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Replacement of fishmeal by spirulina *Arthrospira platensis* affects growth, immune related-gene expression in gibel carp (*Carassius auratus gibelio* var. CAS III), and its challenge against *Aeromonas hydrophila* infection

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ABSTRACT

The present study examined the effect of dietary spirulina, *Arthrospira platensis* on growth performance, blood physiological indices, immune-related gene expressions and resistance of juvenile gibel carp against *Aeromonas hydrophila* infection. Four isonitrogenous (360 g kg^{-1}) and isolipidic (90 g kg^{-1}) diets were formulated with containing different levels of spirulina powder of 0 g (SP0, the control diet), 3.38 g (SP3.38), 6.76 g (SP6.76) and 13.52 g (SP13.52) per 100 g diet to replace 0%, 25%, 50% and 100% of fishmeal protein, respectively. And each diet was randomly assigned to triplicate tanks (150-L capacity per each) and each tank was stocked with 22 fish (15.37 ± 0.06 g). Fish were fed one of the tested diets up to satiation twice a day for 46 days. A challenge test was carried out after the feeding trial by injecting *Aeromonas hydrophila* intraperitoneally for 7 days. The results showed that fish growth, feeding rate in groups SP3.38 and SP6.76 were significantly higher than those of groups SP0 and SP13.52 ($P < 0.05$). Feed efficiency and protein retention rate had no significant difference among all tested groups. Plasma superoxide dismutase and phagocyte activity of blood leukocytes significantly increased in the spirulina-fed fish groups at 12-h post the bacterial challenge ($P < 0.05$). Both pre and post challenge test, plasma lysozyme activities in spirulina-fed groups were significantly higher than that in the control group ($P < 0.05$). Plasma malondialdehyde got the lowest value in the SP13.52 group before and after the challenge test. The transcriptional levels of TLR2 (Toll like receptor 2), myeloid differentiation factor 88 (MyD88), Toll/IL-1 receptor domain-containing adaptor protein (TIRAP), interleukin-1β (IL-1β) and tumor necrosis factor-α1 (TNF-α1) in spleen and kidney significantly increased post the bacterial challenge compared to the pre challenge. And the relative expressions of the immune-related genes of spirulina-fed fish groups were higher than those of the control group before and after the challenge test. The 7-day cumulative survival rate after the bacterial challenge was highest in the SP3.38 group ($P < 0.05$). The present results indicated that low dietary inclusion of spirulina significantly enhanced the immune response of gibel carp partly through TLR2 pathway and 3.38% of dietary spirulina was recommended for the juveniles based on the growth and immune response.

1. Introduction

With the development of aquafeeds industry, fishmeal is extensive used as a preferred protein source for its high contents of essential amino acids, essential fatty acids, mineral elements, bioactive substance and low level of anti-nutritional factors [1]. Although the worldwide fishmeal production reaches a relative high and stable level, the production still could not match the rapid expansion of worldwide aquaculture [2]. Therefore, alternative protein sources for fishmeal have gained more and more attentions [3]. *Arthrospira platensis* is a blue-green microalgae and prokaryotic organism, widely distributed in fresh water and seawater. *A. platensis*, consisting of 55–70% protein, could replace up to 75% of dietary fishmeal protein for *Litopenaeus vannamei* [4] and even 100% of dietary fishmeal protein for catla (*Catla catla*) and rohu (*Labeo rohita*) [5]. Some basic nutrients of *A. platensis* powder were showed in Table 1.

In our previous study, we have found that the activities of plasma immune-related enzymes of gibel carp could be improved even by inclusion of 19.8% dietary spirulina, *A. platensis* powder replacing 20.0% of dietary fishmeal [6]. Macias-Sancho et al. [4] also reported that

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Table 1
The contents of the nutrients in *Arthrospira platensis* powder (g kg^{-1} dry matter).

Chemical composition	*Arthrospira platensis*[a]
Crude protein	721.56
Crude lipid	26.58
Phosphorus	10.12
Linoleic acid	18.80
Linolenic acid	9.13
Flavonoid	0.87
β-carotene	0.80
Vitamin A	1.16
Phycocyanin	101.00–163.00
Essential amino acid	
Thr	27.01
Phe	24.82
Val	30.47
Ile	28.64
Leu	47.65
His	8.45
Met	11.62
Arg	34.43
Lys	24.64
Non-essential amino acid	
Asp	50.57
Ser	25.86
Glu	78.01
Gly	26.03
Ala	39.25
Tyr	21.24
Pro	17.31

[a] *Arthrospira platensis*: the contents of nutrients refer to [6, 33–35].

spirulina increased the percentage of hyaline and granular hemocytes and reduced the apoptotic index of *Litopenaeus vannamei*. Similarly, carp (*Cyprinus carpio*) orally administered with spirulina performed enhanced responses of phagocytic activity, superoxide anion production and the expressions of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) genes [7]. These immune-enhanced effects of spirulina in fishes could be due to the composition of abundant vitamins, minerals, phycocyanin, β-carotene and algal polysaccharides [8–10]. Amar et al. [11] suggested that dietary β-carotene from *Dunaliella salina* could significantly improve serum alternative complement activity, lysozyme activity and the phagocytic rate and index of rainbow trout. It was also indicated that polysaccharide fraction from marine macroalga, *Padina gymnospora*, stimulated the upregulation of the cytokine IL-1β and antimicrobial peptide lysozyme-C of common carp (*Cyprinus carpio*) [12]. Another study on murine splenic B cells showed that the homogenous polysaccharide from *Phoma herbarum* YS4108 can stimulate B cell proliferation and generation of IgM response through a receptor-mediated mechanism, which involves Toll like receptor 2 (TLR2) and TLR4 [13]. Moreover, TLR2 modulates inflammation in zymosan-induced arthritis in mice [14]. Therefore, we assume that dietary inclusion of spirulina could improve the immune response of fish through TLR2 pathway.

TLR signaling pathway is an important and evolutionarily conserved innate immune pathway, which is crudely classified into the MyD88 (myeloid differentiation factor 88)-dependent and MyD88-independent (TRIF-dependent) pathway [15]. TLR2 is one of MyD88-dependent pathways [16]. TLR2 affects the immune response by sensing peptidoglycan (PGN) and lipoteichoic acid (LTA) of bacterial cell wall of invading microbes [17]. In TLR2 signaling pathway, MyD88 recruits IL-1 receptor-associated kinase (IRAK) through interaction of the death domains of both molecules [18]. And Toll/IL-1 receptor domain-containing adaptor protein (TIRAP) activates the downstream MyD88-dependent signaling pathway [19]. IRAK is activated by phosphorylation and then associates with TNF receptor associated factor 6 (TRAF6), and finally leading to the activation of the transcription factor nuclear factor κB (NF-κB), which results in the production of induced inflammatory cytokines [20,21]. MyD88 is an essential Toll/IL-1 receptor (TIR) domain-containing adaptor for the induction of inflammatory cytokines via all the TLRs [22]. TIRAP is a second TIR domain-containing adaptor that specifically mediates the MyD88-dependent pathway via TLR2 and TLR4 [23]. IL-1β and TNF-α are two important inflammatory cytokines which are also good markers for the inflammatory response [24,25]. And two isoforms of TNF-α (TNF-α1 and TNF-α2) identified and characterized in *Carassius auratus* [26].

Aeromonas hydrophila is one of the most common pathogenic bacteria in the freshwater aquaculture [27]. The pathogenicity of *A. hydrophila* is due to the produce of endotoxins and hemolysin and cause the epizootic ulcerative syndrome (EUS) [28,29]. It always results in the large outbreak of septicemia and the high mortalities in gibel carp farming industry [30,31].

Gibel carp (*Carassius auratus gibelio*) CAS III is a main cultured freshwater omnivorous fish in China and its production in 2016 of China was more than 3 million tonnes [32]. The main objective of this study was to evaluate the effect of dietary replacement of spirulina instead of fishmeal on growth performance, immune response, and transcriptional levels of immune related genes including TLR2, MyD88, TIRAP, IL-1β and TNF-α1 of gibel carp, as well as the fish resistance against *Aeromonas hydrophila* infection.

2. Materials and methods

2.1. Experimental diets

In the present study, four isonitrogenous (360 g crude protein kg^{-1} diet) and isolipidic (90 g crude lipid kg^{-1} diet) diets were formulated and their chemical composition was showed in Table 2 where spirulina powder was added at levels of 0 g (SP0, the control diet), 3.38 g (SP3.38), 6.76 g (SP6.76) and 13.52 g (SP13.52) per 100 g diet, which replacing 0%, 25%, 50% and 100% of fishmeal protein respectively. All ingredients of each diet were ground through a 40 mesh, completely mixed and then extruded into 2 mm pellets by a laboratory granulator (SLP-45, Fishery Mechanical Facility Research Institute, Shanghai, China). The pellets were dried using an oven (60 °C) and stored at 4 °C until use. And the amino acid profile of the experimental diets is shown in Table 3.

2.2. Fish and feeding trial

Juvenile gibel carp were collected from the Guanqiao Hatchery of Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, Hubei, China). Two weeks prior to the feeding trial, all fish were acclimated in 6 fiber glass cylinders (150-L) and fed up to satiation twice a day at 8:30 and 15:30 h on the control diet.

The feeding trial was conducted at an indoor recirculating system. At the beginning of the trial, all fish were fasted for 24 h. Apparent healthy and similar size fish (initial body weight: 15.37 ± 0.06 g) were randomly distributed into 12 fiber glass cylinders (150-L) at a density of 22 fish per tank. Triplicate tanks were randomly assigned to each diet. During the trial, fish were fed up to apparent satiation twice a day at 8:30 and 15:30 h for 46 days.

Water flowing rate into each tank was about 2000 ml min^{-1}. The photoperiod was 12 h light: 12 h dark with the light period from 8:00 to 20:00. Water temperature was recorded daily and maintained at 25.5 ± 1.0 °C. Ammonia-N, dissolved oxygen and pH were monitored once every week and the concentration of ammonia nitrogen was recorded below 0.1 mg kg^{-1}, dissolved oxygen was more than 7.5 mg L^{-1} and pH was 6.8–7.0.

2.3. Challenge test

The single colony of *A. hydrophila* used in the challenge test was