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Oral vaccination of tilapia against *Streptococcus agalactiae* using *Bacillus subtilis* spores expressing Sip

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ABSTRACT

Streptococcus agalactiae infections are becoming an increasing problem in aquaculture because of significant morbidity and mortality, which restricts the healthy development of tilapia aquaculture. To seek safe and effective prevention measures, a *Bacillus subtilis* GC5 surface displayed vaccine was prepared and applied orally in tilapia. The study first showed that recombinant spores can engraft in the tilapia intestine. Then, the effect of protection and the immune responses were evaluated. The results of ELISA showed that Sip-specific antibody in the sera of GC5-Sip-immunized fish can be detected after the first oral administration when compared to the phosphate buffer saline (PBS) control group, and the levels of specific IgM gradually strengthened with boosting, so does the specific antibody against bacteria, proving that humoral immunity was induced. Quantitative real-time PCR (qRT-PCR) results showed that the immune-related gene expression of the gut and spleen exhibited a different rising trend in the GC5-Sip group, revealing that innate immune response and local as well as systemic cellular immunity were induced. The outcome of fish immunized with GC5-Sip spores provided a relative percent survival (RPS) of 41.7% against *S. agalactiae* and GC5 group had an RPS of 24.2%, indicating that GC5-Sip was safe and effective in protecting tilapia against bacterial infection. Our study demonstrated that the oral administration of *B. subtilis* spores expressing Sip could cause an effective immune response and offer good resistance to bacterial infection. Our work may lead to the development of new ideas for immunoprophylaxis against *S. agalactiae* infection.

1. Introduction

Streptococcus agalactiae, also referred to group B *Streptococcus* (GBS), is a gram-positive bacterium. It is not just regarded as a mammalian pathogen [1]; it also causes serious infection in many kinds of animals, including fish [2]. The outbreaks in tilapia (*Oreochromis niloticus*) in the summers of 2009 and 2010 in major cultivation areas of southern China [3], which gave rise to considerable morbidity and mortality, led people to pay close attention to this pathogen. Polysaccharide capsules were associated with the pathogenicity of the bacteria [4]. Depending on the type of capsular antigen, *S. agalactiae* can be divided into ten serotypes (i.e. Ia, Ib and II–IX) [5]. An investigation of isolated *S. agalactiae* showed high rates of serotypes Ia, Ib, II and III in both humans and fish [6]. As the multiple functional genes involved in capsular polysaccharide synthesis had different structures [7], they eventually formed capsular antigens with strict specificity. Surface immunogenic protein (Sip)—which is widely distributed in different isolates, highly conservative and easy to contact with the body's immune system to stimulate cross-reactive immunological responses—was discovered by Brodeur [6]. Since then, Sip as a potential antigen has been applied in subunit vaccines to verify its immunogenicity, and it has proven an effective vaccine candidate [8,9].

Vaccination is an alternative method to control streptococcal diseases because of the shortage of other methods, including using chemical therapeutics such as antibiotics whose frequent use might lead to the emergence of antibiotic-resistant *S. agalactiae* or other bacterial

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Table 1
Primers used in this study.

Name	Sequence (5'→3')	Application
Sip-F	CTAGGATCCAAGAAACAGATACGACGTGGA	construction of recombinant plasmid
Sip-R	CCGCTCGAGTTAGTTAAAGGATACGTGAACGTG	
amyE 5'-F	TCACTAGCTAGCTCATTGCTCGGGCTGTATGACTG	
amyE 5'-R	AAGCTTGACTGACCCGGGAATCTCACACCAT	
amyE 3'-F	CCCGGGTCAGTCAAGCTTGAAAAGCCAAATAGGCGATC	
amyE 3'-R	TCAAAAGTACTGTTACACCATCACTGTTCGTTCC	
cotC-F	TTCTAGTCTAGATGTAGGATAAATCGTTTGGGC	
cotC-R	CGGATCC*ACTACCGCCACCTCCACTACCGCCACCTCCACT ACCGCCACCTCC*GTAGTGTTTTTTATGCTTTTTATAC	
CAT-F	ATTCCCGGGAGCACGCCATAGTGACTGGC	
CAT-R	ATCCAAGCTTTCTAGAGGATCCCTCGAGGCGGCCGCG	
	GTACCTTATAAAAGCCAGTCATTAGG	
amyE-F	TCATTGCTCGGGCTGTATGACTG	PCR detection
amyE-R	GTTACACCATCACTGTTCGTTCC	
MHCI-F	TTCTCACCAACAATGACGGG	Real-time PCR
MHCI-R	AGGGATGATCAGGGAGAAGG	
MHCII-F	AGTGTGGGGAAGTTTGTTGGAT	
MHCII-R	ATGGTGACTGGAGAGAGGCG	
CD8-F	ATGGACCAAAAATGGCTTCTG	
CD8-R	GCTGAAAGATCCAATGAATTC	
CD4-F	TTCAGTGGCACTTTGCTCCTAA	
CD4-R	TGGGCGATGATTTCCAACA	
T-bet-F	CCTCCTCATCTTCTACATCAC	
T-bet-R	CCTCTTCTTGTTTTCACCCAC	
GATA3-F	CTGGAGGGGAGCAAAGGAAT	
GATA3-R	CGTGAAGAGGTGTGGACTGG	
TNF-α-F	CTCAGAGTCTATGGGAAGCAG	
TNF-α-R	GCAAACACGCCAAAGAAGGT	
IL-1β-F	AACACTGACAGAACAACTGCGAACA	
IL-1β-R	TCGCAGTTGTTCTGTCAGTGTTGTT	
TGF-β-F	TGGGACTATGAGCAGGAGGG	
TGF-β-R	AACAGCAGTTGTGTGATTGGGT	
IL-10-F	GCTTCCCCGTCAGGCTCAA	
IL-10-R	CTGTCGGCAGAACCGTGTC	
IgM-F	ACGAGGAAGCAGACTCAAGTTAT	
IgM-R	ACAATAGCTCTAGTTGTGTTAACC	
lectin-F	TGAGTTCCGCAGGGGAGACTACCT	
lectin-R	TAAGAGTTTGTGCTCCACCTAGG	
TP 3-F	GGGGAGGCCTTTATTCACCAT	
TP 3-R	CTTGCTGCTGTTGTTGCTGTTT	
TCR-β-F	GGACCTTCAGAACATGAGTGCAGA	
TCR-β-R	TCTTCACGCGCAGCTTCATCTGTT	
β-actin-F	GAGCGTGAGATTGTGCGTGAC	
β-actin-R	TCCATACCGAGGAATGAGGGC	

[a]Restriction sites for plasmid construction are underlined.
[b]Linker $(GGGGS)_3$ are in italics.

agents [10]. Currently, there are many kinds of vaccines to prevent the spread of *S. agalactiae* in tilapia. For instance, live attenuated [11] or inactivated *S. agalactiae* [12–14] were developed as potential vaccines, and they can provide certain protection against challenges with a virulent *S. agalactiae* strain. These vaccines generally use intraperitoneal (IP) or bath immersion (BI) to inoculate. Although IP is considered the most effective inoculation method, the operation is difficult, and it can easily cause damage to fish. Conversely, BI is less efficient and requires a large amount of vaccine to be used. As a result, a safe and effective vaccine administered by the oral route is highly needed.

Bacillus subtilis is regarded as a non-pathogen, the spore form is currently being used as a probiotic. Probiotics are bio-friendly and widely used in both humans and animals [15,16], and gradually applied in aquaculture [17]. The spores of *B. subtilis* have strong resistance properties and can protect antigens in the gastrointestinal tract from degradation. Therefore, the spores have been suggested as an oral vaccine delivery system for recombinant heterologous antigens and can generated effective immune responses in a murine model [18,19]. However, they have rarely been reported as an oral vaccine for bacterial disease in fish, and more studies are needed to prove their feasibility in fish farming.

B. subtilis GC5 have been isolated from the intestinal tract of grass carp (*Cyenopharyngodon idellus)* in our laboratory [20]. In this study, we used the cotC, a major component of the GC5 strain spores, as a fusion partner for the expression of the Sip antigen on the spore coat. The tilapia were immunized by the oral administration of engineered GC5-Sip spores. Then, we evaluated the persistence of spores in the intestinal tract and the immune response by ELISA and qRT-PCR, which suggested that an immune response could be elicited and that this may contribute to effective protection against *S. agalactiae* infection to some extent. This work suggested that GC5-Sip could be used as an oral vaccine candidate and laid a foundation for future study on the exact mechanism in fish.

2. Materials and methods

2.1. Fish

Healthy and *S. agalactiae*-free tilapia (Genetic Improvement of Farmed Tilapia from Nile tilapia (*O. niloticus*), GIFT) with a mean weight of 22 ± 2 g were purchased from Five Longgang Aquatic

Product Development Company (Guangdong Province, China) and housed at Xiantao Fishery (Hubei Province, China). The fish were reared in tanks and given a commercial diet twice a day throughout the study. Water was partly replaced daily, and the temperature was maintained at approximately 28 °C. The fish were acclimated two weeks before the formal experiments.

2.2. Bacterial strains and growth conditions

B. subtilis GC5 strains isolated from the gut of grass carp were used to make competent cells and produce the spores with recombinant plasmid. Plasmid amplification for nucleotide sequencing, subcloning experiments and transformation of *Escherichia coli*-competent cells were performed in the *E. coli* strain DH5α. *B. subtilis* GC5 strains and *E. coli* DH5α were routinely cultivated aerobically in Luria-Bertani (LB) broth at 37 °C. The pathogenic bacteria *S. agalactiae* XQ-1 strains (kindly given by Professor Ai-Hua Li, Institute of Hydrobiology, Chinese Academy of Sciences) were cultured in Brain Heart Infusion (BHI) medium at 28 °C to infect tilapia and determine the amount of bacteria required to achieve a mortality rate of 50% as well as used for finally challenge.

2.3. Construction of recombinant plasmid

For displaying recombinant antigen proteins on bacillus spore surface, a vector for restructuring into *B. subtilis* genome was constructed. A homologous arms amylase gene, which was used to double-crossover into the *B. subtilis* genome, was amplified using genomic DNA from the *B. subtilis* GC5 strain (primers used in this study are listed in Table 1). This gene was synthesised by PCR-driven overlap extension to introduce SmaI and HindIII into the appropriate position of the amylase gene, and about 1000 bp-amplified product was ligated to a T19 simple vector named PamyE. The cotC protein (about 500 bp) was chosen as the anchor protein to display the heterologous antigen on the spore surface. The sequence encoding the cotC and its promoter was amplified, and the product was inserted into the PamyE vector. The gene encoding the Sip of *S. agalactiae* was produced using the *S. agalactiae* chromosome as a template and Sip-specific primers, and the purified product was fused to the C-terminal portion of the cotC. Followed Sip was terminate sequence of cotC, which was cloned into the C-terminal end of chloramphenicol derived from the Pht10 vector (kindly given by Wuhan University). The resulting plasmid was named PamyE-CAT-cotC-Sip. All the constructs were confirmed by complete sequencing (TSINGKE Biological Technology).

2.4. Bacillus subtilis transformation and chromosomal integration

The plasmid PamyE-CAT-cotC-Sip was used to transform *B. subtilis* strain GC5-competent cells using the chemical method, and then the substance was smeared on LB plates containing 5 μg/mL chloramphenicol. After incubation at 37 °C for nearly 20 h, several chloramphenicol-resistant positive colonies were grown. The double-crossover event at the amylase locus generated the GC5-Sip strain. Integration of cotC-Sip at the amyE locus was conducted on LB plates with 1% starch. The plates were stained with iodine to examine the amylase activity, and then chromosomal DNA was made for PCR as well as the *B. subtilis* GC5 as a control. Primer pairs Sip and amyE were respectively used to detect the correct insertion of the exogenous gene.

2.5. Preparation of Bacillus subtilis spores

To prepare spores, *B. subtilis* was first grown in LB medium for 4 h to the logarithmic phase and then transferred to Difco-Sporulation medium (DSM) to produce spores using the exhaustion method. Sporulating cultures were harvested after the initiation of spore formation. Viable spores were titrated to determine the number of cfu/mL and then stored at −80 °C until use.

2.6. Western blot and immunofluorescence microscopy analysis of surface display

Western blot and immunofluorescence microscopy were applied to detect the expression of Sip protein in recombinant GC5-Sip spores. Spore coat proteins were extracted from suspensions of spores using an SDS-DTT extraction buffer as described in detail elsewhere [21]. Electrophoresis in denaturing gels and the Western blot assays were performed as described elsewhere [22] using Sip-specific polyclonal antibodies (pAbs) and goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (HRP) (Thermo Scientific). Reactive bands were detected with Immobilon™ Western Chemiluminescent HRP Substrate (Millipore), and the pictures were taken by an Image Quant LAS 4000 system (GE Healthcare).

To analyze the surface exposure of cotC-fused Sip molecules, sporulating cells of wild type GC5 and the isogenic recombinant strains GC5-Sip were analyzed by immunofluorescence microscopy with Sip-specific primary pAbs and FITC-conjugated goat anti-rabbit IgG (Thermo Scientific) as the secondary antibody. The images were acquired using a confocal microscope (Zeiss).

The pAbs against Sip of *S. agalactiae* was produced by immunizing rabbits with prokaryotic-expressed Sip as described in a previous report [23]. The IgG fraction of the rabbit antiserum was purified using a HiTrap protein G column (GE Healthcare). Thereafter, the specific pAbs were purified by affinity chromatography using the recombinant Sip coupled to NHS-activated Sepharose 4B (GE Healthcare).

2.7. Analysis of viable spores in intestinal tissues

Groups of tilapia were inoculated intragastrically with 100 μL suspensions of 2×10^9 GC5-Sip spores using a ball-ended feeding needle. About 1-cm samples of hindgut were collected at various time points and homogenized in PBS. They were then centrifuged at 500 rpm for 5 min, and before plating serial dilutions on LB agar plates containing chloramphenicol (5 μg/mL), the supernatants were heat treated at 80 °C for 20 min to kill any germinated spores. The plates were incubated for 12 h at 37 °C for enumeration.

2.8. Vaccination and challenge

Immunization trials were conducted on *S. agalactiae*-free tilapia, which were randomly divided into three groups, with 100 fish per group. One group was immunized with GC5-Sip spores via gavage at a dosage of 10^9 cfu/100 μL per fish, and the other two respectively were given the same amount of GC5 spores and PBS as control. As shown in Fig. 6A, the fish were boosted once more, using the same method used in the first vaccination at week 3. Five tilapia in each group were euthanized with 100 μg/mL of tricaine methanesulfonate (MS-222; Sigma-Aldrich) at 3, 7, 14 and 21 days after the first immunization to collect hindgut and spleen tissues for RNA extraction, which were stored in RNAlater (Qiagen) at −80 °C and ready for qRT-PCR. Furthermore, the fish were bled five times at week 2, 3, 5, 6 and 8. Sera were collected and kept at −80 °C for ELISA.

At week 6 post-vaccination, fish vaccinated with GC5 spores (n = 20), GC-Sip spores (n = 22) as well as the PBS group (n = 21) were challenged by IP injection with *S. agalactiae* (mortality above 50%). Fish mortality was monitored daily for 14 days, and dead fish were removed on a daily basis. The relative percent survival (RPS) was calculated by the following formula: RPS = [1-(% mortality of vaccinated fish/% mortality of control fish)] × 100%.

2.9. Induction of immune-related genes

Total RNA of examined tissues was extracted by TRIZOL Reagent

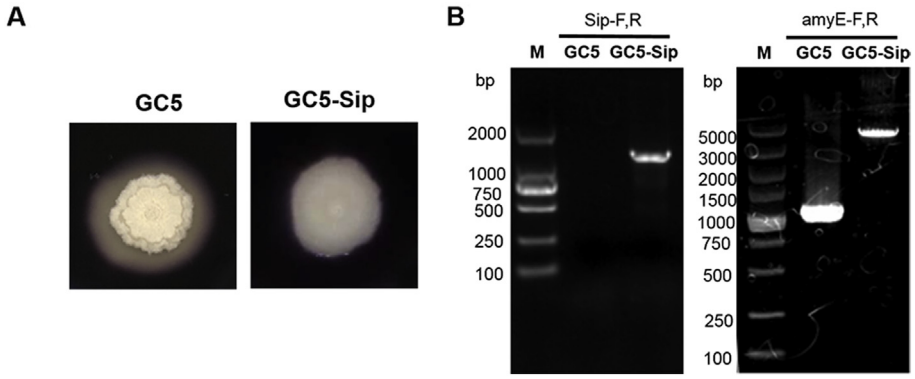

Fig. 1. Identification of transgenic *B. subtilis*. (A) Analysis of amylase activity. *B. subtilis* GC5 wild-type and GC5-Sip recombinant strains on starch-containing LB plate stained by iodine. (B) PCR analysis of *B. subtilis* genome, primer pairs used in PCR are labelled above.

(Invitrogen). cDNA was synthesised using PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara) to eliminate contaminated genomic DNA. The expression of reference gene β-actin as well as a set of immune-related genes was detected by qRT-PCR in a DNA Engine Chromo 4 real-time system (BioRad) with SYBR Green Supermix (BioRad). PCR conditions were as follows: 95 °C for 5 min and then 45 cycles of 95 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s. The expression of genes was calculated as relative expression to β-actin using the $2^{-\Delta\Delta Ct}$ method, samples were analyzed in triplicate and all data were reported as relative mRNA expression compared to the value of the PBS control group. The primers for each gene are given in Table 1.

2.10. Indirect ELISA for detection of antigen-specific serum antibody

Plates were coated with 100 μL of recombinant Sip protein (10 μg/mL) or *S. agalactiae* (10^9 cfu/mL) in carbonate-bicarbonate buffer per well and incubated at 4 °C overnight. After blocking with 5% BSA in PBS for 1 h at 37 °C, serum samples were applied as a 1/50 dilution in PBS for 3 h at 37 °C. After washing with PBS three times, the plates were incubated with mouse anti-tilapia IgM for 1 h at 37 °C. Then, plates were incubated with HRP-conjugated goat anti-mouse IgG (Thermo Scientific) diluted at 1:2000 for 1 h at 37 °C and next reacted with tetramethylbenzidine (TMB) substrate (Beyotime) for 10 min. Finally, the reaction was stopped by 2 M H_2SO_4, and the absorbance of each well was measured in a precision microplate reader (Bio-Rad iMark) at 450 nm.

2.11. Statistical analysis

Data were analyzed by one-way ANOVA with Dunnett's post-hoc test (SPSS Statistics, version 19, IBM). In all cases, differences were considered statistically significant when $p < 0.05$.

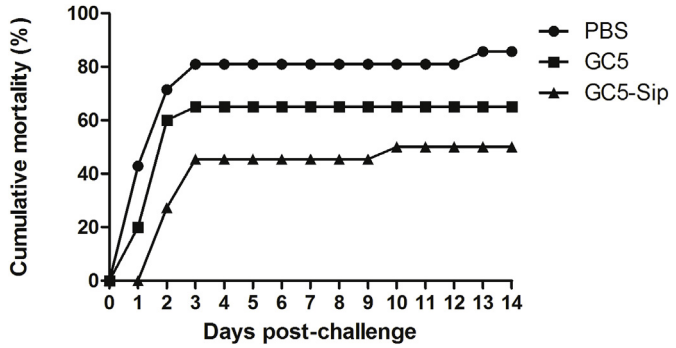

Fig. 3. Cumulative mortality of tilapia orally vaccinated with GC5-Sip spores after challenged by *S. agalactiae*; native GC5 spores and PBS as controls.

3. Results

3.1. Construction and chromosomal integration of cotC-Sip gene

The strategy to obtain recombinant *B. subtilis* spores expressing antigen proteins Sip of *S. agalactiae* on their surface was based on the use of the carrier protein cotC and its promoter for the construction of translational fusions and on chromosomal integration of the cotC-Sip gene fusions into the coding sequence of the amylase gene by double-crossover recombination events. For the analysis of amylase activity, GC5-Sip mutant and *B. subtilis* GC5 wild type strains on the starch-containing LB plate were stained by iodine. The integration of cotC-Sip can disrupt the amylase gene, causing an amylase negative phenotype. Thus, a blue color was produced by a starch-iodine reaction around the *B. subtilis* GC5-Sip colonies, while the wild type strain showed a big white halo around the colony because the secretion of amylase resulted

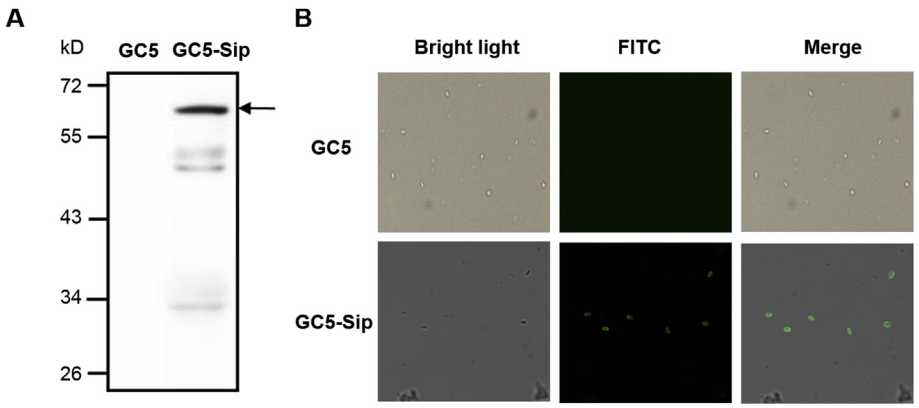

Fig. 2. Expression of *S. agalactiae* Sip protein on GC5 strain spore surface. (A) Western blot analysis of proteins extracted from *B. subtilis* GC5 and GC5-Sip spores. Fusion protein cotC-Sip was detected by Sip-specific antibody. Arrows point to fusion proteins. Molecular weight markers are indicated. (B) Immunofluorescent detection of recombinant spores.

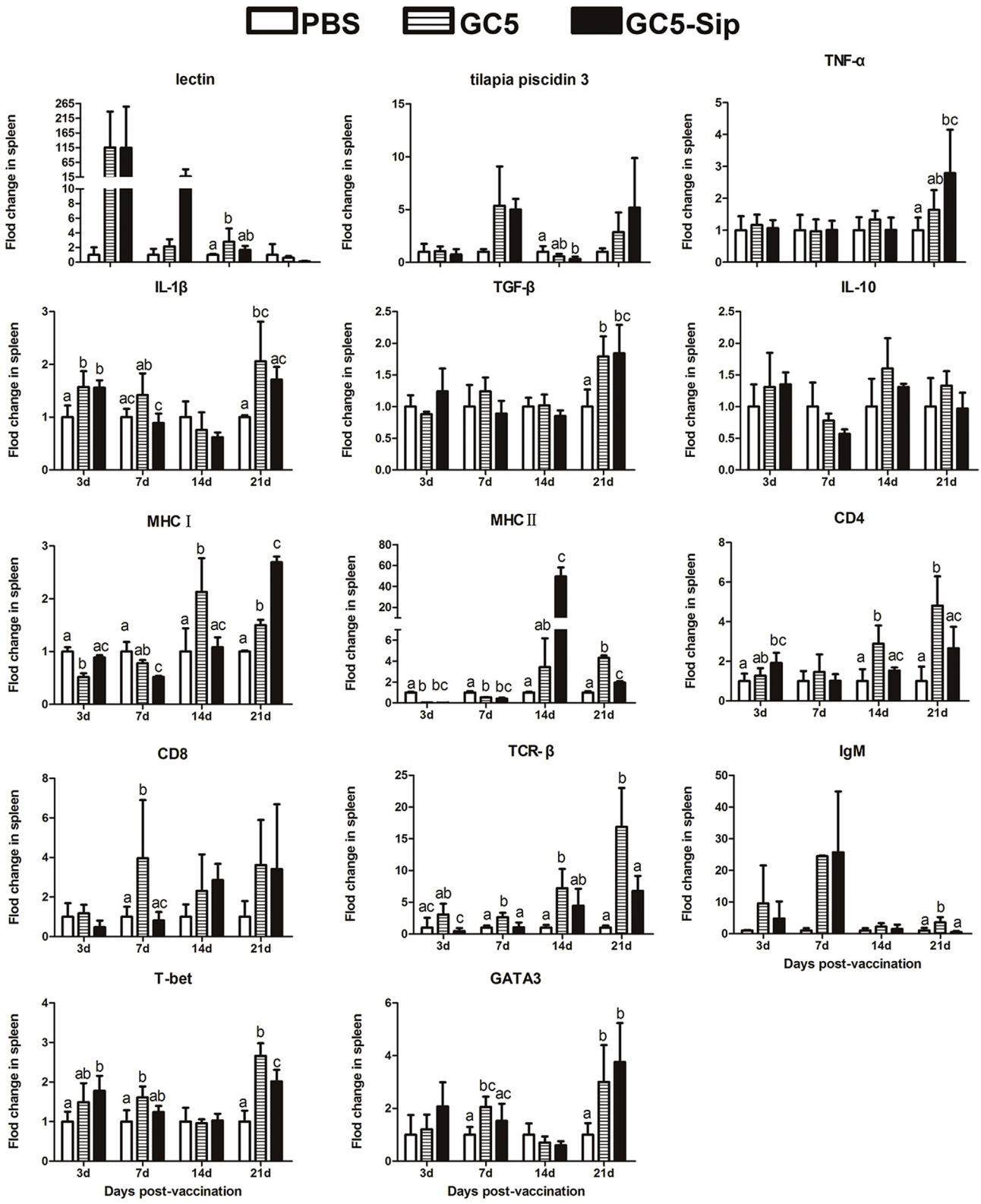

Fig. 4. qRT-PCR analysis of expression of immune-related genes in tilapia immunized with different formulations in spleen after first vaccination. The letters a, b and c indicate that the differences in gene expression at the same time in the PBS, GC5 and GC5-Sip groups are significant ($p < 0.05$). The same letters appearing means no significant change. Data are means for four individual fish and presented as means ± SD.

in the hydrolysis of the starch on the plate (Fig. 1A).

Then, validation of genomic DNA integration was performed. The fragment of the Sip gene (about 1200 bp) was able to amplify from the *B. subtilis* GC5-Sip genome with specific primers but not in the wild type *B. subtilis* GC5 strain. The full length of amylase gene amplified products was about 1000 bp when wild type *B. subtilis* GC5 genomic DNA was used as a template. However, it was about 4200 bp including the amylase gene, chloramphenicol resistance genes, cotC and its promoter,

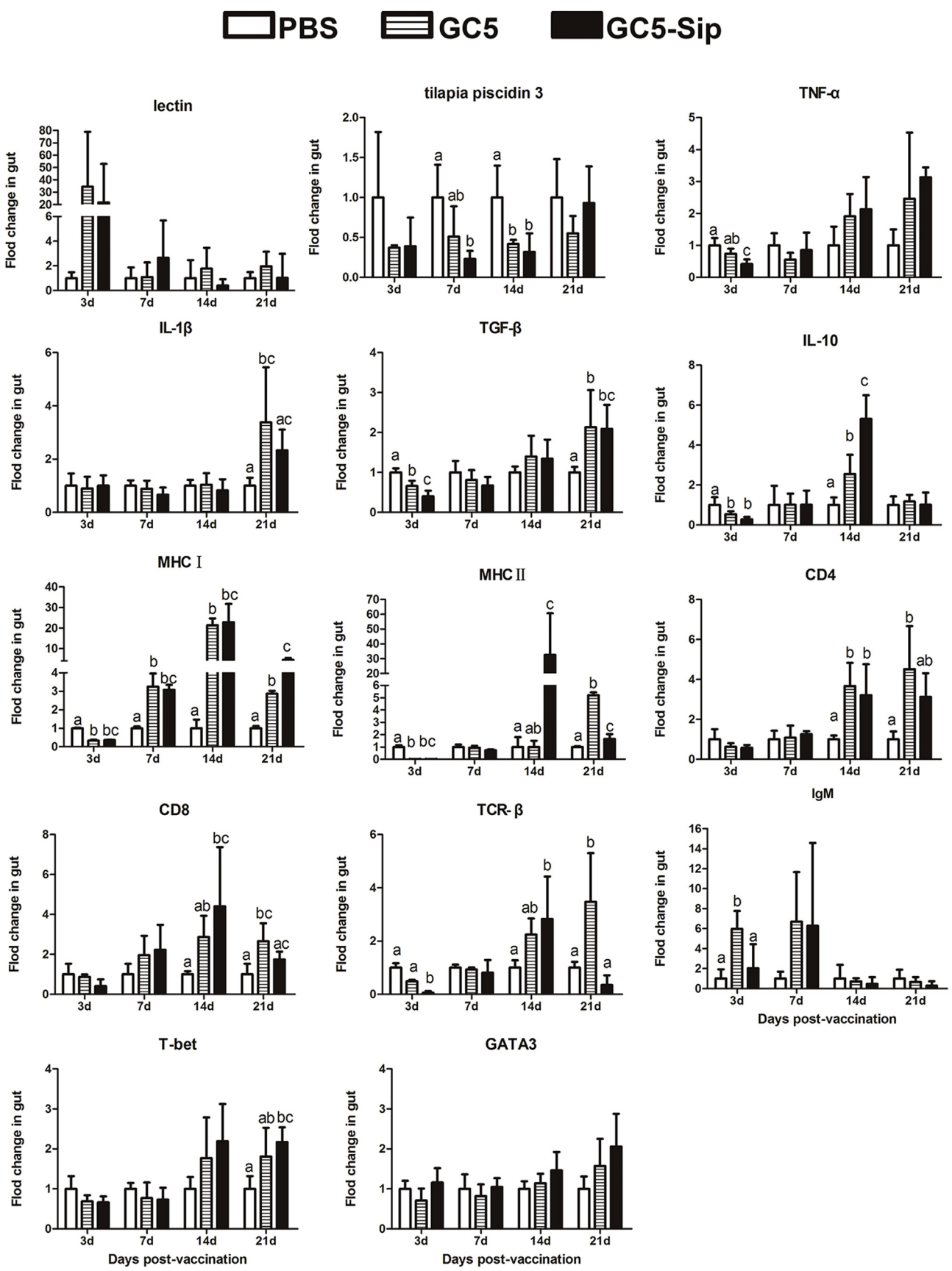

Fig. 5. qRT-PCR analysis of expression of immune-related genes in tilapia immunized with different formulations in gut after first vaccination. The letters a, b and c indicate that the differences in gene expression at the same time in the PBS, GC5 and GC5-Sip group are significant ($p < 0.05$). The same letters appearing means no significant change. Data are means for four individual fish and presented as means ± SD.

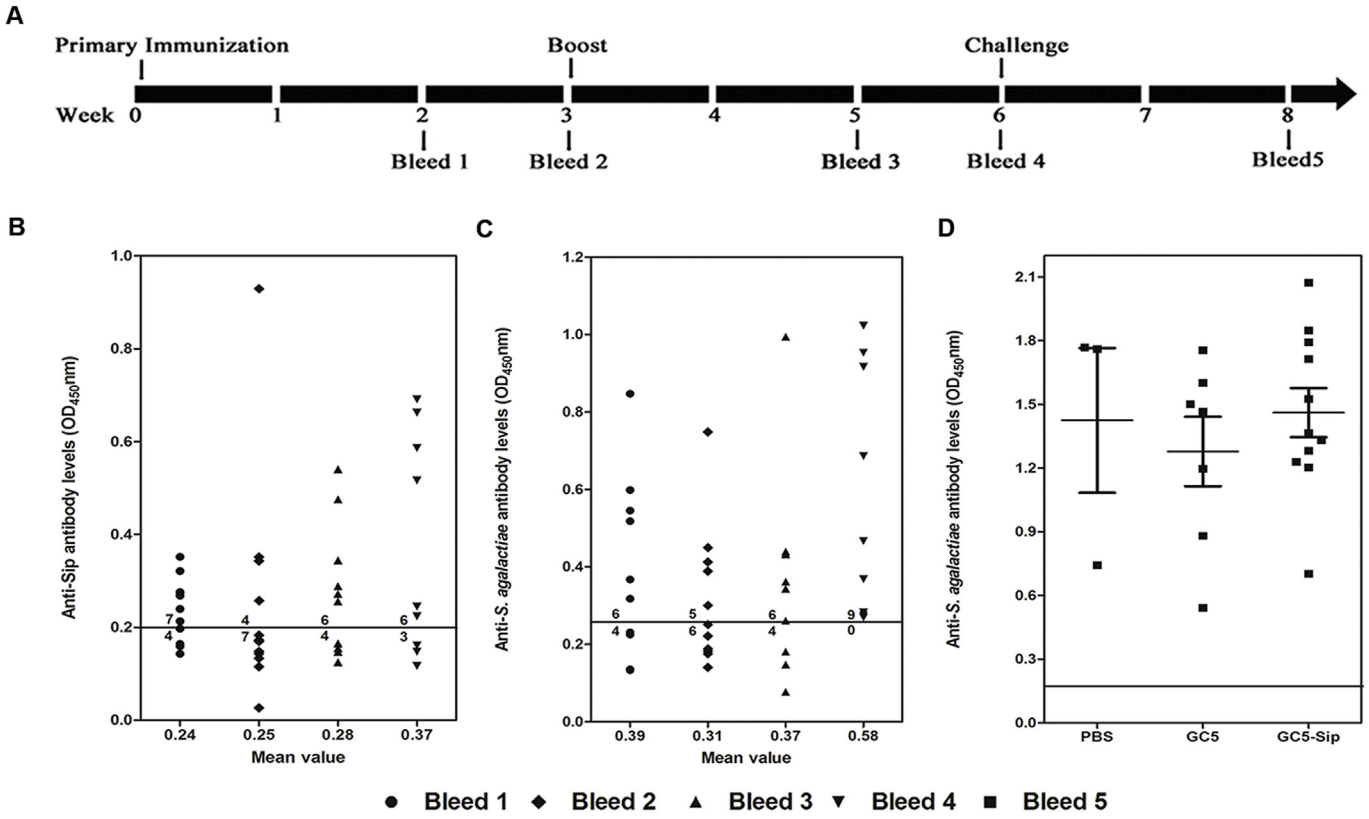

Fig. 6. ELISA detection of serum antibody levels in tilapia immunized with different formulations. (A) Strategy of oral administration of tilapia and sampling. (B) Detection of Sip-specific antibody levels by ELISA, the line on 0.2 indicates antibody levels in the PBS control group. (C) Detection of *S. agalactiae*-specific antibody levels by ELISA, the line on 0.26 indicates antibody levels in the PBS control group. (D) Detection of *S. agalactiae*-specific antibody levels in the survivors after challenge by ELISA. The line on 0.38 represents antibody levels in the PBS control group in the last bleed (bleed 4) before challenge. Each dot represents the OD_{450} value of ELISA for corresponding fish. Figures above the line show the number of immune responders, and figures below the line show the number of immune nonresponders.

Table 2
Survey of *B. subtilis* spores in tilapias hindgut (including feces).

	Spore count in different time (h)						
	3	12	24	48	72	96	120
Mean ± SD	1.28×10^4 4×10^3	1.2×10^7 2.86×10^6	7.83×10^5 2.75×10^5	1.2×10^5 5.29×10^4	5.49×10^4 8.25×10^4	8.08×10^4 6.02×10^4	1.71×10^4 5.9×10^3

and antigen gene Sip could be detected from GC5-Sip transformants' genomic DNA (Fig. 1B). These results suggest the successful integration of tandem genes into the *B. subtilis* GC5 genome.

3.2. Expression of Streptococcus agalactiae Sip protein on GC5 strain spore surface

Spore coat proteins were extracted to detect heterologous Sip expression by Western blot. As shown in Fig. 2A, a clear band corresponding to the size of the cotC-Sip fusion protein (about 65 kDa) was revealed in the samples from GC5-Sip but not in those with the wild type GC5 control, indicating the successful expression of the heterologous Sip protein on the recombinant GC5-Sip spore surface.

To further validate the expression of Sip on the spore coat of *B. subtilis*, immunofluorescence microscopy was then done. As shown in Fig. 2B, distinct fluorescence signals were captured in the recombinant GC5-Sip spores, while no specific fluorescence signals were detected in the wild type GC5 spores stained with the isotype antibody. This result further suggested that the Sip protein was displayed on the surface of the recombinant GC5-Sip spores.

3.3. Persistence of Bacillus subtilis spores in the intestinal tract

To verify whether spores for heterologous antigen delivery were present in the intestinal lumen and mucosa after intragastric inoculation, about 1-cm fragments of the hindgut containing fecal content were collected at different time intervals to determine the number of spores presenting. Spore counts showed viable GC5-Sip was present in the hindgut at 3 h in low levels, and it reached maximum levels at 12 h. By 24 h, the GC5-Sip spore count was still considerably high, and it persisted steadily to 120 h (Table 2). Therefore, this data showed that although spore counts gradually dropped over time, considerable numbers of spores were detectable at each time point.

3.4. Vaccine efficacy

Three weeks after the second vaccination, three groups of tilapia vaccinated with GC5-Sip spores or with the GC5 native spores or PBS control were challenged by *S. agalactiae*. Mortality occurred on the first day after bacterial infection in the PBS/*S. agalactiae* and GC5/*S. agalactiae* groups, while it occurred one day later in the case of the GC5-

Sip/*S. agalactiae* group. The mortality was mainly concentrated in the first three days, and it reached the peak at that time. The challenge data showed greater differences in protective effects in the three groups, and cumulative mortality rates were 85.7% for the PBS/*S. agalactiae* group, 65% for the GC5/*S. agalactiae* group and 50% for the GC5-Sip/*S. agalactiae* group (Fig. 3). Hence, the corresponding RPS of GC5 and GC5-Sip was 24.2% and 41.7% with PBS as a control. In conclusion, oral vaccine GC5-Sip spores could increase the survival rate of tilapia against *S. agalactiae*. In addition, the samples collected from the brain, visceral liquid, liver, cranial kidney and eye from moribund fish in each group during and at the end of the experiment were subjected to a bacteriological examination. *S. agalactiae* used for the challenge was isolated and confirmed.

3.5. Immune-related gene expression

At 3, 7, 14 and 21 d after the first oral administration, qRT-PCR was carried out to analyze the expression of immune-related genes in the spleen and hindgut to reflect the changes in the immune responses of the fish. In the spleen, the transcriptional levels of innate immune-related genes lectin and tilapia piscidin 3 (TP3) in spore-immunized groups increased a lot at the early stages. A rise in IgM and IL-1β gene expression also occurred earlier. The gene expression of TNF-α and TGF-β in the GC5-Sip group did not change at first when compared to the GC5 group or PBS group, whereas it was significantly up-regulated at 21 d. The profile of MHC I and MHC II expression decreased first and then increased significantly, along with the rise of TCR-β. The increasing response of T-bet and GATA3 was significant, although with variations in response time. Although CD4, CD8 and IL-10 transcription was also detected, the levels did not show a significant change in each group (Fig. 4). As for the hindgut, the relative levels of IL-10 and TGF-β in GC5-Sip were slightly up-regulated, and the gene expression of lectin, MHC I, MHC II, TCR-β and IgM had a change trend similar to that seen in the spleen. Unlike in the spleen, in the gut, mRNA expression of CD4 and CD8 was found to be up-regulated, while the expression level of TP3 was relatively low. T-bet in GC5-Sip was significantly higher than that in the control group. However, the gene expression of GATA3, TNF-α and IL-1β was not statistically significant (Fig. 5).

3.6. Measurement of antibodies in serum by ELISA

To evaluate the ability of recombinant spores to induce systemic humoral immune responses, sera from tilapia immunized with GC5-Sip spores were used to test the appearance of Sip-specific antibody and this antibody was then used to validate the ability to recognized bacteria. As shown in Fig. 6B, compared to the PBS group, in the same antibody titer (1:50), the Sip-specific antibody was detected at week 2 (bleed 1) after primary immunization, and the antibody response level as well as the proportion of responder fish increased and peaked at week 6 (bleed 4) after the booster vaccination. No specific antibody responses were detected in the GC5 group (data not shown). The antisera from responder fish could also recognize the pathogen (Fig. 6C) and antibody responses to *S. agalactiae* of all survivor groups (including PBS,GC5, and GC5-Sip group) after challenge were positive (Fig. 6D).

4. Discussion

There are few efficacious and safe oral vaccines currently approved for use in humans, let alone in fish. In this work, recombinant *B. subtilis* spores displaying the antigen Sip on the surface were restructured and used as an oral vaccine. *B. subtilis* was selected as the antigen delivery vehicle because of its many advantages. First, *B. subtilis* is widely used as a probiotic in aquaculture [24]. In tilapia fed *B. subtilis*-supplemented diets for a certain period of time, weight gain significantly increased and innate immune parameters were enhanced [25]. Additionally, some pathogens including *S. agalactiae* can be inhibited by *B. subtilis* [20]. Second, genetic manipulations are easy to implement. There are many reports about the use of cotC, a major component of the *B. subtilis* spore coat, as a fusion partner for the expression antigens on the *B. subtilis* spore coat [26,27], and this fusion does not affect the structure or function of spores [26]. Third, and most importantly, *B. subtilis* spores show immunogenicity and efficient adjuvant activity [28]. Orally administered *B. subtilis* seem to generate not only humoral immunity but also cellular immune responses. These facts made *B. subtilis* the best choice for a vaccine delivery system.

The dissemination of *B. subtilis* spores in the tilapia hindgut showed that *B. subtilis* viable spores are still detectable in 5 days (120 h) after a single oral immunization. This suggests that spores are not transient passengers of the gastrointestinal tract, because they may adhere to mucus and epithelium to colonize in the intestinal mucosa [29]. The same phenomenon has been observed previously in murine models [30,31]. Consequently, spores, as better presentation of the passenger antigen, may protect the surface antigen through the gastrointestinal tract environment [32]. In addition, a reasonably timed booster immunization can extend the antigen retention time in the hindgut where mucosal immunity mainly takes place, reasoning that enough to stimulate strong and long-lasting immune responses.

As fish reside in the aquatic environment, most pathogens initiate their infections from the mucosal surface, including the gills and skin [33], or via the gut [34]. Some pathogens including *S. agalactiae* can enter the circulatory system and reach other organs [35]. Therefore, a complex and effective immune response always consist of interactions between innate and acquired immune systems, which would help to resist the invasion of pathogens. In this work, gut (representing local mucosal tissue) and spleen (representing systemic lymphoid organ) were chosen to detect the immune-related gene expression. The innate immune system is the first line of host, in which lectin and antimicrobial peptides (AMPs) are important factors. In the present study, lectin and TP3 [36,37] in *B. subtilis* group(both GC5 and GC5-sip)were up-regulated to a large extent at 3 d, although TP3 transcript in gut did not increase, this still indicates that *B. subtilis* can induce the innate immune response. As for TP3 expression in gut, the same case occurred in a previous study [37], this might because piscidin family expression shows substantial differences among tissues. Based on the results in the current study, the acquired immune is more conducive than innate immune to a specific antigen. Unlike mammals, teleost fish lack Peyer's patches in the gut [38]. However, fish possess a strong antigen uptake and transport capacity in the second gut segment containing diffusely scattered lymphoid cells [39–41], such as macrophages, B cell and T cell [42]. When spores protect antigens pass through the hostile gastrointestinal environment and reach the second segment of the intestine, they can be taken up and transported from lumen to underlying tissues. They are then processed by intraepithelial macrophages [43], in which spore showed breakage of the electron-dense outer coat [28] that carries the antigen. In this work, MHCII and downstream of the combinative cell marker CD4 were highly expressed in both the gut and spleen. This accounts for the occurrence of antigen presenting and excitation of the helper T cell response. At the same time, the induction of pro-inflammatory cytokine IL-1β and TNF-a produced by macrophages further illustrated the activation of macrophages. Accompanied by the expression of a pro-inflammatory factor increase, the anti-inflammatory cytokines IL-10 and TGF-β subsequently increased to keep the dynamic balance between the inflammatory response and avoid causing unnecessary damage to the host. Besides macrophages, the up-regulated mRNA transcripts of IgM indicates that the function of B lymphocytes are also activated. B cell may act as antigen-presenting cells (APCs) and may also play a role in innate immunity, as teleost fish B cells have been proved to have potent phagocytic activities and microbicidal abilities [44,45]. In this study, the expression level of MHCI and CD8 also increased, suggesting that other cells were involved in antigen presentation in addition to professional APCs. Since spores may be taken up by cells such as enterocytes in the gut [43,46], or cross-presentation may

occur and then present antigens to T-killer cells to attack cells invaded by pathogens, the generation of mature cytotoxic T cells can be enhanced by helper T cells. However, it is not yet clear whether Th1 and Th2 cells are equally efficient [47]. TCR-αβ molecules recognize an antigen-derived peptide presented by APCs that express MHC class I or class II, whereas the targets recognized by γδ-TCRs are not fully understood [48,49],this is corresponding to the result of increasing expression level of TCR-β in this work. Two transcription factors are responsible for the shift of $CD4^+$ T cells into the Th1 or Th2 phenotype: T-bet for Th1 and GATA-3 for Th2 [50,51]. Thus, the increased mRNA level of T-bet and GATA-3 after immunization in the spleen and gut naturally suggested that the vaccines triggered reactive Th1/Th2 immune responses. Overall, the GC5 group showed a trend similar to that of the GC5-Sip group, likely because *B. subtilis* as probiotics has strong immunogenicity and may serve as a vaccine adjuvant [32,52]. This was similar to previous research in the murine model indicating that *B. subtilis* can promote active lymphocyte proliferation within Peyer's patches and the production of cytokines in mesenteric lymph nodes and in the spleen [52]. Thus, making spores as carriers to display the antigen Sip can help strengthen the immune response, especially in antigen presenting. From what has been discussed above, both innate and acquired immune responses were indeed elicited by the GC5-Sip vaccine following oral administration. This is consistent with previous research [53].

Furthermore, spores seem to generate not only cellular immune responses but also humoral immunity. Systemically delivered immune protection that induces humoral immune protection is highly desirable to efficiently protect against pathogen invasion. Due to the lack of an IgG homolog and a class switch mechanism in teleost fish, the main types of antibody responses in serum are mainly mediated by IgM [54]. This work showed that GC5-Sip could make the fish produce specific IgM in the sera which demonstrated that an effective humoral immune response was induced. More importantly, the sip-specific antibody from the tilapia immunized with oral vaccines could effectively recognize the pathogen. The antibodies could facilitate phagocytosis of pathogens by opsonization and activate the classical complement pathway in fish, providing a favourable factor against pathogen invasion [55]. This is corresponds with the activation of Th2 and IgM expression mentioned above, which can efficiently induce responses in populations of small, resting B cells to secrete immunoglobulin [56]. In addition, bleeds 3 and 4 showed that a booster vaccination is necessary to enhance the specific antibody level. The antibody levels of all surviving fish were positive, illustrating again the importance of antibody against pathogenic bacteria.

The most meaningful aspect of this work was that oral immunization with recombinant spores was able to confer protection against the infection of *S. agalactiae* at lethal levels. Compared to PBS-vaccinated fish, the RPS of native spore-vaccinated fish was 24.2%, which might because that the probiotic properties of *B. subtilis* were effective to strengthen the innate immune responses and could reduce mortality from bacterial challenge [57,58]. On this basis, the RPS of recombinant spore-vaccinated fish achieved 41.7%. This strongly suggests that protective immunity against *S. agalactiae* was indeed elicited. In combination with the qRT-PCR and ELISA results, we speculated that this was probably due to the activation of humoral and cellular immunity after vaccination.

In summary, this study utilized *B. subtilis* GC5 spores as a delivery vector to display conserved Sip of *S. agalactiae* on the spore surface, thereby generating a safe and effective vaccine candidate. Our results showed that the levels of specific antibodies and immune-related genes were significantly higher in the GC5-Sip group than in the GC5 group or PBS group, suggesting that the orally administrated vaccine created with *B. subtilis* spores offers high immunogenicity and sufficient protective immunity to resist bacterial invasion.

Conflicts of interest

The authors have no conflicting commercial or financial interest in publishing this paper.

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