Fish and Shellfish Immunology 87 (2019) 627–637

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Oral immunization with recombinant *Lactobacillus casei* expressing flaB confers protection against *Aeromonas veronii* challenge in common carp, *Cyprinus carpio*

Yi-Di Kong[a,1], Yuan-Huan Kang[a,1], Jia-Xin Tian[a], Dong-Xing Zhang[a], Lei Zhang[a], Luo-Tao Tao[a], Tong-Lei Wu[b], Ying Li[a], Gui-Qin Wang[a,**], Xiao-Feng Shan[a,*]

[a] College of Animal Science and Technology, Jilin Agricultural University, Changchun, 130118, China
[b] Provincial Key Laboratory of Preventive Veterinary Medicine, Hebei Normal University of Science and Technology, Qinhuangdao, 066004, China

ARTICLE INFO

Keywords:
Lactobacillus casei
Flagellin
Immune protection
Aeromonas veronii
Live vector vaccine
Common carp

ABSTRACT

Aeromonas veronii is an important type of gram-negative pathogen of human-livestock-aquatic animal and causes great economic losses in the aquaculture industry. Vaccination is an effective method of defence against *A. veronii*. There are many factors that restrict the use of vaccination, and the development of new oral vaccines is urgently needed. The selection of suitable antigens is of great significance for the development of aquaculture vaccines. Bacterial flagellin can specifically bind to TLR5 and induce the release of cytokines from the organism, which could be used in the development of vaccines. In this study, we constructed two recombinant *Lactobacillus casei* (*L. casei*) (surface-displayed or secretory) expressing the flaB of *A. veronii* and evaluated the effect of immune responses in common carp. The flaB gene (900 bp) of *A. veronii* was subcloned into the *L. casei* expression plasmids pPG-1 (surface-displayed) and pPG-2 (secretory). Western blot and immunofluorescence assays confirmed the expression of the recombinant flaB protein. Common carp immunized with Lc-pPG-1-flaB and Lc-pPG-2-flaB via oral administration route exhibited induction of antibody expression and innate immune responses. The results indicated that Lc-pPG-1-flaB and Lc-pPG-2-flaB can induce high levels of IgM, ACP, AKP, LZM and SOD activity in organisms, and Lc-pPG-1-flaB can induce even higher levels. The recombinant *L. casei* may effectively induce humoral immunity and increase the serum immunological index. Furthermore, leukocytes phagocytosis percentage and index of the recombinant *L. casei* were enhanced. The results of qRT-PCR showed that recombinant *L. casei* can significantly increase the expression of IL-10, IL-β, IFN-γ and TNF-α in the tissues of immunized common carp, compared with control groups. Viable recombinant *L. casei* strains, which were delivered directly survived throughout the intestinal tract. Common carp that received Lc-pPG-1-flaB (66.7%) and Lc-pPG-2-flaB (53.3%) exhibited higher survival rates than the controls after challenge with the pathogen *A. veronii*. Our work indicated that Lc-pPG-1-flaB and Lc-pPG-2-flaB had beneficial effects on immune response and enhanced the disease resistance of common carp against *A. veronii* infection. The combination of flaB delivery and the Lactic acid bacteria (LAB) approach may be a promising method for the development of oral vaccines for treating *A. veronii*. In future research, we will focus on the colonization ability of LAB in the intestines and on the impact of these bacteria on intestinal flora.

1. Introduction

Aeromonas veronii is an extremely important communicable pathogen of humans, animals and aquatic organisms. This pathogen is widely found in aquatic environments and most strains exhibit high pathogenicity [1,2]. There have been increasing reports of aquatic animal diseases caused by *A. veronii* in China, which has led to enormous economic losses to the freshwater aquaculture industry [3]. According to Qin G M et al., *A. veronii* is pathogenic and can be infected alone, causing the onset of disease and death of common carp [4]. A large number of deaths were reported to have occurred among common carp cultured on a farm, and the pathogen was identified as *A. veronii*

* Corresponding author.
** Corresponding author.
E-mail addresses: wgqjlau@alyun.com (G.-Q. Wang), sxf1997@163.com (X.-F. Shan).
[1] Yi-Di Kong and Yuan-Huan Kang, equally contributed to this study.

https://doi.org/10.1016/j.fsi.2019.01.032
Received 17 November 2018; Received in revised form 13 January 2019; Accepted 23 January 2019
Available online 30 January 2019

[5]. Moreover, the number of cases of human diarrhea caused by *A. veronii* is also on the rise, which is a serious to public health safety. Due to frequent problems of quality and safety in aquatic products, vaccination is considered an effective strategy for prevention of infection [6]. Hence, the development of novel vaccines against *A. veronii* infection is urgently required. Several studies have established that multiple virulence factors of *A. veronii* play a significant role in invasion of the defence system, colonization and spreading [7,8]. The currently known virulence factors of *A. veronii* were reported by Song et al., including lipopolysaccharides (LPS), outer membrane proteins (OMPs), aerolysin (Aer), flagellin, etc [9]. Flagellin, as a favorable immune antigen, has broad prospects for the development of vaccines, which can immunize hosts individually, cause high levels of cytokine expression and play a crucial role in the immune response [10,11]. Mohammad et al. have reported that vaccination with recombinant fused with flagellin enhances cellular and humoral immunity against urinary tract infection in mice [12]. Vaccination with flagellin conferred protection against colonization by *Escherichia coli* (*E. coli*) in a murine model, as reported by Koushik et al. [13].

Notably, mucosa is the first barrier encountered by foreign pathogens that come into contact with fish, which local immune response also has a protective effect on the body. Compared with traditional immunization, mucosal immunity produces high levels of antibodies that strongly activate host T cells and stress responses [14]. Particularly, mucosal immunity is highly suitable for the prevention of large-scale infectious diseases among animals. The pathway of mucosal immunity in digestive tract has become a hot topic in current research [15]. Therefore, oral vaccines prepared by mucosal immunization are suitable methods for treatment of aquatic diseases such as *A. veronii* infection in fish. Zhang D X et al. have reported that OmpAI can be expressed in *L. casei* successfully and exhibits favorable immune effects as an oral vaccine in common carp infected with *A. veronii* [16].

As protein delivery vectors for mucosal immunization, LAB can induce an effective immune response and immune tolerance in the body, which has recently become a hot topic research [17]. An efficient mucosal vaccine carrier should prevent immunogens from being degraded by gastric acid or digested in the intestine [16]. In this context, LAB has been widely recognized as vaccine vectors that can withstand the adverse environment of the intestine and complete the colonization and antigens presentation. LAB are non-pathogenic microorganisms that have produce a series of beneficial effects on the body. The most typical effect is improvement of the composition of the intestinal flora and regulation of the intestinal micro-ecological balance. It has been reported that LAB can form a protective layer in the intestine to resist colonization by foreign pathogens [18]. In previous studies, many *Lactobacillus*-associated model vaccines have been developed. For instance, the recombinant *L. casei* expression system can induce a mucosal immune response and a systemic immune response in mice [19]. *Lactobacillus* species can be effectively colonized specific areas of mucosal tissue and successfully induced local mucosal immunity [20]. In a word, LAB is suitable candidates for delivery of heterologous antigens to mucosal sites. However, to date, there have been few studies on recombinant *L. casei* strains that produce a protective antigen against *A. veronii* as a candidate vaccine.

In this study, we successfully expressed the *A. veronii* antigen flaB in the *L. casei* CC16 strain with natural antigenicity. Common carp immunized with recombinant *L. casei* via oral administration route exhibited a significant specific antibody response. In summary, our work suggests that the oral vaccine based on *L. casei* may be a promising way for the control of bacterial infections in aquaculture and possesses very broad development prospects.

2. Materials and methods

2.1. Fish

Clinically healthy common carp specimens weighting 56.0 ± 1.0 g were purchased from a commercial fish farm (Changchun, Jilin, China) and maintained in 160 L tanks with aeration at 25 ± 1 °C, and allowed to acclimatize for two weeks. The common carp were fed a commercial diet approximately 1% of their body weight twice daily. All the animal experimental procedures and immunizations were preformed in accordance with the Regulations for Animal Experimentation of Jilin Agricultural University.

2.2. Bacterial strains, plasmids and growth conditions

A. veronii TH0426 was isolated from *Pelteobagrus fulvidraco* (yellow catfish) as described previously [16] and grown in Rimler-Shotts (RS) medium (Luqiao, China). *L. casei* CC16 was isolated from the intestine of common carp and cultured anaerobically in MRS medium (Solarbio, China) at 30 °C. For plasmid cloning, competent *E. coli* MC1061 cells were grown in Luria-Bertani (LB) medium at 30 °C with shaking. Chloramphenicol, (*Cm*; Sigma, USA; final concentration, 10 μg/ml) was added to the medium. The *E. coli-Lactobacillus* shuttle vector pPG-1, a type of cell-surface expression plasmid containing an anchoring matrix-encoding pgsA gene derived from *Bacillus subtilis* behind the target gene, was used. To ensure normal target protein secretion, the pPG-1 and pPG-2 harbour the ssUSP secretion signals before the target gene.

2.3. Construction of recombinant L. casei-flaB

The flaB gene (900 bp) of the *A. veronii* TH0426 strain (GenBank: CP012504.1) was amplified by PCR. The forward primer was designed as 5′ AACCCGGGATGGC

CATGTTCATTAACACTAATA 3′ containing a *Sma*I restriction site (underlined), and the reverse primer was designed as 5′ CCGATATCT TAACCAAGCAGAGATA

GTGCTGAT 3′ containing an *Eco*RV restriction site (underlined); additionally, a forward primer was designed as 5′ CGGGATCCATGGC CATGTTCATTAACACTA

ATA 3′ containing a *BamH*I restriction site (underlined), a reverse primer was designed as 5′ CCCTCGAGTTAACCAAGCAGAGATAGTGC TGAT 3′ containing a *Xho*I restriction site (underlined). The purified PCR product of the flaB gene was digested with *Sma*I/*Eco*RV or *BamH*I/*Xho*I restriction endonucleases and inserted into the corresponding sites of the pPG-1 or pPG-2 expression vector respectively, to obtain as pPG-1-flaB or pPG-2-flaB, respectively. The pPG plasmids have been described in a previous study, and the recombinant pPG-1-flaB and pPG-2-flaB were transformed into *L. casei* CC16 by electroporation [21]. The positive clones of Lc-pPG-1-flaB and Lc-pPG-2-flaB were verified by DNA sequencing. As a vector control, *L. casei* containing the pPG plasmid without the flaB gene (Lc-pPG) was used for the subsequent experiments.

2.4. Western blot analysis and immunofluorescence assay

As described previously, expression of the recombinant flaB gene in *L. casei* strains was checked by Western blot analysis [22]. Concisely, Lc-pPG-1-flaB, Lc-pPG-2-flaB and Lc-pPG were grown in basal MRS medium containing 10 μg/ml of chloramphenicol. Xylose was added to the culture medium (final concentration, 10 g/l) to induce antigen expression. After induction at 30 °C for 10 h, pellets containing

approximately 1×10^8 cells were analyzed by SDS-PAGE and the proteins were electrotransferred onto a nitrocellulose membrane. The immunoblots were blocked with PBS containing 5% skimmed milk and incubated with mouse anti-flaB serum (1:100 dilution), which was prepared in our laboratory, as a primary antibody, followed by HRP-conjugated goat anti-mouse IgG (Sigma, USA) at a dilution of 1:200 as a second antibody. Finally, the immune-complexes were detected using the Western ECL substrate (Thermo Scientific) in an Amersham Imager 600 (GE Healthcare, UK). The surface-localized flaB protein from Lc-pPG-1-flaB was detected by immunofluorescence as described previously [18]. Briefly, Lc-pPG-1-flaB cells were cultured and induced in MRS broth with xylose overnight at 37 °C. Pellets containing 1×10^5 Lc-pPG-1-flaB cells were washed using PBS containing 1% bovine serum albumin (BSA) and then incubated with mouse anti-flaB serum (1:100) at 37 °C for 1 h. Subsequently, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma, USA) secondary antibodies containing 1% Evans blue at 37 °C for 2 h and analyzed with a confocal microscope (Zeiss LSM710). Lc-pPG was used as a negative control.

2.5. Growth and hereditary stability of recombinant L. casei strains

The growth and hereditary stability of recombinant *L. casei* strains was determined as described previously [16]. In short, recombinant *L. casei* strains (1:100) were transferred into MRS medium with 2% xylose (*Cm*, 10 μg/ml), and the OD 600 was examined at time of induction and 5 h after induction. Plasmids were extracted from the cells, and the presence of flaB fragments in each strain was verified using specific primers for the flaB gene by PCR.

2.6. Immunization and sample collection

For oral administration, recombinant *L. casei* were grown overnight in MRS medium containing *Cm* (10 μg/ml) supplemented with xylose, were mixed thoroughly with a commercial basal diet, and then oven-dried at 40 °C for 6 h. Finally, an average of 2×10^9 cells of each recombinant *L. casei* strains and the diet were kept at 4 °C until feeding. Two experiment groups (n = 80 each) of fish were fed diets containing Lc-pPG-1-flaB and Lc-pPG-2-flaB respectively, and under the same conditions, two control groups (n = 80 each) of fish were fed diets containing Lc-pPG and PBS separately. Oralvaccination was conducted on days 0, 14, 28, 42, 56, according to the immune protocol, and the vaccine was administered on three consecutive days at days 0–2 (prime vaccination), 28–29 (booster vaccination) and 58 (challenger) (Fig. 10A). On days 0, 16, 30, 44, 58 after the first immunization, serum were prepared from the blood samples collected from the tail vein and the liver, spleen, head kidney and intestine were rapidly collected from five fish from each group. All samples were stored at −80 °C until subsequent analysis.

2.7. Enzyme-linked immunosorbent assay (ELISA)

As described previously, the flaB-specific antibody IgM titers in the serum were determined by enzyme-linked immunosorbent assay (ELISA), and 5 μg/ml recombinant flaB protein was used as a coating antigen [23]. Ninety-six well ELISA plates were blocked with 2% BSA for 2 h at 37 °C. Five microliters of serum samples were added per well in triplicate and incubated for 1 h at 37 °C. Then, HRP-conjugated anti-common carp IgM monoclonal antibody (Stirling, Scotland) was used to detect the bound antibodies by incubation for 1 h at 37 °C, and the absorbance was examined at 450 nm. Endpoint titers were expressed as the highest dilution that yielded an OD ≥ 2 times greater than the mean value of the blank. The activity of acid phosphatase (ACP), alkaline phosphatase (AKP), lysozyme (LZM) and Superoxide dismutase (SOD) were evaluated using ELISA kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. All analyses were conducted in triplicate.

2.8. Leukocytes phagocytosis experiment

Anti-coagulant (0.2 ml) was mixed with inactivated *Staphylococcus aureus* suspension (0.1 ml) and incubated for 1 h at 37 °C, followed by centrifugation for 15 min at 1000 r/min. Then, the white blood cells were smeared at the interface between plasma and red blood cells. The cells were dried, fixed with methanol for 10 min and stained for 30 min with Giermsa stain. Eventually, 100 white blood cells were randomly selected under the microscope to calculate the leukocytes phagocytosis percentage (PP) and phagocytic index (PI).

2.9. Gene expression analysis

Total RNA from the liver, spleen, head kidney and intestine were extracted using the High Pure RNA Tissue Kit (Takara, Japan), and RNA quality was determined by visualization on a 1.0% agarose gel. The concentrations of all the RNA samples were detected using a Nanodrop 2000c (Thermo Scientific, USA) and cDNA was synthesized for quantitative reverse transcription PCR (RT-qPCR) using the Reverse Transcriptase M-MLV Kit (Takara, Japan) according to the manufacturer's instructions. qPCR was performed with the THUNDERBIRD SYBR qPCR Mix Kit (TOYOBO, China), and in a Stratagene MxPro system (Stratagene Mx3005P, USA) in 96-well reaction plates. All target and reference genes used in this study were selected on published information, and are shown in Table 1. The selected immune-related genes were IL-10, IL-1β, IFN-γ and TNF-α. The total volume of each RT reaction was 20 μl containing 1000 ng of total input RNA sample, 2 × SYBR Green Master Mix (Takara, Japan), and specific primers at a final concentration of 1 μM. All qPCRs were performed three times. The specificity of the reaction was verified by analysis of melting curves, and the data were analyzed by Stratagene MxPro software (Stratagene Mx3005P, USA).

2.10. Survival of recombinant L. casei in fish intestine

After prime immunization (at day 1), the whole intestine was collected for plating of serially diluted samples and homogenized in sterile PBS (1% fetal calf serum). The homogenates of each sample were subjected to 10^4-fold dilution and 100 μl of each dilution was plated on MRS-*Cm* agar plates and incubated under anaerobic conditions at 30 °C for 24 h. Ten single colonies were selected randomly from each sample, and the survival of recombinant *L. casei* was confirmed by direct colony PCR as described previously [24]. The housekeeping gene dnaA of *L. casei* [25] was amplified with the primers pair 5′-TCTGTTTATTTATG GTGGCG-3' (upper) and 5' -CTGCGGTCATCAAGTTTCA-3' (lower); the pPG-1-specific primer pair using a forward primer: 5′ AGGAGGAAGC TAGCACATGAAGAAA 3′ and a reverse primer: 5′-TCATATAAAATTTG GTAAAAATGTA-3′; pPG-2-specific primer pair as following: forward primer: 5' -AGGAGGAAGCTAGCACATGAAGAAA-3'; reverse primer: 5′-CACTCGAGAAGCTTGAGCTCTCTAG-3′. The PCR conditions were as follows: 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 54 °C (dnaA) or 62 °C (pPG-1 and pPG-2) for 1 min and 72 °C for 1 min. PCR products were verified by DNA sequencing analysis.

2.11. Challenge test

On day 58 post-immunization, all the vaccinated fish were injected intraperitoneally with 200 μl of the *A. veronii* TH0426 strain (LD $50 = 5 \times 10^6$ CFU/ml). The PBS group was used as the negative control. The fish challenged with *A. veronii* were monitored for 28 days, and survival rate was analyzed in all the groups post challenge.

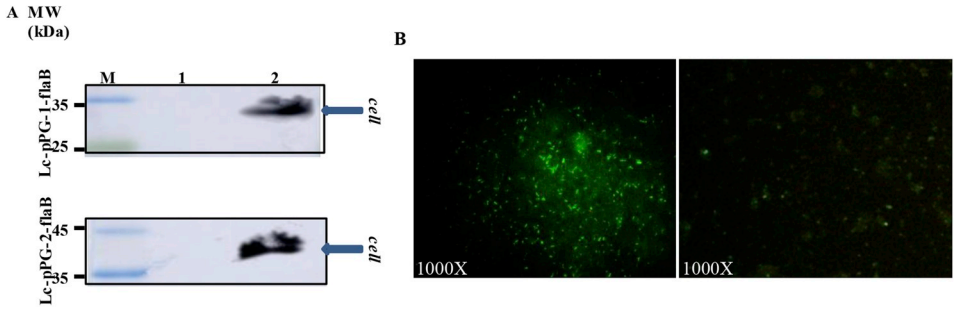


Fig. 1. Expression of flaB protein on *L. casei*. (A) Western blot analysis. Cellular extracts (Cell) was analyzed with western blotting. MW indicates the molecular mass markers (kDa). Gray and black arrows indicated the recombinant *L. casei* secreted flaB (32 kDa) in cell lysates and supernatants, respectively. (B) Immunofluorescence microscopy analysis. Lc-pPG-1- flaB (left) and Lc-pPG (right), magnification: × 1000. There was green fluorescence on the surface of Lc-pPG-1-flaB and no immunofluorescence reaction on the Lc-pPG cell surface.

2.12. Statistical analysis

Statistical analysis was performed using SPSS v.22.0 software and GraphPad PRISM v7.0. For multiple comparisons one-way ANOVA were preformed, followed by Tukey's test. Data was presented as the mean ± SD (standard deviation). In all cases, differences were considered to be significant at $p < 0.05$.

3. Results

3.1. Construction of L. casei vaccine and expression of flaB protein

The recombinant plasmids pPG-1-flaB and pPG-2-flaB were constructed and introduced by electroporation into *L. casei*. The immunoreactive band corresponding to the flaB protein was detected by Western blotting. A 32-kDa immunoreactive band of (Fig. 1A lane 2) corresponding to the flaB protein was detected in the cell lysates of Lc-pPG-1-flaB and Lc-pPG-2-flaB, but this band was not detected in the Lc-pPG lysate (Fig. 1A, lane 1). The results demonstrated that flaB protein expression and secretion can be induced by xylose in recombinant *L. casei*. Moreover, the surface localization of the flaB antigen of Lc-pPG-1-flaB was determined by an immunofluorescence assay. The results indicated that the flaB protein adhered to the surface of *L. casei*, as demonstrated by the triggering of spontaneous green fluorescence (Fig. 1B, left), while Lc-pPG exhibited no significant adhesion ability (Fig. 1B, right). This finding suggested that Lc-pPG-1-flaB cells could react with the polyclonal mouse anti-flaB antibody.

3.2. Characteristics of recombinant L. casei expressing flaB

We screened the recombinant *L. casei* by PCR and sequencing techniques and analyzed the growth and hereditary stability of the cells. As shown in Fig. 2A, recombinant plasmids were stably inherited over 50 generations. Bands shown in white (900 bp) were observed by sequencing techniques and matched the target sequences. L: DNA ladder (bp), lanes 1–3: PCR product of recombinant *L. casei* flaB gene after 50 generations. Moreover, we examined the impact of flaB protein expression on *L. casei* growth, and the growth of Lc-pPG-1-flaB and Lc-pPG-2-flaB showed no significant difference compared to that of Lc-pPG (Fig. 2B), indicating that expression and secretion of heterologous antigen had negligible limiting effects on cell growth.

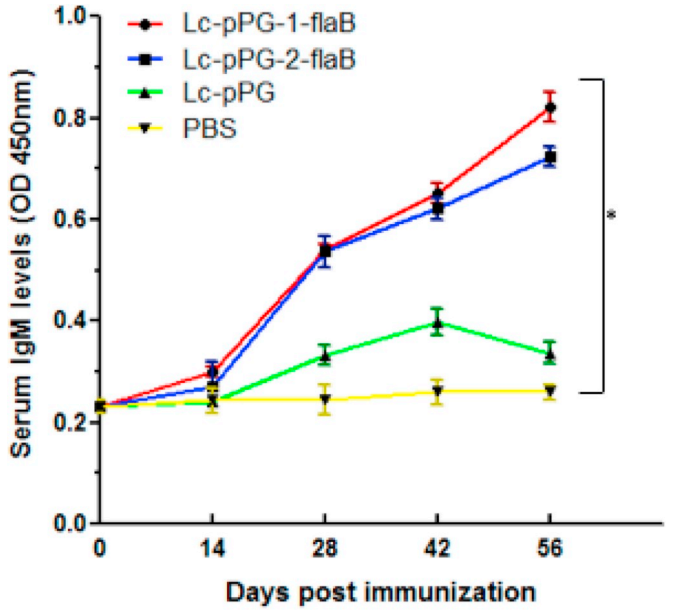


Fig. 3. The flaB-specific IgM antibodies in the serum (n = 5 fish/group) following vaccination by Lc-pPG-1-flaB, Lc-pPG-2-flaB, Lc-pPG and PBS. Data are presented as mean ± SD fold increase relative to PBS control. *: $p < 0.05$.

3.3. Humoral immune parameters

As shown in Fig. 3, the fish that received the recombinant *L. casei* exhibited increased level of specific IgM in the serum (at day 28) compared to the controls ($p < 0.05$). Meanwhile, the IgM levels of the

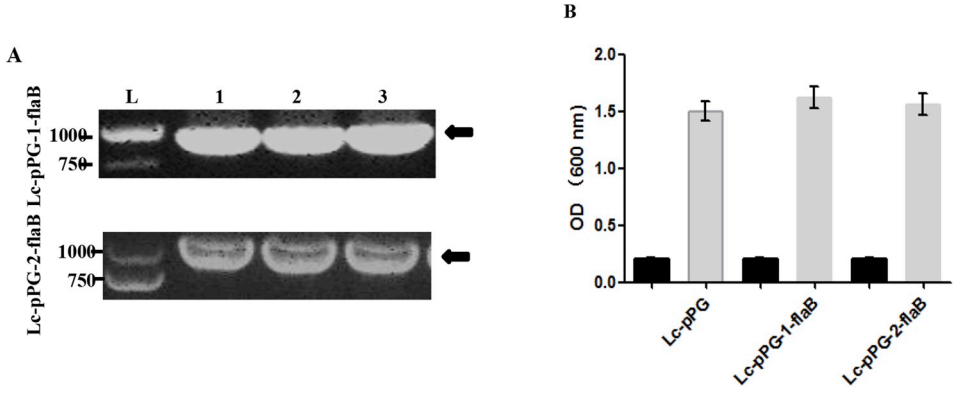


Fig. 2. Characteristics of recombinant *L.casei* expressing flaB antigen. (A) Hereditary stability of recombinant *L.casei* strains were subject to PCR with flaB-specific primer pairs confirming that cells were genetically stable after 50 generations. Bands indicated by white (900 bp) were analyzed by DNA sequencing and were consistent with the putative sequences. L: DNA ladder (bp), Lane 1-3: PCR product of recombinant *L.casei* flaB gene after 50 generation. (B)Growth of *L.casei* producing flaB antigen. The OD 600 was measured at the induction point (black bars) and 5 h after induction (gray bars) for recombinant *L.casei* strains. Data are indicated as mean ± standard deviation (SD).

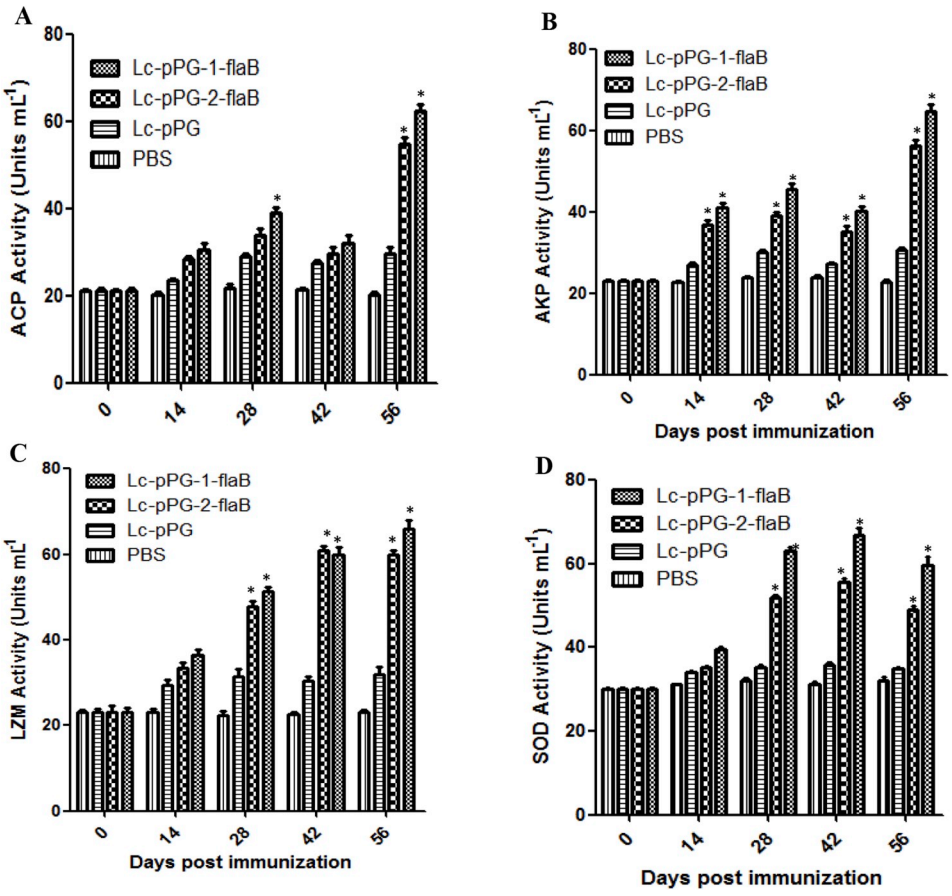


Fig. 4. Humoral immune responses elicited by recombinant *L.casei.*Changes of the activity serum of acid phosphatase (ACP) (A) alkaline phosphatase (AKP) (B), lysozyme (LZM) (C), and superoxide dismutase (SOD) (D) in peripheral blood of common carp (n = 5 fish/group) after oral immunization. Data are presented as mean as mean ± SD fold increase relative to PBS control. *: $p < 0.05$.

Lc-pPG group did increase, but there was no difference compared with the PBS group ($p > 0.05$). The levels of specific IgM in the Lc-pPG-1-flaB and Lc-pPG-2-flaB groups increased continuously, suggesting that the IgM levels in the recombinant *L. casei* groups peaked on day 56, and these peaks levels were significantly higher than those of the PBS control ($p < 0.05$).

As the results showing (Fig. 4A), the effects of recombinant *L. casei* on serum ACP activity were stronger than those of the controls. The ACP activity in all the groups increased at day 14 and increased gradually after booster immunization (at day 28). However, there was no significant difference between the Lc-pPG and PBS groups ($p > 0.05$). The highest serum ACP activity was observed in fish that received recombinant *L. casei* at day 56 compared to the controls ($p < 0.05$). Similarly, as shown in Fig. 4B, the AKP activity of Lc-pPG-1-flaB and Lc-pPG-2-flaB peaked at day 56, and the highest AKP activity was observed in the Lc-pPG-1-flaB group. On day 14, the AKP activity in the recombinant *L. casei* groups gradually increased and was significantly ($p < 0.05$) higher than that in the control group. The serum LZM activity is shown in Fig. 4C, and there were no differences ($p > 0.05$) compared to the control group at day 14. However, the activity were significantly ($p < 0.05$) higher in the Lc-pPG-1-flaB and Lc-pPG-2-flaB than in the control after booster immunization (at day 29). Likewise, as shown in Fig. 4D, SOD activity also exhibited the same increasing trend as LZM activity in all the immunization periods.

3.4. Analysis of leukocytes phagocytosis after vaccination

The results of leukocyte phagocytosis detection in immune fish serum are shown in Fig. 5. The serum of fish that received recombinant *L. casei* exhibited increase leukocytes phagocytosis ability. The PP and PI in the Lc-pPG group increased, but there was no difference compared with the PBS group ($p > 0.05$). The leukocyte phagocytosis ability in the Lc-pPG-1-flaB and the Lc-pPG-2-flaB groups increased by degrees, peaking at 56 days, and this peak value was significantly higher than that of the PBS group ($p < 0.05$).

3.5. Expression of immune-related genes

IL-10 expression in the serum is shown in Fig. 6. In liver, spleen and kidney, the Lc-pPG-1-flaB and Lc-pPG-2-flaB groups demonstrated significantly increased IL-10 expression more rapidly than the control groups ($p < 0.05$) after prime immunization (at day 1). However, there were no significant differences in intestine compared to the control group on day 14. IL-10 expression in the intestine increased quickly after booster immunization (at day 28), peaking at 56 days. There was no significant difference between the Lc-pPG and PBS groups. As shown in Fig. 7, IL-1β expression in the serum of all the groups was significantly different from that of the controls ($p < 0.05$) after booster immunization (at day 28). In kidney and intestine, IL-1β expression reached a two-fold upregulation at days 42 and 56. Likewise, as the results showing (Fig. 8), IFN-γ expression in serum was significantly increased at day 28 in all of samples compared with the control groups ($p < 0.05$) and peaked at 56 days. TNF-α expression analysis showed (Fig. 9) that the fish that received recombinant *L. casei* exhibited a rapidly increase in TNF-α expression in kidney at day 14 as compared to the controls ($p < 0.05$). In liver, spleen and intestine, almost no difference was observed between days 0 and 14 ($p > 0.05$), whereas the levels increased gradually after booster immunization (at day 28) compared with the controls ($p < 0.05$).

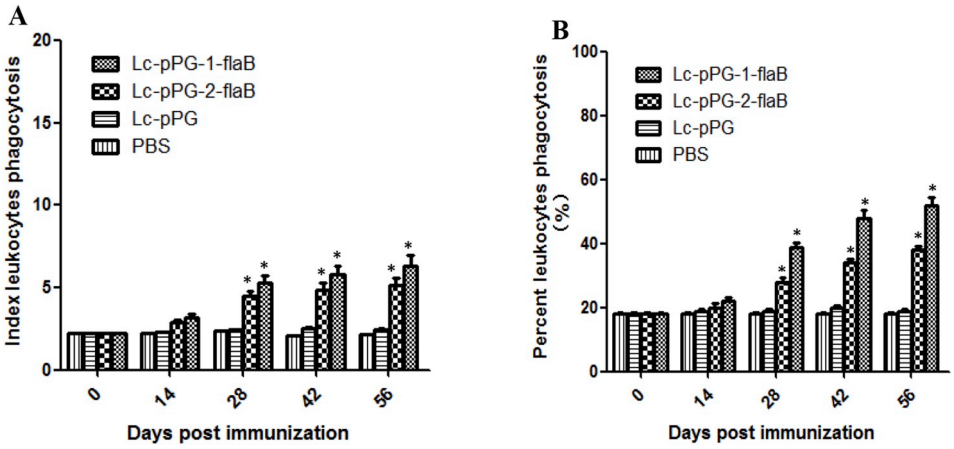


Fig. 5. Leukocyte phagocytosis percentage and leukocyte phagocytic index in the serum (n = 5 fish/group) following vaccination by Lc-pPG-1-flaB, Lc-pPG-2-flaB, Lc-pPG and PBS. Data are presented as mean ± SD fold increase relative to PBS control. *: $p < 0.05$.

3.6. Survival of recombinant L. casei in the fish intestine

As shown in Fig. 10B, colony growth of genetically modified *L. casei* was observed, and the recovery of Lc-pPG-1-flaB and Lc-pPG-2-flaB colonies from the intestine was similar. Relatively, few colonies of Lc-pPG were also observed on the plates, while no colonies were observed for the PBS controls. As shown in Fig. 10C, colonies were randomly screened, and a 615 bp PCR product was obtained by amplification of the housekeeping gene dnaA. Colonies recovered from the Lc-pPG-1-flaB (2150 bp) and Lc-pPG (1250 bp) groups were confirmed using the pPG1-specific primer pair, and those from Lc-pPG-2-flaB (2364 bp) group was verified using the pPG2-specific primer pair. All products were subjected to DNA sequencing and the obtained sequences were aligned with the putative sequences.

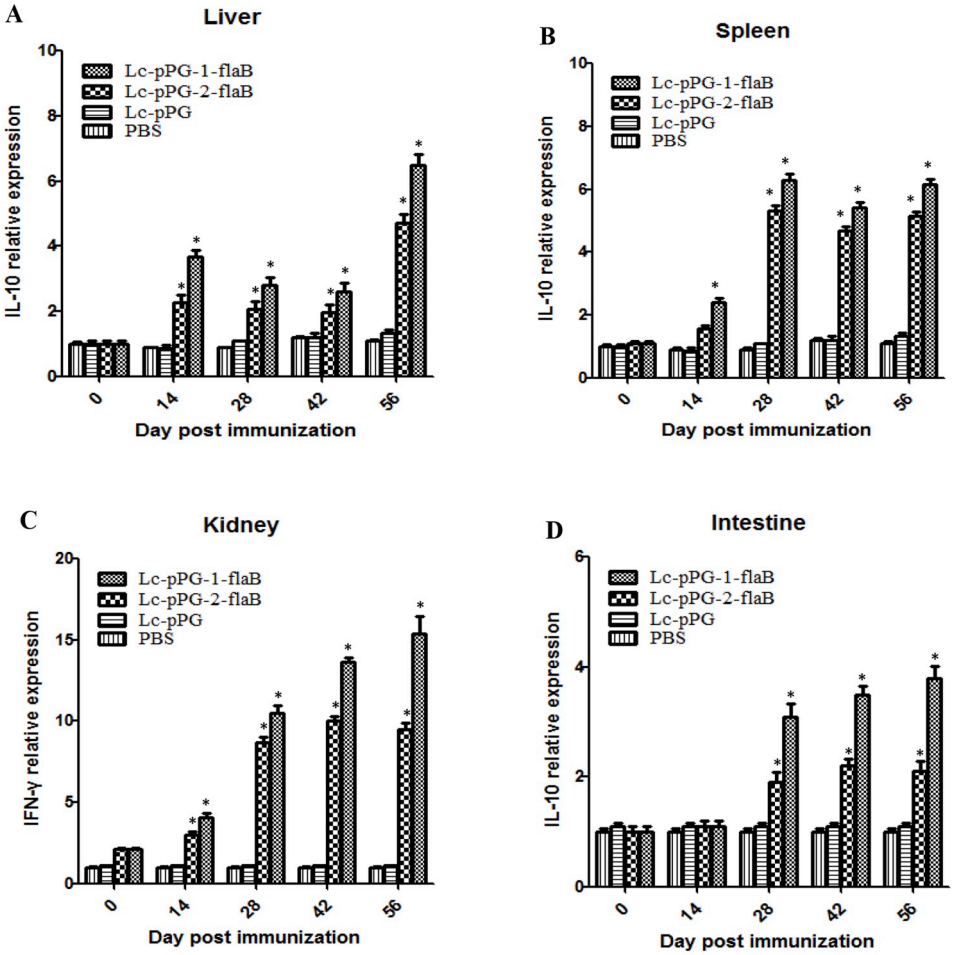


Fig. 6. qRT-PCR analysis of the expression of IL-10 in liver (A), spleen (B), kidney(C) and intestine (D) of common carp (n = 5 fish/group) after immunization. Data are presented as mean ± SD fold increase relative to PBS control. *: $p < 0.05$.

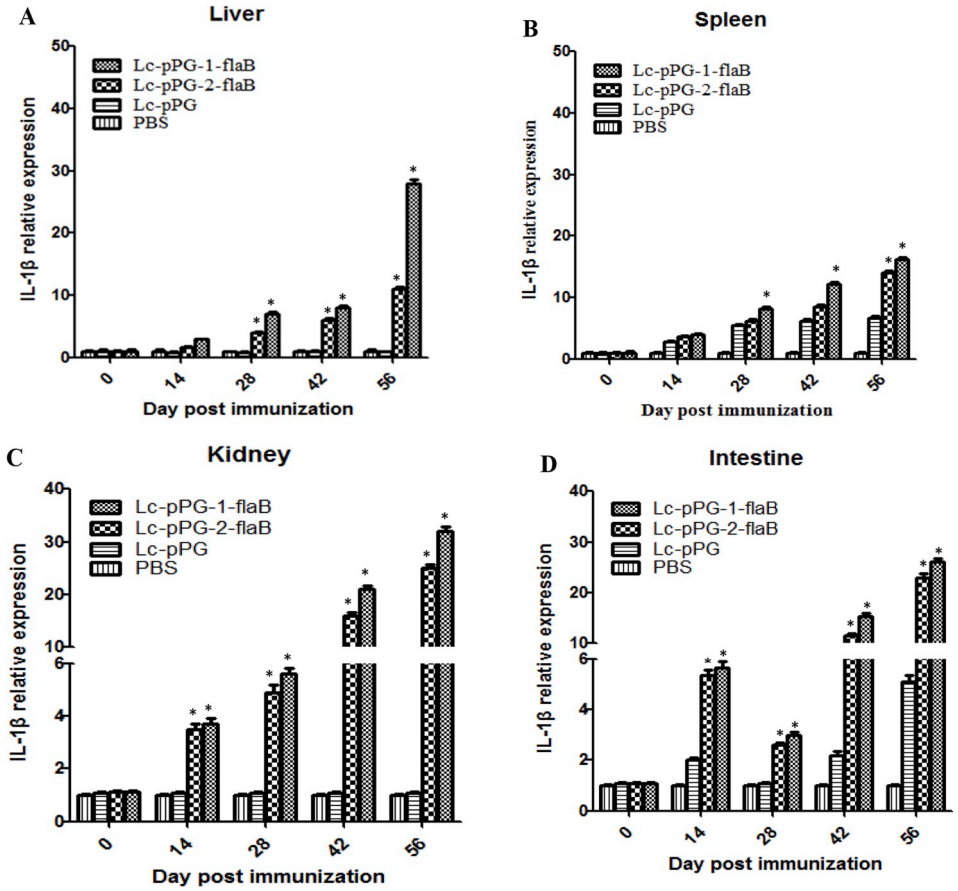


Fig. 7. qRT-PCR analysis of the expression of IL-1β in liver (A), spleen (B), kidney(C) and intestine (D) of common carp (n = 5 fish/group) after immunization. Data are presented as mean ± SD fold increase relative to PBS control. *: $p < 0.05$.

3.7. Protection against A. veronii challenge

A bacterial challenge experiment was performed to evaluate the immunity protection provided by recombinant *L. casei* vaccination against *A. veronii* TH0426 infection. As shown in Fig. 11, the relative survival percentage was 66.7% in Lc-pPG-1-flaB, 53.3% in Lc-pPG-2-flaB and 30.0% in Lc-pPG. Mortality of PBS controls started on the first day and reached 100% on day 12 post-challenge of *A. veronii*. The main clinical symptoms included increased amounts of mucus on the body surface, bleeding and festering on skin and swelling of the abdomen, etc. To sum up, the survival rate of Lc-pPG-1-flaB is higher than that of Lc-pPG-2-flaB and Lc-pPG after challenge with a lethal dose of *A. veronii*.

4. Discussion

In recent years, the aquaculture industry has faced many problems, among which diseases of aquatic animals cause great economic loss, and the use of antibiotics causes water pollution and affects the safety of aquatic food products, which have seriously restricted the development of Chinese aquaculture industry [26]. *A. veronii* is one of the main pathogens in aquaculture, and reports of aquatic animals infected with this pathogen on the rise [27]. However, there remains a lack of efficient and safe treatments for diseases caused by *A. veronii*. As of now, vaccination is the most economical and effective measure to protect the fish from disease, and this approach has many advantages, such as convenience, high efficiency and eco-friendliness. To date, inactivated vaccines, subunit vaccines and DNA vaccines have been reported to provide protection against *Aeromonas* [27]. Therefore, it is necessary to focus on aquaculture vaccines to against *A. veronii*. In aquaculture, the development of vaccines is limited by multiple factors. Different immunization methods can also lead to immune effects. The immune effect of oral immunization is not worse than that of injection-based immunization. Oral vaccines of fish prepared by mucosal immunization are more advantageous than traditional injectable vaccines. For instance, administration of microcapsules that directly present antigens can save time [28]. Meanwhile, oral vaccines can induce local mucosal immune responses, producing specific antibodies, effectively resisting invading pathogens and eliminating invading pathogens. Furthermore, oral immunization is highly suitable for the prevention and control of large-scale animal diseases, this method can be used to complete the immunization process while saving time. However, the activity of the antigen is affected by the environment of the digestive tract. To avoid these effects, a suitable delivery vector is necessary to be selected [29]. LAB are recognized as probiotics for vaccine carriers [30], which have certain effects on the growth performance, immunity and water quality of aquatic animals [31–34]. In this study, the strains of *L. casei* expressing flaB of *A. veronii* with two xylose-induced expression systems were developed and immunogenicity as a vaccine delivery vehicle to induce immune responses against *A. veronii* in fish was evaluated.

The expression system of *Lactobacillus* has widely studies, and some reports have investigated the relationship between the antigens expression site and mucosal immunity [35]. In our study, we observed that antigen could be expressed on *L. casei* stably. Detection of flaB in the cell pellet indicated that the expression system also enabled the *L. casei* to secrete or surface-display the flaB antigen (Fig. 1). Analysis of the properties of the recombinant *L. casei* strains showed that, although overproduction of heterologous proteins often hampers bacterial growth, the flaB-expressing strains grew well and genetically stable (Fig. 2). These data indicate that the immunogenicity of flaB based on *L.*

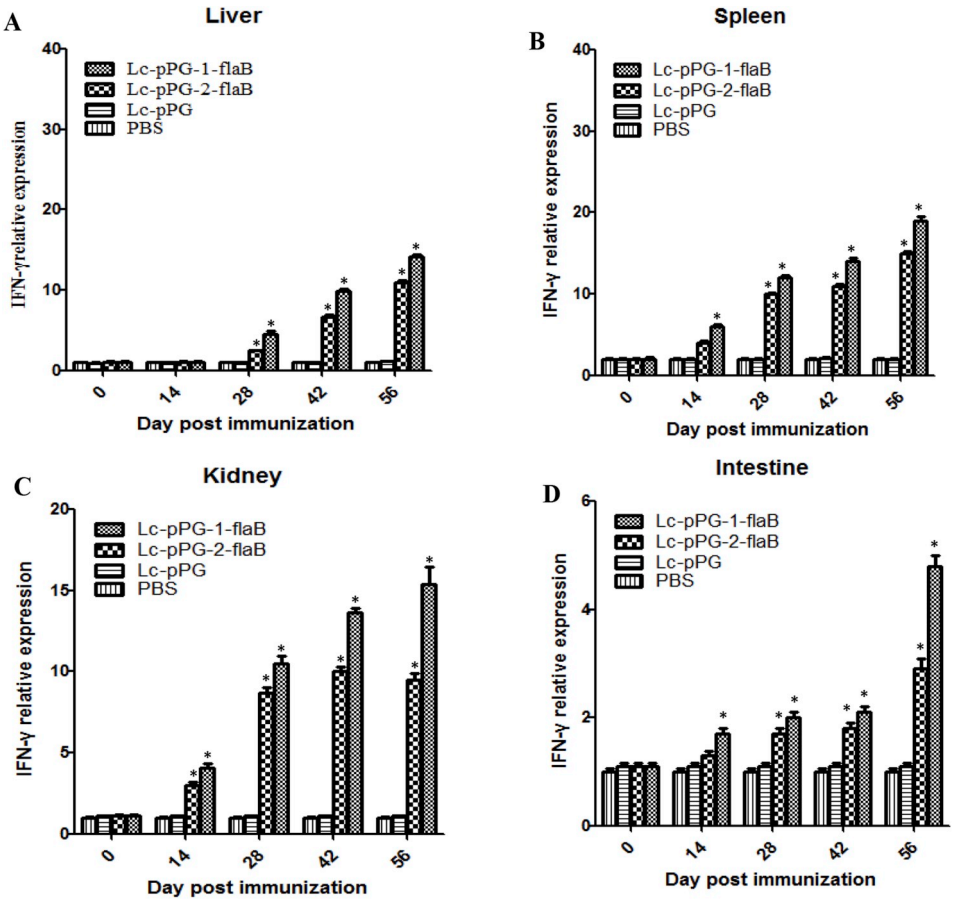


Fig. 8. qRT-PCR analysis of the expression of IFN-γ in liver (A), spleen (B), kidney(C) and intestine (D) of common carp (n = 5 fish/group) after oral immunization. Data are presented as mean ± SD fold increase relative to PBS control. *: $p < 0.05$.

casei expression system can be further investigated by oral administration in the fish model. These results are similar to those reported by Zhang DX et al. [16].

Flagellin is the main component of bacterial flagella, mainly including flaA and flaB, which exhibits good immunogenicity and can also be used as a basis for serological identification. Flagellin also has broad application prospects in the research and development of oral vaccines [36]. Delaney KN et al. has reported that immunization with OMP (OprF and OprI) in the presence of flagellins enhances the killing of nonmucoid *Pseudomonas* strains in a serum mediated manner [37]. Fish serum contains various antibacterial substances. The results of analyzes showed that the flagellin protein was immunogenic against *Aeromonas* infection. In this regard, the highly conserved flaB of *A. veronii* is an attractive candidate antigen for oral vaccines. The IgM of fish is an important component of the humoral immune system. IgM is a typical immune indicator in fish that can resist foreign pathogens in a timely manner. In our study, periodic blood sampling was used to measure IgM levels, Lc-pPG-1-flaB and Lc-pPG-2-flaB were analyzed that both groups induced high levels of IgM expression in the body. Our results showed that the levels of flaB-specific IgM antibodies in serum of fish immunized against *A. veronii* were significantly increased after prime immunization in Lc-pPG-1-flaB group, which demonstrated slightly higher levels than Lc-pPG-2-flaB group. When fish are invaded by foreign pathogens, leukocytes can inoculate the invading pathogens non-specifically, so that the phagocytic ability also reflects the body's immunity level in fish. In our study, the activity of phagocytic leukocytes was analyzed by detection of PP and PI. The PP and PI values showed that the leukocytes phagocytic activity in recombinant *L. casei* was enhanced rapidly after booster immunization (at day 28). Our data also revealed that oral vaccination with recombinant *L. casei* likely contributes to increased serum levels of non-specific immune parameters. ACP, AKP, LZM and SOD are rapidly secreted during pathogenic invasion, resisting and decomposing pathogens in a timely manner. Via oral immunization with recombinant *L. casei*, the body's activity of ACP, AKP, LZM and SOD in the fish was increased. Our results indicated that recombinant *L. casei* can benefit the improvement of humoral immunity. Lc-pPG-1-flaB can induce higher levels of SOD and LZM than Lc-pPG-2-flaB, and these enzymes are essential for humoral immunity. This finding is consistent with studies in which probiotics have been shown to significantly increase ACP, AKP, LZM, and SOD activity in aquatic animals [36,37].

When the body is stimulated by the external environment, a variety of cytokines, are produced that have various biological functions, such as functions associated with damaged tissue repair, adaptive immunity and cell growth [38,39]. IL-10 is an anti-inflammatory factor that is effective at relieving inflammation. IL-1β is widely accepted as a pro-inflammatory factor, which is used as a reference gene in studies of immune regulation [40]. Moreover, TNF-α is plays an essential role in response to bacterial and viral invasion [41]. IFN-γ can play a defensive role in response to invasion by pathogens and serves as the first line of defence against viral infection [42]. In our study, immunization with recombinant *L. casei* were found by qRT-PCR to strongly induce the expression of IL-1β, TNF-α and IFN-γ in the liver, spleen, kidney and intestine after booster immunization, which is consistent with previous report of up-regulation was observed in response to administration of *Lactobacillus rhamnosus* and *Lactococcus lactis* in *Oreochromis niloticus* [43]. Our results showed a significant upregulation of IL-1β and TNF-α in spleen and kidney, which triggered an early pro-inflammatory response. Similarly, a study conducted by Pirarat N et al. suggested that the induction of TNF-α cytokines by *L. rhamnosus* served as an

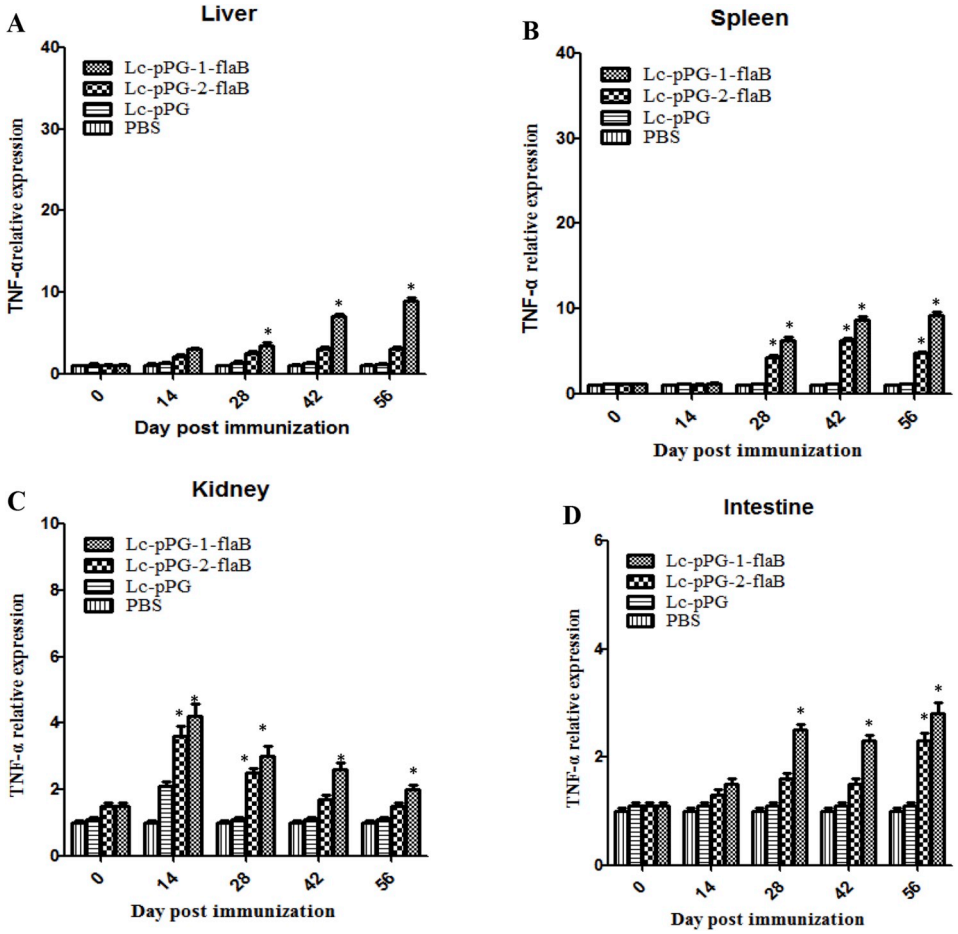
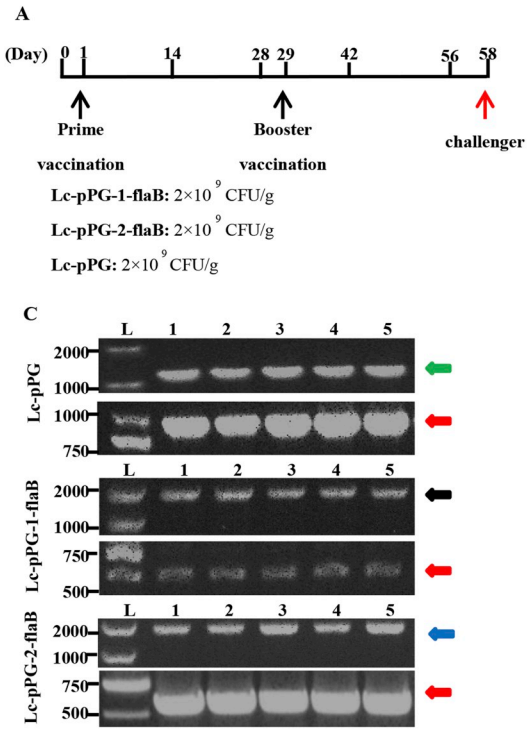


Fig. 9. qRT-PCR analysis of the expression of TNF-α in liver (A), spleen (B), kidney(C) and intestine (D) of common carp (n = 5 fish/group) after oral administration. Data are presented as mean ± SD fold increase relative to PBS control. *: $p < 0.05$.



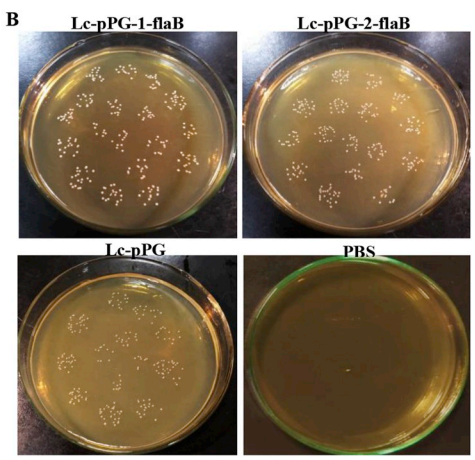


Fig. 10. Survival of the flaB-expressing *L.casei* in the intestine of fish. (A) Experimental schedule. (B) Homogenates of the entire intestine were plated on MRS cm agar. (C) Ten single colonies were randomly picked from each plate of intestine collected from individual fish subjected to colony-direct PCR with pPG1-specific, pPG2-specific (upper images of each group) or *L.casei* dnaA-specific (lower images of each group) primer pairs. Bands indicated by green (1250 bp), black (2150bp), blue (2364 bp), and red arrows (615 bp) were further analyzed by DNA sequencing and were consistent with the putative sequences. L: DNA ladder (bp),Lane 1-5: PCR product of recombinant *L. casei* using specific primer pairs.

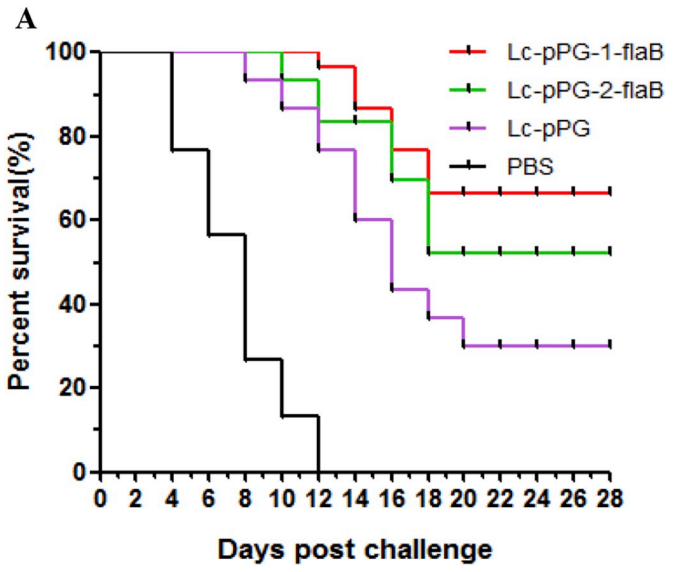


Fig. 11. Survival rate of fish immunized with Lc-pPG-1-flaB, Lc-pPG-2-flaB, Lc-pPGand PBS following challenge with the *A. veronii* TH0426 strain on the day 58 post-immunization. 30 fish/group were used to record percent survival for 28 days.

important regulator of intestine associated immune systems [44]. In our study, the recombinant *L. casei* groups displayed high anti-inflammatory cytokine IL-10 gene expression in the liver, spleen, kidney and intestine of fish. Overall, IL-10 levels first increased and then decreased, indicating that the inflammatory response decreased with time, and the production of IL-10 prevented body damage and weakened the inflammatory response. Similarly, enhanced expression of IL-10 has been observed in *Epinephelus coioides* subjected to oral administration of *Lactobacillus plantarum* [45]. To sum up, the upregulated cytokine could provide early protective immunity after challenge, indicating the capability of *L. casei* expressing flaB to elicit an immuonostimulatory response. The results of plate counting showed that the colonization capability of recombinant *L. casei* was good, which may be associated with the function of the flaB protein, and may explain the significant increase in various immune indexes in the intestine [37].

The survival rate of fish that received recombinant *L. casei* was higher than that of the PBS controls, and Lc-pPG-1-flaB provided strong protection (66.7%) against *A. veronii* (TH0426 strain) challenge to common carps. Although Lc-pPG-1-flaB provided protection of 53.3%, the effect was slightly weaker for the detection of various indicators than the effect of Lc-pPG-1-flaB. The results demonstrated that the recombinant *L. casei* can protect common carp against *A. veronii*, which may delay the infection of bacteria, acute symptoms and protect the intestinal mucosa by alleviation of the inflammatory response. In future research, we will evaluate the effects of recombinant *L. casei* on mucosal and systemic immune responses.

5. Conclusions

In summary, this study has used *L. casei* as an antigen-presenting vector to immunize fish via oral administration. Expression of various immune enzymes and IgM can be induced, which is essential for humoral immunity in common carp. Additionally, it can also cause leukocytes differentiation, leading to enhanced phagocytic activity of cells and increased cytokines levels. Our research has initially determined that recombinant *L. casei* can colonize the intestine of common carp and enhance the resistance to bacteria, having considerable immune protective effects. Thus, the optimal condition and immune dosage of recombinant *L. casei* that yields improved immune efficacy need to be explored. Further investigations of the detailed mechanisms are necessary to examine the suitability of *Lactobacillus* as a potential oral vaccine. The development of *Lactobacillus* oral vaccines that continuously express antigenic proteins is essential for the treatment of aquatic diseases.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgements

This work was supported by the National Key Research and Development Program of China (Project No. 2017YFD0501001), the earmarked fund for Modern Agro-industry Technology Research System (CARS-46) and the National Natural Science Foundation of China (No. 31372540), the Natural Science Foundation of Science and Technology Department of Jilin Province (project No. 20170101016JC) and the Project of Jilin Provincial Education Department (Project No. JJKH20180694KJ).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.01.032.

References

[1] Y.H. Kang, D.X. Zhang, B.T. Yang, H.L. Zhang, X.F. Shan, A.D. Qian, Latest research progress on *Aeromonas veronii*, Chin. J. Zoonoses 34 (2018) 452–465.
[2] T.L. Wu, X.F. Shan, Q.F. Meng, W.S. Guo, W.L. Wang, A.D. Qian, Advances in *Aeromonas veronii*, Chin. J. Vet. Drug 45 (2011) 41–44.
[3] W. Zhang, C. Zhang, C.L. Gong, R.Y. Xue, Y.F. Liu, Isolation, identification and drug sensitivity test of pathogenic *Aeromonas veronii* from *Alosa sapidissima*, Aquaculture 38 (2017) 12–15.
[4] G.M. Qin, X.J. Zhang, C.Z. Chen, H. Fang, B.L. Yan, Infection of *Aeromonas veronii* from diseased *Cyprinus carpio L* and the biological characteristics of its pathogen, J. Anhui Agric. Sci. 36 (2008) 8115–8117.
[5] K.M. Yang, R. Dang, Z.S. Xie, J.X. Ma, C.R. Duan, A.M. Guo, C. Yue, Isolation, identification and antibiotic sensitivity of *Aeromonas veronii* from *Cyprinus carpio*, Freshw. Fish. 47 (2017) 47–52.
[6] A.K. Dhar, S.K. Manna, F.T. Allnutt, Viral vaccines for farmed finfish, Virus Dis. 25 (2014) 1–17.
[7] S.W. Yi, M.J. You, H.S. Cho, C.S. Lee, J.K. Kwon, G.W. Shin, Molecular characterization of *Aeromonas* species isolated from farmed eels (*Anguilla japonica*), Vet. Microbiol. 164 (2013) 195–200.
[8] J. Sun, X. Zhang, X. Gao, Q. Jiang, Y. Wen, L. Lin, Characterization of virulence properties of *Aeromonas veronii* isolated from diseased gibel carp (*Carassius gibelio*), Int. J. Mol. Sci. 17 (2016) 496–497.
[9] M.F. Song, D.X. Zhang, H.P. Zhang, et al., Research advances of virulence factors in *Aeromonas veronii*, Chin. Vet. Sci. 48 (2018) 1038–1042.
[10] A.T. Gewirtz, T.A. Navas, S. Lyons, et al., Bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression, Immunology 167 (2001) 1882–1885.
[11] R. Alexander, G. Tom, M.S. Hans, Toll-like receptor signaling in bony fish, Vet. Immunol. Immunopathol. 134 (2009) 139–150.
[12] R.A.K. Mohammad, O. Mana, M. Mehdi, et al., Vaccination with recombinant *FimH* fused with flagellin enhances cellular and humoral immunity against urinary tract infection in mice, Vaccine 31 (2013) 1210–1216.
[13] R. Koushik, H. David, M.O. Marguerite, M.F. James, Vaccination with EtpA glycoprotein or flagellin protects against colonization with enterotoxigenic *Escherichia coli* in a murine model, Vaccine 76 (2009) 4601–4608.
[14] K.M. Nutsch, C.S. Hsieh, T cell tolerance and immunity to commensal bacteria, Curr. Opin. Immunol. 24 (2012) 385–391.
[15] J.H. Rombout, V. Kiron, Mucosal vaccination of fish, Fish Vaccin. 32 (2014) 56–67.
[16] D.X. Zhang, Y.H. Kang, L. Chen, C.F. Wang, A.D. Qian, Oral immunization with recombinant *Lactobacillus casei* expressing OmpAI confers protection against *Aeromonas veronii* challenge in common carp, *Cyprinus carpio*, Fish Shellfish Immunol. 72 (2018) 552–563.
[17] S.C. Ren, C.Q. Ai, Q.X. Zhang, H. Zhang, W. Chen, Advances in the field of lactic acid bacteria as delivery vehicles for mucosal recombinant vaccines, J. Food Saf. Qual. 5 (2014) 983–989.
[18] S. Nikoskelainen, A. Ouwehand, G. Bylund, et al., Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*Lactobacillus rhamnosus*), Fish Shellfish Immunol. 15 (2003) 443–452.
[19] J.W. Ge, Y.P. Jiang, M. Wang, X.Y. Qiao, M. Liu, L.J. Tang, Y.J. Li, Induction of immune response after oral inoculation of mice with *Lactobacillus casei* surface-

displayed porcine epidemic diarrhea viral N protein, Chin. J. Biotechnol. 25 (2009) 813–818.
[20] X.Y. Yao, H.M. Wang, D.J. Li, et al., Inoculation of *Lactobacillus* expressing hCGβ in the vagina induces an anti-hCGβ antibody response in murine vaginal mucosa, J. Reprod. Immunol. 63 (2004) 63111–63122.
[21] X.L. Hou, L.Y. Yu, J. Liu, G.H. Wang, Surface-displayed porcine epidemic diarrhea viral (PEDV) antigens on Lactic acid bacteria, Vaccine 26 (2007) 24–31.
[22] Y.G. Xu, X.T. Guan, Z.M. Liu, C.Y. Tian, L.C. Cui, Immunogenicity in swine of orally administered recombinant *Lactobacillus plantarum* expressing classical swine fever virus E2 protein in conjunction with thymosin α-1 as an adjuvant, Appl. Environ. Microbiol. 81 (2015) 3745–3752.
[23] K.N. Delaney, J.P. Phipps, J.B. Johnson, et al., A recombinant flagellin-poxvirus fusion protein vaccine elicits complement-dependent protection against respiratory challenge with vaccinia virus in mice, Viral Immunol. 23 (2010) 201–210.
[24] Q.C. Cheung, Z. Yuan, P.W. Dyce, D. Wu, K. Delange, J. Li, Generation of epidermal growth factor-expressing *Lactococcus lactis* and its enhancement on intestinal development and growth of early-weaned mice, Am. J. Clin. Nutr. 89 (2009) 871–879.
[25] Q. Bao, Y. Song, H. Xu, J. Yu, W. Zhang, B. Menghe, et al., Multilocus sequence typing of *Lactobacillus casei* isolates from naturally fermented foods in China and Mongolia, J. Dairy Sci. 99 (2016) 5202–5213.
[26] H. Huang, Z.H. Qi, L.J. Yu, Q.C. Wu, Status quo of study on aquatic vaccine in China, Hunan Agric. Sci. 21 (2010) 136–138.
[27] M.F. Song, Y.H. Kang, D.X. Zhang, L. Chen, J.F. Bi, H.P. Zhang, L. Zhang, A.D. Qian, X.F. Shan, Immunogenicity of extracellular products from an inactivated vaccine against *Aeromonas veronii* TH0426 in koi, *Cyprinus carpio*, Fish Shellfish Immunol. 81 (2018) 176–181.
[28] J.H. Yu, W.S. Ji, H.S. Xu, Oral vaccination of seaperch (*Lateolabrax japonicus*), against *Vibrio anguillarum* using bioencapsulated vaccine, High-tech Commun. 3 (2001) 15–18.
[29] Q.F. Meng, Z. Xu, L.W. Wang, Research progress of the live vector vaccine, Heilongjiang Anim. Sci. Vet. Med. 19 (2013) 28–31.
[30] M.V. Asseldonk, G. Rutten, M. Oteman, et al., Cloning of usp 45, a gene encoding a secreted protein from *Lactococcus lactis*, subsp. lactis, MG1363, Gene 95 (1990) 155–160.
[31] V.M. Son, C.C. Chang, M.C. Wu, et al., Dietary administration of the probiotic, *Lactobacillus plantarum*, enhanced the growth, innate immune responses, and disease resistance of the grouper *Epinephelus coioides*, Fish Shellfish Immunol. 26 (2009) 691–698.
[32] H. Harada, H. Ishikawa, Probiotic effect of *Lactobacillus* sp. DS-12 in flounder (*Paralichthys olivaceus*), J. Gen. Appl. Microbiol. 43 (1997) 305–308.
[33] Y. Chen, F.Q. Wang, Z.T. Shao, B. Wang, Effects of lactic acid bacteria on the microflora in water and larvae guts of Japanese flounder (*Paralichthys olivaceus*), Mar. Fish. Res. 27 (2006) 37–41.
[34] C. Suzer, D. Çoban, H.O. Kamaci, et al., *Lactobacillus*, spp. bacteria as probiotics in gilthead sea bream (*Sparus aurata, L.*) larvae: effects on growth performance and digestive enzyme activities, Aquaculture 280 (2008) 140–145.
[35] L. Song, Y.X. Guo, H. Xu, D. Xiong, Z.M. Pan, X.A. Jiao, Progress on bacterial flagellin as an adjuvant, Prog. Vet. Med. 36 (2015) 92–95.
[36] F. Hayashi, K.D. Smith, A. Ozinsky, T.R.Y. Hawn, et al., The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5, Nature 410 (2001) 1099–1103.
[37] H. Ramadan, E.N. Wael, M.A. Abeer, S. Mona, I.K. Hany, M.A. Youssif, Immunization with outer membrane proteins (OprF and OprI) and flagellin B protects mice from pulmonary infection with mucoid and nonmucoid *Pseudomonas aeruginosa*, Microbiol. Immunol. Infect. 51 (2018) 312–320.
[38] H.H. Seyed, R. Zahra, H. Abdol, et al., The effects of *Lactobacillus* 7 cidophilus as feed supplement on skin mucosal immune parameters, intestinal microbiota, stress resistance and growth performance of black swordtail, Fish Shellfish Immunol. 42 (2005) 533–538.
[39] H.H. Zhou, H.M. Ma, W.B. Zhang, W. Xu, F.Z.G. Liu, K.S. Mai, Effects of potential probiotics on growth performance and immune response of the juvenile sea cucumber (*Apostichopus japonicus*), J. Fish. China 34 (2010) 955–963.
[40] C. Low, S. Wadsworth, C. Burrells, et al., Expression of immune genes in turbot (*Scophthalmus maximus*) fed a nucleotide-supplemented diet, Aquaculture 221 (2003) 23–40.
[41] Z.J. Yang, C.H. Li, H. Zhang, L. Miao, J. Chen, Molecular cloning, characterisation and immune-related expression of tumor necrosis factor α (tnf-α) gene in ayuplecoglossus altivelis, Oceanol. Limnol. Sinica 46 (2015) 1380–1389.
[42] L.G. Bermúdezhumarán, N.G. Cortesperez, R. L'Haridon, et al., Production of biological active murine IFN-gamma by recombinant *Lactococcus lactis*, FEMS Microbiol. Lett. 280 (2008) 144–149.
[43] Y. Xia, M.X. Lu, G. Chen, J.M. Cao, F.Y. Gao, M. Wang, Z.G. Liu, D.F. Zhang, H.P. Zhu, M.M. Yi, Effects of dietary *Lactobacillus rhamnosus* JCM1136 and *Lactococcus lactis* subsp. *lactis* JCM5805 on the growth, intestinal microbiota, morphology, immune response and disease resistance of juvenile Nile tilapia, *Oreochromis niloticus*, Fish Shellfish Immunol. 76 (2018) 368–379.
[44] N. Pirarat, K. Pinpimai, M. Endo, T. Katagiri, A. Ponpornpisit, V. Chansue, M. Maita, Modulation of intestinal morphology and immunity in nile tilapia (*Oreochromis niloticus*) by *Lactobacillus rhamnosus* GG, Res. Vet. Sci. 91 (2011) 92–97.
[45] V.M. Son, C.C. Chang, M.C. Wu, et al., Dietary administration of the probiotic, *Lactobacillus plantarum*, enhanced the growth, innate immune responses and disease resistance of the grouper *Epinephelus coioides*, Fish Shellfish Immunol. 26 (2009) 691–698.