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Astragalus polysaccharides, chitosan and poly(I:C) obviously enhance inactivated *Edwardsiella ictaluri* vaccine potency in yellow catfish *Pelteobagrus fulvidraco*

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ABSTRACT

The yellow catfish (*Pelteobagrus fulvidraco*) is an economically important fish in China, but *Edwardsiella ictaluri*, an intracellular pathogenic bacterium, causes great losses to the culture industry. Currently, vaccination is the most promising strategy to combat the infectious diseases, while adjuvant can provide effective assistant for vaccines to enhance immune responses. In the present study, inactivated *E. ictaluri* vaccine was prepared, then Astragalus polysaccharides (APS), chitosan and poly(I:C) were employed as adjuvants to evaluate the effect on boosting immune responses and protecting yellow catfish against *E. ictaluri*. The survival rate was obviously improved after vaccination with APS, chitosan or poly(I:C) respectively, in addition, these three adjuvants could clearly protect the target tissue (intestine) by pathological sections in infectious experiments. In sera, total protein levels increased throughout the immunization stages, total superoxide dismutase levels continued to raise after vaccination, and lysozyme activity levels improved at different periods, examining by the commercial kits. Moreover, checking by real time quantitative RT-PCR assays, in both spleen and head kidney tissues which were the major immune organs, mRNA expressions of inflammatory cytokine *IL-1β* increased in the early stage of immunity, typical Th1 immune response cytokines *IL-2* and *IFN-γ2* rose up in the whole immune period, and *IgM* significantly enhanced in the adjuvant supplementation groups. The results demonstrated the good efficiency of APS, chitosan or poly(I:C) as adjuvant, and provided more options for the fish adjuvants.

1. Introduction

Yellow catfish, *Pelteobagrus fulvidraco*, has been recognized as an important economic culture freshwater fish, is widely farmed in southern China because of its delicious meat and high market value [1]. However, with the rapid development of intensive farming in recent years, a parallel increase of infectious diseases caused by several kinds of bacteria [1,2]. *Edwardsiella ictaluri*, one of the most destructive pathogens to yellow catfish, leads to “crack-head disease”, seriously limits the development of this industry [2,3].

Vaccination is a common strategy to prevent diseases, protective efficacy of vaccines correlates well with their ability to activate immune responses [4–6]. Many types of vaccines induce both innate and adaptive immune responses to some extent. But at the same time, it also reveals several deficiencies in extensive clinical experiments, the major shortcoming is that these vaccines against microbial infections lack sufficient immunogenicity to reach the level of protection [7]. Currently, most vaccines rely on adjuvants, which improve humoral and/or cellular immune responses. Adjuvants have the advantage of enhancing the immunogenicity of non-replicative antigens; by reducing the quantity of antigens required per dose and forming depots at injection sites, they reduce the number of boosters required to induce long-term protective immunity [8]. Hence, more potent adjuvants enabling the use of a lower antigen dose and reducing side effects would advantageously be used for vaccines.

Astragalus polysaccharides (APS) is isolated from a traditional Chinese medicinal herb, *Astragalus mongholicus*. APS have multitudinous biological and pharmacological activities including

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antioxidant, antitumor, immunomodulatory, antiviral effects [9]. Evidences have indicated the importance of APS in the modulation of immune functions in both human and experimental animals. Several reports have also shown immune stimulatory activity of APS in a range of fish [9–13]. Chitosan is a natural biodegradable polysaccharide obtained from crustacean shells, as a cationic polysaccharide, has gained increasing attention in pharmaceutical field due to its favorable biological properties, such as non-toxicity, biodegradability, mucoadhesive properties, etc [14]. It has been reported as an adjuvant capable of driving potent cell-mediated immunity, when animals received chitosan with antigen, the innate immune responses was markedly improved, and DC maturation was also be promoted. Moreover, conjugation vaccine with chitosan augmented humoral and cellular immune responses, which significantly enhanced specific antibody (Ab) titer and prolonged duration of specific Ab titer [15–17]. poly(I:C), a synthetic analog of double-stranded RNA, is known to stimulate the production of inflammatory cytokines and type I interferon (IFN) [18–21]. These findings imply that APS, chitosan or poly(I:C) may be promising candidate adjuvants for vaccines to prevent fishes from microorganism infection.

In this study, to research the effect of APS, chitosan or poly(I:C) as adjuvants on the immune responses in yellow catfish against *E. ictaluri*, we divided the fish into five groups, intraperitoneally injected with vaccine only, APS/vaccine mixture, chitosan/vaccine mixture and poly (I:C)/vaccine mixture, normal saline (0.65% NaCl) respectively, challenged with *E. ictaluri* on 15 days post injection (dpi). Survival rate was measured firstly, and the effect of adjuvants on the target organ of *E. ictaluri*, intestine tissue, was observed. Serum samples at different time points were collected to measure the change trend of total protein (TP), total superoxide dismutase (TSOD), lysozyme activity (LA). Besides, we analyzed *IL-1β*, *IL-2*, *IFN-γ2* and *IgM* mRNA expressions in the main immune tissues (spleen and head kidney). This work will provide a better understanding of APS, chitosan or poly(I:C) as promising candidate commercial adjuvants for fish vaccines.

2. Materials and methods

2.1. Fish

Yellow catfish (20 g mean weight) were obtained from a fish farm in Huangshi (Hubei province, China), and were acclimated at 25 ± 1 °C. The fish were fed twice daily with commercial pellets for two weeks before experiments.

2.2. Preparation of vaccine, vaccination, challenge and sampling

The *E. ictaluri* was expanded to 10^9 cfu/mL, then 0.3% formaldehyde was used to inactivate the bacteria for 48 h. Coated the inactivated bacteria to BHI culture plate for 4 days, if there was no bacterial growth, then the inactivated vaccine was prepared.

To determine the 50% lethal concentration of *E. ictaluri*, four groups of yellow catfish (n = 15) were injected intraperitoneally with 5×10^4, 10^5, 5×10^5 and 10^6 cfu/mL, and control group was injected with normal saline. The LD_{50} value was determined at 2×10^4 cfu, then it was used for experimental challenge.

For the formal experiment, yellow catfish in control group were intraperitoneally injected with normal saline, and four experimental groups were intraperitoneally injected with 200 μL inactivated *E. ictaluri* vaccine, vaccine formulated with 100 μg APS, 100 μg chitosan or 100 μg poly(I:C) per fish on Day 0 (D0) respectively. 115 fish were used in each group, 50 of them were fed separately to measure mortality, and the remaining 65 were used for sampling. All fish were challenged with *E. ictaluri* on 15 dpi. Five fish of each group were sacrificed for harvesting serum, spleen and head kidney on Day 1, Day 3, Day 7, Day 14, Day 16, Day 18, Day 22, Day 29 post injection, and the intestine tissues were randomly collected from each group on 29 dpi.

2.3. Survival assay/protective efficacy studies

Five groups of yellow catfish (n = 50) were injected with 200 μL normal saline, inactivated *E. ictaluri* vaccine, vaccine mixed with 100 μg APS, vaccine mixed with 100 μg chitosan, or vaccine mixed with 100 μg poly(I:C), fed and changed water normally. On 15 dpi, all of them were challenged with 200 μL *E. ictaluri* (10^5 cfu/mL), and then, death events were monitored for the next 14 days.

2.4. Hematoxylin and eosin (HE) staining

The intestine tissues were dissected and fixed immediately in 10% neutral buffered formalin for 24 h, dehydrated, paraffin-embedded, and sectioned. 4 μm sectioned samples were mounted on aminopropyltriethoxysilane-coated slides. Following the deparaffinization in xylene, sections were rehydrated, stained with hematoxylin and eosin (HE), and mounted with neutral gum, then the images were captured.

2.5. Serum biochemistry indexes

The yellow catfish were anesthetized with 3-Aminobenzoic acid ethyl ester methanesulfonate (MS222). Blood samples were collected from the caudal vein and were placed for 1 h at room temperature. After centrifugation at 4500 rpm, 4 °C for 15 min, the serum were gathered and stored at −80 °C. The serum biochemical indexes of TP, TSOD and LA were assayed by the corresponding commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.6. qRT-PCR of immune gene expressions

The total RNAs of head kidney and spleen tissues were isolated with TRIzol reagents (Aidlab, China) according to the instruction, the quantity of RNA was determined by measuring absorbance at 260 and 280 nm, and its integrity was tested by electrophoresis in 2% agarose gel. mRNAs were reverse-transcribed into cDNAs respectively with M-MLV reverse transcriptase, RNase inhibitor (Thermo Fisher Scientific, USA), hexamer random primer. The primers for qRT-PCR analyses were listed in Table 1 (The sequence information were obtained from http://hzaugenelab324.vicp.io/viroblast/viroblast.php). All reactions were performed in duplicate and each assay was repeated for three times. *β-actin* was used as an internal control gene, and the relative mRNA expression of gene was calculated with the $2^{-\triangle\triangle CT}$ method.

2.7. Statistical analysis

The results were reported as means ± SE post data preparation and statistical analysis using GraphPad Prism 7.0 software. Statistical significance was assessed using student's two-tailed *t*-test in each experiment group relative to vaccine group. Significance (*P*-value) is indicated as: *($P < 0.05$); **($P < 0.01$).

Table 1
Primer sequences in this study.

Gene name	Primer direction	Primer sequence (5′-3′)	Size (nt)
IL-1β	Forward	GGCTGGTTTGCTGATGTGTC	101
	Reverse	CTCGCTGAACACCTTCGAGT	
IL-2	Forward	GTGCCAGACTGAATACCAGCAT	126
	Reverse	TTCACCTCCTCTTCACGCTTC	
IFN-γ2	Forward	CAGAGCTGCTTCTTTCTAAATGGA	159
	Reverse	AACAAAACGTCGCTTTGTTTGT	
IgM	Forward	ACTCAGTCTAAAGAGGCGGC	117
	Reverse	GCACACGAGTTCACCACTTC	
β-actin	Forward	TTCGCTTGGAGATGATGCT	136
	Reverse	CGTGCTCAATGGGGTACT	

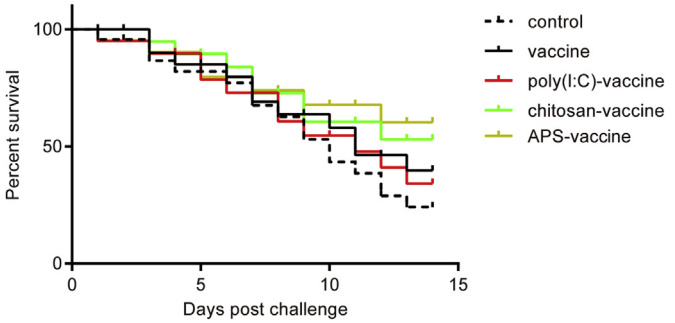

Fig. 1. Survival rates of yellow catfish against *E. ictaluri* infection. Animals were peritoneally injected with normal saline, vaccine only, APS/vaccine mixture, chitosan/vaccine mixture or poly(I:C)/vaccine mixture on day 0. On day 15, fish in each group (n = 50) were challenged with *E. ictaluri*, and death events in each group were monitored on the next 14 days.

3. Results

3.1. APS, chitosan or poly(I:C) could improve the survival rate of yellow catfish

To assess whether APS, chitosan or poly(I:C) could assist the vaccine to provide better protection to yellow catfish, fish in different groups were injected with normal saline, vaccine only, APS/vaccine, chitosan/vaccine mixture or poly(I:C)/vaccine mixture, then they were challenged with *E. ictaluri* on day 15. Fish survival was monitored and counted over the next 14 days (Fig. 1). Compared with the control group, the vaccine could be found to provide a certain protective effect to yellow catfish. Furthermore, after vaccination with APS or chitosan, the percent survival were notably improved. However, the percent survival of poly(I:C) group was lower than the vaccine group, but still higher than the control group. These results revealed that APS, chitosan or poly(I:C) could protect yellow catfish to some extent.

3.2. APS, chitosan or poly(I:C) could protect intestine tissue from the violation of E. ictaluri

To estimate whether APS, chitosan or poly(I:C) could provide a protective effect on the target organ of *E. ictaluri*, the intestine tissues were dissected, fixed and sectioned for HE staining on 29 dpi (Fig. 2). Totally healthy intestine tissue exhibited complete mucous layer, submucous layer, muscular layer and serous membrane (Fig. 2A). By contrast, the control group showed abnormal intestine tissue after challenge, including shorter villi, bleeding, and muscular layer injury (Fig. 2B). However, the group injected with vaccine just showed a little abnormality of shorter villi (Fig. 2C). At the same time, the intestine tissues appeared to be relatively normal in injected vaccine with three novel adjuvants (Fig. 2D, E, F). These results showed that APS, chitosan or poly(I:C) could obviously protect the intestine tissue of yellow catfish.

3.3. Serum total protein, total superoxide dismutase and lysozyme activity could be positively improved by APS, chitosan or poly(I:C)

To analyze the effects of APS, chitosan or poly(I:C) on serum biochemistry, TP, TSOD, LA were assayed by the specific commercial kits (Fig. 3). Our data showed that after vaccination with three novel adjuvants, TP levels were significantly improved in almost the whole experimental periods compared to the vaccine or control group (Fig. 3A). On the other hand, during the immunization with three novel adjuvants, TSOD levels were higher than vaccine or control group, and they continued to increase from 1 dpi to 29 dpi (Fig. 3B). LA levels increased significantly. In addition, after vaccination and challenge, they increased first and then decreased a little (Fig. 3C). It is worth mentioning that APS could improve more serum biochemistry levels than chitosan and poly(I:C) in TP, TSOD or LA (Fig. 3). These results indicated that APS, chitosan or poly(I:C) could positively improve serum physiological levels.

3.4. APS, chitosan or poly(I:C) triggered the early inflammatory response

To evaluate the effects of APS, chitosan or poly(I:C) on early inflammation, typical early inflammatory cytokine *IL-1β* was examined by qRT-PCR (Fig. 4). In head kidney, mRNA expressions of *IL-1β* were rapidly up-regulated in contrast with vaccine or control group on 1 dpi 3 dpi, and went down after that, and presented the highest fold change on 3 dpi (Fig. 4A). Similarly, in spleen, mRNA expressions of *IL-1β* were up-regulated in APS, chitosan or poly(I:C) group on 1 dpi and 3 dpi, and then it declined (Fig. 4B). They slightly increased on 16 dpi, infected on 15 dpi. These results suggested an early inflammatory immune response

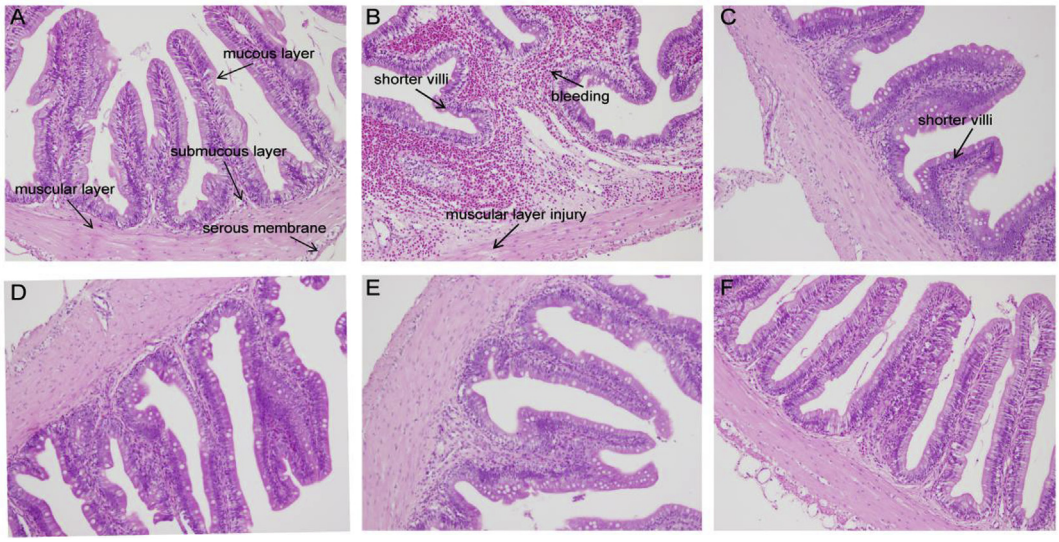

Fig. 2. Histopathologic photographs of yellow catfish intestine tissue after *E. ictaluri* infection. Yellow catfish were peritoneally injected with normal saline (B), vaccine only (C), poly(I:C)/vaccine mixture (D), chitosan/vaccine mixture (E), APS/vaccine mixture (F) on day 0, challenged with *E. ictaluri* on day 15, intestine tissues were collected on day 29, then treated by HE staining. Healthy intestine tissue of untreated yellow catfish was used as blank control (A). Each group was parallel three times, one was selected for presentation.

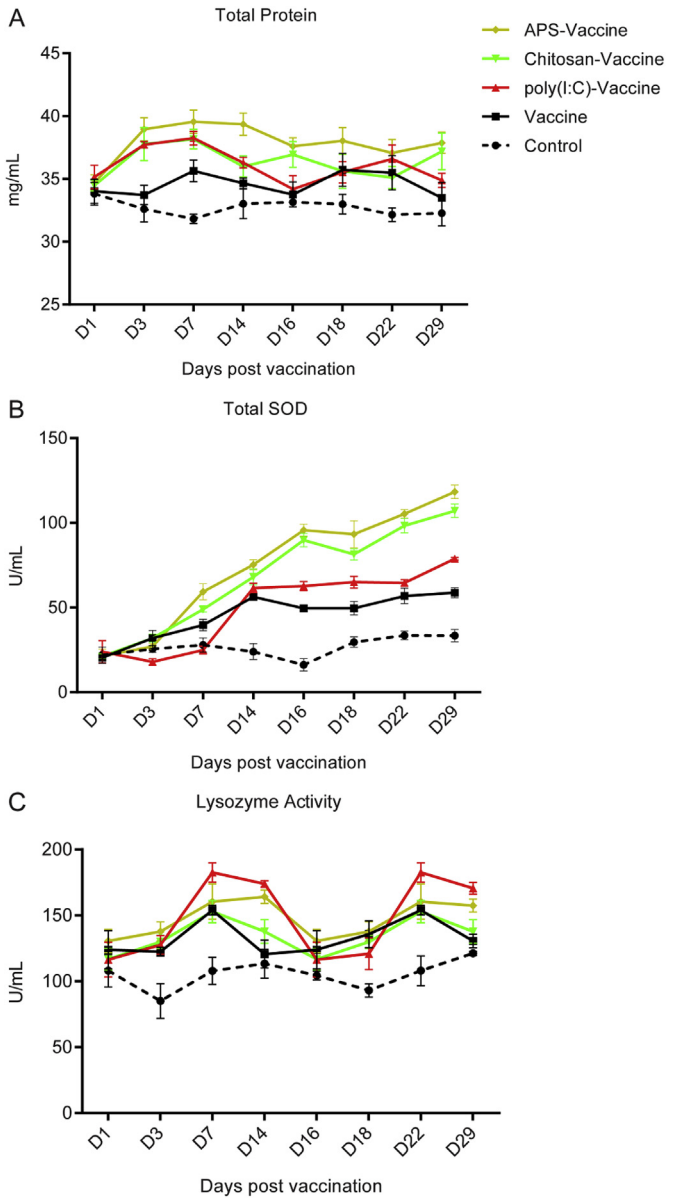

Fig. 3. Serum biochemical indexes of TP, TSOD and LA. Total protein (A), superoxide dismutase (B), and lysozyme (C). Serum biochemical indexes of TP, TSOD, LA were determined by their specific commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Each data was repeated three times, data were presented as means ± SE (n = 4).

could obviously triggered by APS, chitosan or poly(I:C).

3.5. Th1 immune response was induced via APS, chitosan or poly(I:C)

To study the influence of APS, chitosan or poly(I:C) on cellular immune responses, *IL-2* and *IFN-γ2* (typical Th1 immune response cytokines) were determined by qRT-PCR (Fig. 5). After vaccination with three novel adjuvants, in both head kidney and spleen, mRNA expression levels of *IL-2* slightly augmented at almost all stages except for the last two points (Fig. 5A and B). Meanwhile, in head kidney, after vaccination with three novel adjuvants, mRNA expressions of *IFN-γ2* appreciably rose in the whole experimental period. And the same trend was observed in spleen, with the difference that fold changes were lower than those in head kidney. In addition, mRNA expression levels of *IFN-γ2* reached maximum on 7 dpi in both head kidney and spleen (Fig. 5C and D). These results demonstrated that these three novel

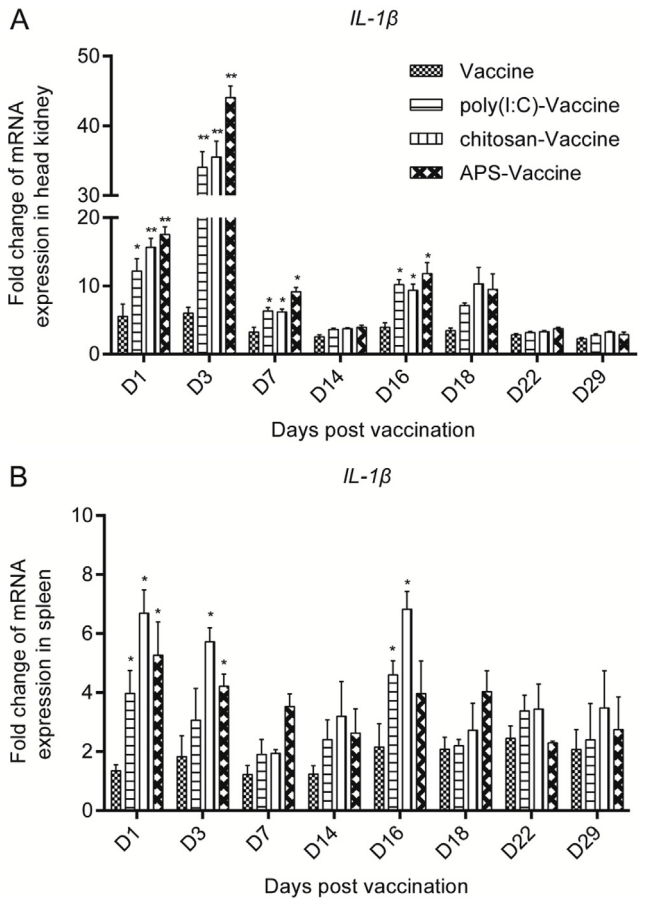

Fig. 4. mRNA expressions of *IL-1β* in head kidney and spleen. They were determined by qRT-PCR. Head kidney (A), and spleen (B). *β-actin* gene was used as a reference gene. Data were presented as means ± SE (n = 4). Statistical analysis was performed by unpaired student's *t*-test (*$P < 0.05$ and **$P < 0.01$).

adjuvants could induce Th1 immune responses.

3.6. APS, chitosan or poly(I:C) enhanced mRNA expressions of IgM in the mid-to-late immunity

To determine whether mRNA expressions of *IgM* could be strengthened by APS, chitosan or poly(I:C) in head kidney and spleen which were the major immune tissues, mRNA expressions of *IgM* were examined by qRT-PCR (Fig. 6). After vaccination with three novel adjuvants, mRNA expressions of *IgM* were significantly improved after 7 dpi compared with vaccine group in head kidney, and continued to ascend except for 16 dpi, when there was a great decrease after infection (Fig. 6A). In spleen, mRNA expressions of *IgM* showed great improvement after 3 days post vaccination with three novel adjuvants, and they remained stably high with a sharp drop on 16 dpi, which was the first day post challenge (Fig. 6B). These results indicated that evidently humoral immune responses were enhanced by APS, chitosan or poly(I:C).

4. Discussion

Vaccination is the most effective technique in prevention of pathogen infection. Adjuvant can enhance the immunization efficacy via innate and adaptive immunity [22]. In earlier studies, many researchers demonstrated APS, chitosan or poly(I:C) could be used as vaccine adjuvants to elevate efficacy of vaccine in protecting from pathogen invasion [17,22,23]. In the present study, we immunized yellow catfish

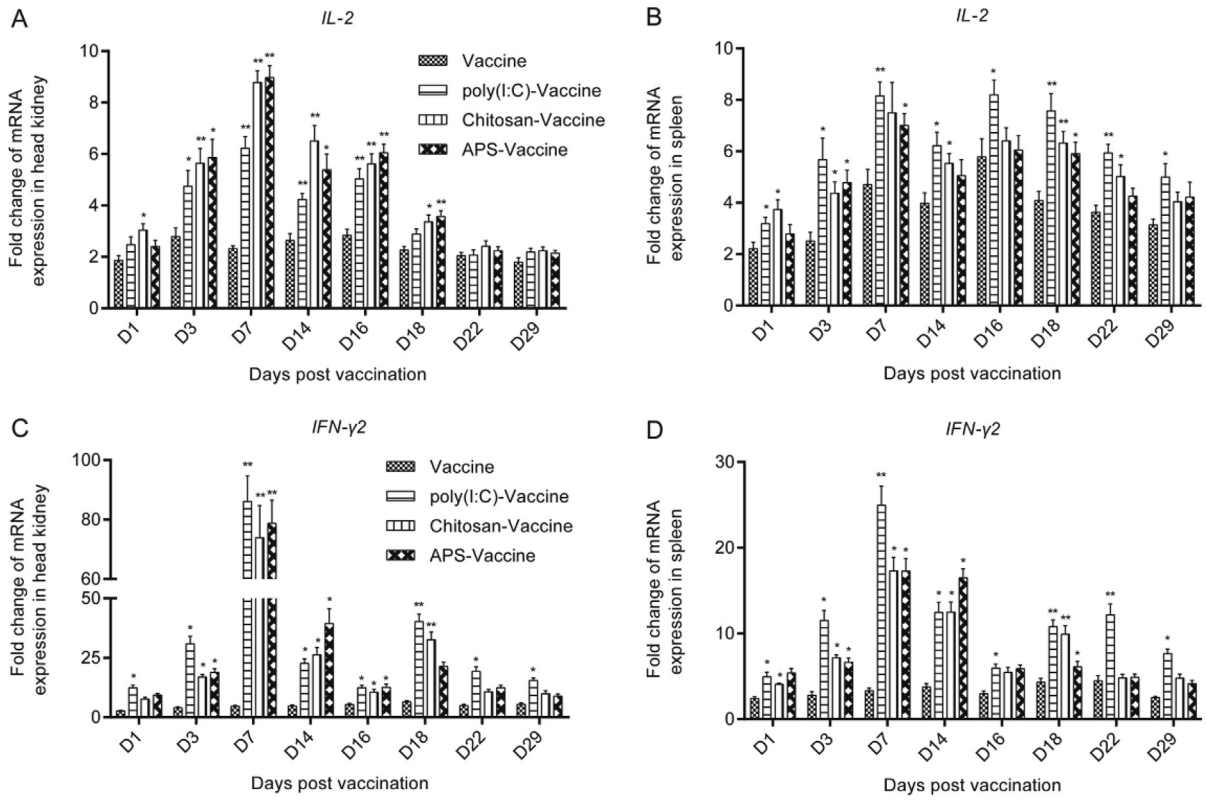

Fig. 5. The mRNA expression patterns of *IL-2* and *IFN-γ2* in head kidney and spleen tissues. mRNA expressions of *IL-2* (A),(B) and *IFN-γ2* (C),(D) in head kidney and spleen were determined by qRT-PCR. *β-actin* gene was used as an internal control gene. Data were presented as means ± SE (n = 4). $*P < 0.05$ and $**P < 0.01$.

by inactivated *E. ictaluri* vaccine with APS, chitosan or poly(I:C) respectively, confirm the efficacy of these three novel adjuvants in yellow catfish against *E. ictaluri*.

The protection rate can comprehensively reflect the protective effect on fish. Our results showed that APS or chitosan can significantly improve the survival rate of fish compared to the vaccine or control group, however, the survival rate of poly(I:C) group is lower than that in vaccine group, which may be related to the slight toxicity of poly(I:C) itself [10]. Histopathologic slide can intuitively reflect the pathological injury of pathogenic infection. Intestine is the major target tissue of *E. ictaluri* in yellow catfish. In the present study, several pathological symptoms including shorter villi, bleeding, and muscular layer injury occurred in the intestine tissue in control group post challenge, the vaccine group showed only shorter villi, on the other hand, APS, chitosan or poly(I:C) groups presented normal intestine tissues. These results support that these three novel adjuvants can improve the protective rates of vaccine against *E. ictaluri* infection.

Measurement of serum biochemical indexes is wildly used in clinical diagnosis of fish physiology to determine the general status of health [24]. Total protein is one of the most important factors in blood, and its clinical significance has been considered as an indicator of health, stress, and welfare in both terrestrial and aquatic organisms [25]. Its value usually changes in different physiological and pathological condition. The increase in serum total protein might be correlated with an increase of proteins like serum lysozyme, complement component, acute phase proteins, cytokines, lectins and bactericidal peptides [25]. Our results showed high levels of TP after vaccination with APS, chitosan or poly(I:C), which were consistent with the results of survival rates. Superoxide dismutase is a very important antioxidant enzyme in body, which mainly removes the superoxide in animal body fluids or tissues, playing a role in reducing or removing the superoxide oxidative damage to animal cell membranes or the reducing active components in cells. Studies have shown that the activity of SOD is closely related to the immune level in organism [26]. In this study, serum TSOD levels increased, suggesting that these three adjuvants could stimulate the production of a large amount of oxidative free radicals, and thus improved the antioxidant and immune capacity in yellow catfish. Lysozyme is mainly produced by monocytes and granulocytes, which can not only destroy and eliminate invaders, but also improve the digestive function of macrophages and enhance the immunity [25]. In the present study, serum LA was significantly higher at different immune time points, which indicated that these three novel adjuvants could stimulate the secretion of lysozyme to enhance the immunity of fish. All of these results suggest that APS, chitosan or poly(I:C) can protect yellow catfish by actively improving the serum biochemical environment.

Innate immunity is the first line of defense against pathogen invasion. Interleukin-1β (IL-1β) is one of the pivotal early pro-inflammatory cytokines, which plays an important role in regulating immune response and inducing a series of inflammatory reactions to infections [27,28]. In the present study, mRNA expressions of *IL-1β* improved rapidly after vaccination and then dropped to normal, and it rose again on the early days after challenge, suggesting that innate immune and inflammation responses were potently elicited when APS, chitosan or poly(I:C) was used as adjuvant.

The adaptive immunity includes humoral and cellular immunity. From evolutionary perspective, teleosts possess functioning humoral immune response, humoral immunity of classical molecular components presents in teleost fishes, but the spectrum of Ig class is narrow in fish and predominates with IgM Ab [29]. Our results showed that mRNA expressions of *IgM* in spleen and head kidney increased in APS, chitosan or poly(I:C) group, and they improved rapidly after challenge, which resulted from memory B cells. In previous studies, APS enhanced

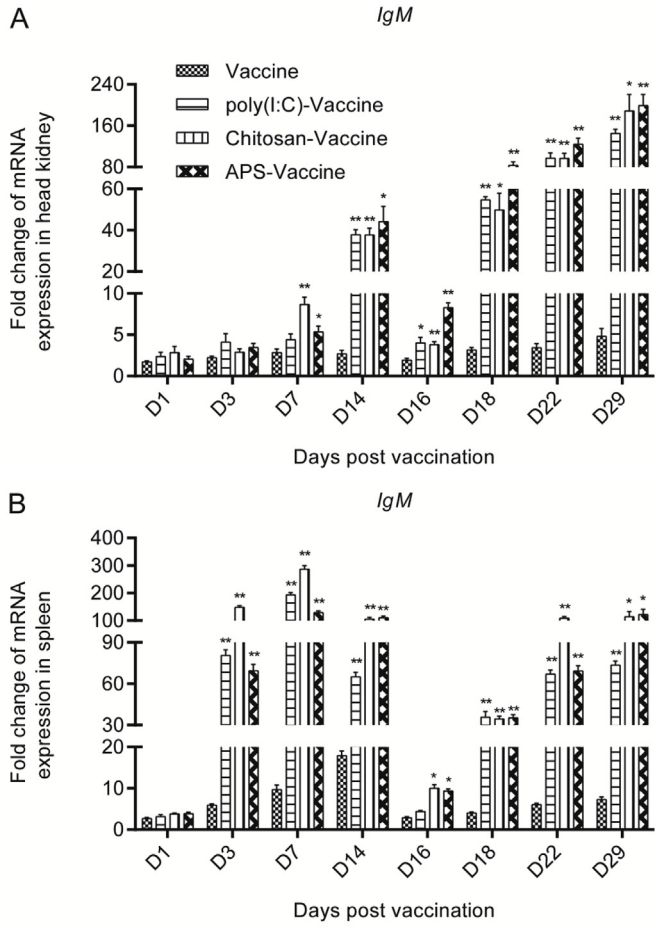

Fig. 6. The mRNA expression profiles of *IgM* in head kidney and spleen. mRNA expressions of *IgM* in head kidney (A) and spleen (B) were determined by qRT-PCR. *β-actin* gene was used as a reference gene. Data were presented as means ± SE (n = 4). *$P < 0.05$ and **$P < 0.01$.

adaptive immune responses in *Micropterus salmoides* [9], chitosan as vaccine adjuvant in mice also significantly up-regulated levels of serum Ab [17,22,30], and poly(I:C) as adjuvant enhanced both humoral and cellular immune responses in *Rhesus macaques* [31], which corresponded to our results. These data demonstrated these three novel adjuvants can enhance humoral immunity.

Although Abs are thought to be the primary correlation with protection against pathogens, and previous studies also demonstrated the efficient clearance of pathogens was mediated by Abs, they can not enter cells to eliminate intracellular microorganisms due to high molecular weight [32]. *E. ictaluri* is a kind of intracellular bacteria, hence, cellular immunity is also important in the defense. In higher vertebrates, IFNγ is marker of Th1 immune responses, and Th1 cells ($CD4^+$ helper T (Th) lymphocytes) lead to eradication of intracellular pathogens by mediating Th1 cell immunity [33]. It was proposed that conservation of $CD4^+$ Th cell functioned among teleost fishes [34–37], and the structure and function of teleost IFNγ2 is similar to mammalian IFNγ [37–39]. IL-2 can promote the differentiation and proliferation of B cells induced by the Th cells, namely $CD4^+$ Th cells, improve the immune level of B cells, promote the killing effect of $CD4^+$ Th cells in assisting $CD8^+$ Th cells, enhance the activity of NK cells, and thereby improve the cellular immune level [40–42]. In this study, to determine the efficacy of vaccine with adjuvants in cellular immunity, we investigated mRNA expressions of *IFN-γ2* and *IL-2* in spleen and head kidney by qRT-PCR, the results showed that mRNA expressions of *IL-2* improved after vaccination with APS, chitosan or poly(I:C), and after challenge with *E. ictaluri*, it also maintained at a high level in both spleen and head kidney. As to mRNA expressions of *IFN-γ2*, they were up-regulated significantly and stayed at high expression levels after challenge, indicating that these three novel adjuvants could enhance cellular immunity.

In conclusion, inactivated *E. ictaluri* vaccine, formulated with APS, chitosan or poly(I:C) as adjuvant, can improve the survival rate of yellow catfish after challenge, and protect intestine tissue from the injury of *E. ictaluri*. Serum biochemical indexes are positively improved to resist bacteria. Furthermore, they can enhance innate immune responses and adaptive immunity which involves in humoral and cellular immunity. These three novel adjuvants can improve the physiological state of intestine, blood, spleen and head kidney to resist *E. ictaluri*. These results suggest that APS, chitosan or poly(I:C) will be emerging potential aquatic adjuvant.

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