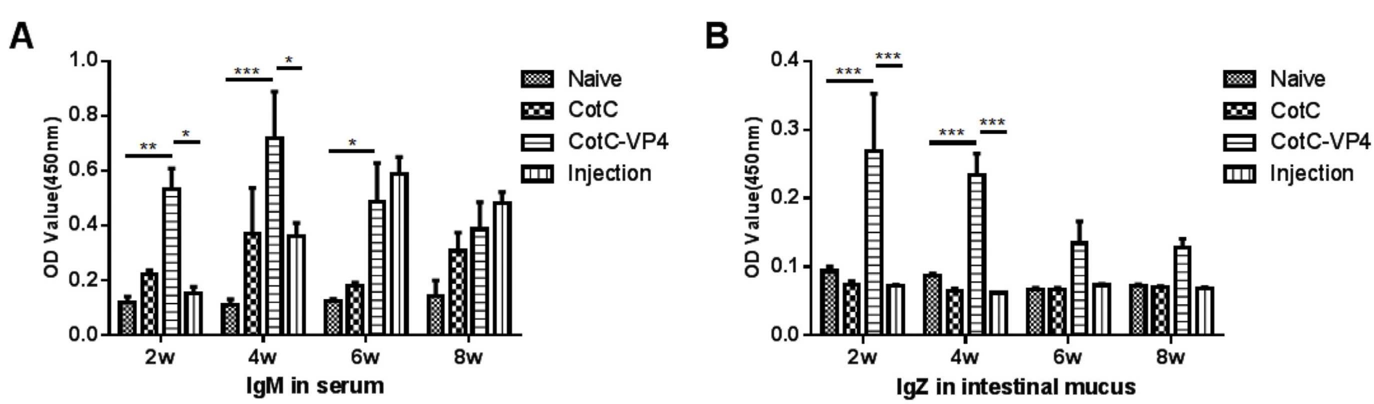

Fig. 8. Detection of specific antibody levels by ELISA. The levels were indicated with OD_{450} value. **(A)** Serum. **(B)** Intestinal mucus. Data were presented as the means ± SD. Comparison between the CotC-VP4 group with the Naive group or the Injection group was shown. Values that were significantly different from the control were indicated by asterisks (one-way ANOVA, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

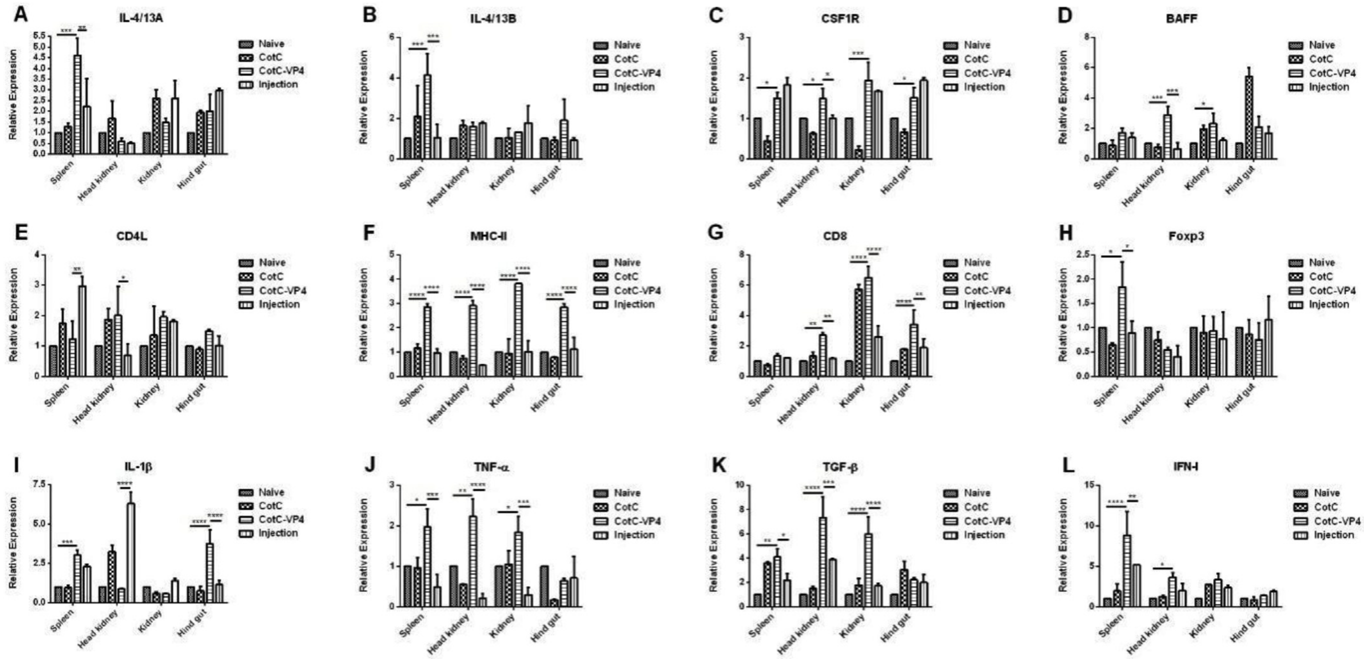

Fig. 9. qRT-PCR analysis of the expression of immune-related genes in different tissues of grass carp. (A) IL-4/13A. **(B)** IL-4/13B. **(C)** CSF1R. **(D)** BAFF. **(E)** CD4L. **(F)** MHC-II. **(G)** CD8. **(H)** Foxp3. **(I)** IL-1β. **(J)** TNF-α. **(K)** TGF-β. **(L)** IFN-I. Samples were collected from the grass carp at 6 weeks post oral administration. Data were presented as the means ± SD. Comparison between the CotC-VP4 group with the Naive group or the Injection group was shown. Values that were significantly different from the control were indicated by asterisks (one-way ANOVA, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

expression of certain immune-related genes [3]. To check the immune response in immunized fish, we examined 28 immune-related genes expression by qRT-PCR in spleen, head kidney, kidney and hind gut tissues at week 6 post the first oral administration. The 28 immune-related genes included 3 innate immune genes (IL-4/13A, IL-4/13B and CSF1R), 5 adaptive immune genes (BAFF, CD4L, MHC-II, CD8 and Foxp3), 3 inflammation-related genes (IL-1β, TNF-α and TGF-β), IFN-I and its 16 related signal pathway genes (RIG-I, MDA5, LGP2, TLR3, TLR7, TLR8, TLR9, TLR21, TLR22, IPS-1, IRF3, IRF7, NF-kB, Mx1, Mx2 and Mx3). We chose the four tissues because spleen and head kidney

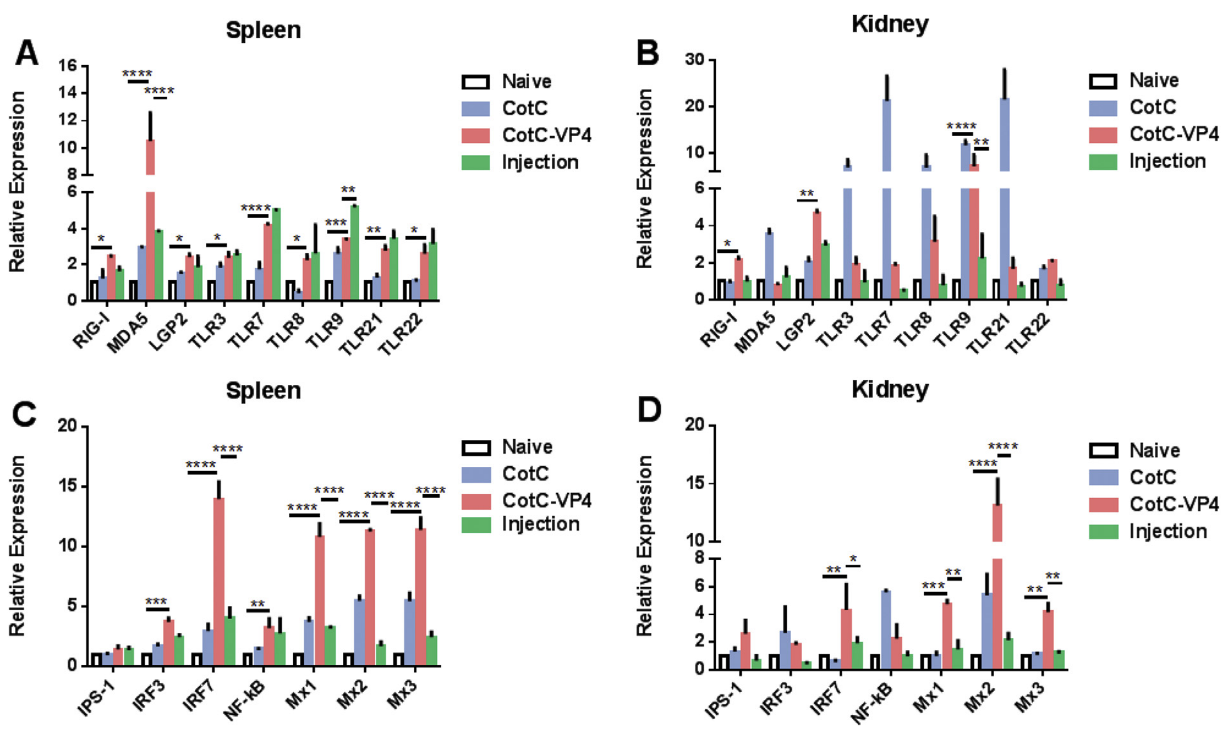

Fig. 10. qRT-PCR analysis of the expression of IFN-I related signaling pathway genes in spleen and kidney of grass carp. (A) and **(C)** Spleen. **(B)** and **(D)** Kidney. Samples were collected from the grass carp at 6 weeks post oral administration. Data were presented as the means ± SD. Comparison between the CotC-VP4 group with the Naive group or the Injection group was shown. Values that were significantly different from the control were indicated by asterisks (one-way ANOVA, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

served as major peripheral immune organs in fish [40–42]. Kidney was one of the target organs of GCRV. And hind gut was the main position for intaking antigen in grass carp [43].

We detected the expression of three innate immune-related genes (IL-4/13A, IL-4/13B and CSF1R) first. As we known, IL-4/13 plays important role in inducing alternative activation phenotype in teleost macrophages [44]. CSF1R is a specific surface marker of grass carp mononuclear macrophage [45]. Results have shown that IL-4/13 were significantly higher in the CotC-VP4 group than that in the Naive group in spleen, and that CSF1R was induced to a significantly high level in the CotC-VP4 group in all the tissues. The results revealed that oral vaccination could induce a non-specific immune response in grass carp.

We also examined the expression of adaptive immune-related genes—BAFF, CD4, MHC-II, CD8 and Foxp3. BAFF is a B cell activating factor that reflects the activation of the B cell [46]. Our results showed that BAFF was significantly higher in the CotC-VP4 group than that in the Naive group in head kidney/kidney and that BAFF had a rising trend in spleen, suggesting B cells were activated. The result was consistent with antibodies production. CD4 and CD8 are critical for defining the T helper cell subset and the cytotoxic T lymphocyte subset respectively in vertebrates [47,48]. They provide the basis for addressing the involvement of T cells [49]. MHC II molecules play a vital role in adaptive immune response by presenting antigenic peptides to $CD4^+$ T lymphocytes [50]. All these three genes were induced in almost all tissues in CotC-VP4 group, implying that a specific cellular immunity was evoked by oral administration in grass carp. Foxp3 is a T cell-specific transcription factor which plays a key role in the development of Treg cells and in the immune regulatory process during inflammation [51]. In our results, there were almost no differences among all groups, suggesting that the immunity was not suppressed in the experimental group. Taken together, the above results suggested that the vaccine could induce not only innate immunity, but also humoral and cellular immunity in immunized fish. Similar results were observed in previous study by Chen DD et al. [20]. In their study, the expression of a series of genes including CD4 and CD8α was up-regulated in muscle and spleen of grass carp immunized with pC-S6 DNA vaccine. Likewise, significantly higher expression of MHC I was also found in the head kidney and spleen of grass carp immunized with recombinant VP35 protein [52]. Combined with their results, ours suggested that the adaptive immunity elicited by CotC-VP4 spores could help grass carp fight against the viral infection.

Next, we detected the expression of inflammatory cytokines (IL-1β, TNF-α, TGF-β) and IFN-I. As we know, IFN response is the key components of innate immune response and the first line of host-defense mechanism against virus infection [8]. Our study confirmed IFN-I was induced; moreover, the mechanism of signal pathways involved triggered our interest as IFN-I and its related signal pathways are of much concern in antiviral immune. The reason why we chose the following molecules in defining the signal pathways was discussed. It is recognized that fish innate immunity plays an essential role in protecting the host against invading pathogens [53]. Fish also possess evolutionarily conserved pattern recognition receptors (PRRs) like mammals, which are responsible for sensing the presence of pathogen-associated molecular patterns (PAMPs). PRRs can be divided into four different classes: Toll-like receptors (TLRs), retinoic acid-inducible gene (RIG) I-like receptors (RLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs) [54]. Among these PRRs, TLRs and RLRs play a crucial role in the recognition of viruses or viral PAMPs [54,55]. Up to now, at least 19 TLR types were discovered [53,56–58]. Among them, TLR3, TLR7, TLR8, TLR9, TLR21, TLR22 played important roles in antiviral immune. RLR family consists of three members: RIG-I, MDA5, and LGP2 [53]. In grass carp, RIG-I, MDA5, and LGP2 acted as positive role molecules in anti-GCRV innate immune [59–62]. Upon activation by viral components, TLRs or RLRs transmit signals to the downstream adaptor molecules, which induce a large-scale amplification of signaling cascade to activate interferons (IFNs) or nuclear factor-kB (NF-kB) pathways via IFN regulatory factors (IRFs) [56,63]. Among all the IRFs, IRF3 and IRF7 are the key regulators of IFN-I expression upon viral infection. Subsequently, IFN-I along with IFN-stimulated genes (ISGs), including Mxs are important effect molecules in host antiviral innate immunity and mediate the first antiviral defense [64]. Hence in our study, we detected RIG-I, MDA5, LGP2, TLR3, TLR7, TLR8, TLR9, TLR21, TLR22, IPS-1, IRF3, IRF7, NF-kB, Mx1, Mx2, Mx3 in spleen and kidney. Most genes were up-regulated in the CotC-VP4 group in spleen. However, only TLR9, IRF7, Mx1, Mx2, Mx3 pathways were activated in CotC-VP4 group in kidney. This might because spleen was the main immune organ while kidney was not. Previous studies showed that the pC-S6 DNA vaccine and recombinant VP35 protein of GCRV could promote induction of IFN-I, Mx1 and TLR22 in grass carp to defense against GCRV infection, and the upregulation of IRF7 was also reported a similar effect [20,52,65], Thus, we suspected that the activation of relevant signaling pathways of immune organs and tissues in immunized fish would be beneficial to resist viral infection after challenged with GCRV. Further research was expected to study the mechanism immune protection in the vaccines. Knowingly, the Injection group did not induce significant differences in the gene expression in most of the tissues might because of the late detection time point.

Though we explored the mechanism involved in immunized fish, more studies should be done to understand the thorough mechanisms, protective duration, and safety of the oral vaccine. Furthermore, immunization strategy needed to be optimized for commercial applications.

In summary, we constructed CotC-VP4 recombinant spores and determined the protective efficacy and immunity after oral administration. Our results indicated that oral delivery of *Bacillus subtilis* spores expressing GCRV VP4 protein was protective against GCRV infection. In our observation, we also found CotC-VP4 spores were able to promote not only innate immunity, but also humoral and cellular immunity post oral immunization. Considering the convenience, safety and protective effect of CotC-VP4 vaccine, we should regard this recombinant oral vaccine as a potential candidate in preventing GCHD. This work also highlighted *Bacillus subtilis* spores as a powerful oral vaccine delivery system, which could be widely used for other oral vaccines. Future applications are promising and may be employed in veterinary and medical fields.

Conflicts 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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