Effects of dietary administration of *Chlorella* on the immune status of gibel carp, *Carassius auratus gibelio*

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Abstract

In the present study the effects of dietary *Chlorella* on the immune status of gibel carp, *Carassius auratus gibelio*, were evaluated. A total of 540 healthy fish were divided into 6 groups, with one control group fed with *Chlorella*-free diet and five experimental groups fed diets supplemented with 0.4 to 2.0% *Chlorella*. The whole trial lasted 60 days. At the end of the trial, the weight gain and immune parameters of fish were analysed. Results showed that *Chlorella* could increase the levels of immunoglobulin M and D, interleukin-22 and chemokine (C-C motif) ligand 5 in some tissues, which indicated that dietary *Chlorella* can be involved in regulating adaptive and innate immunity.

Introduction

Microalgae are a vast group of photosyntheticic heterotrophic organisms which contain essential amino acids, protein, minerals, vitamins, chlorophylls and some kinds of antioxidants and bioactive substances (Yamaguchi, 1996; Kwak et al., 2012). Due to these properties, microalgae had been applied in areas of food and medicine. Recently, the immunostimulating properties of microalgae have attracted the interest of researchers. The *Chlorella* could protect the mice against *Listeria monocytogenes* infection by increasing T-helper-1 cell (Hasegawa et al., 1994; Dantas et al., 1999). Tanaka et al. (1998) found that the *Chlorella* extraction could increase the CD4+ cell number to inhibit the neoplasm metastasis and progression. In addition, *Chlorella* could induce the activation and maturation of human monocyte-derived dendritic cells through NF-κB and PI3K/MAPK pathways (Chou et al., 2012).

Studies in mammals have provided great references for the application of microalgae in fish farming. Amar et al. (2004) found that microalgae extraction could enhance the innate immunity of rainbow trout (*Oncorhynchus mykiss*). Cerezuela et al. (2012) found that three orally administered microalgae (*Nannochloris gaditana*, *Tetraselmis chuii* and *Phaeodactylum tricornutum*) could enhance gilthead seabream (*Sparus aurata*) defence activity. Our previous study found that *Chlorella* could be used as a good additive and could promote the growth performance and physiological parameters of gibel carp (*Carassius auratus gibelio*) (Xu et al., 2014). However, the effects of *Chlorella* on the immunity of aquaculture species remain limited.

Thus, based on our previous study, we conducted the present research with the aim of investigating the effects of dietary supplement of *Chlorella* on the immune status of gibel carp. These results are intended to provide more information for further studying the properties of microalgae and their application in fish diets.

Materials and methods

**Fish**

Healthy gibel carps (average weight 29.91±0.11 g) were purchased from a local fish farm and transferred to our laboratory using a car equipped with automatic aerator. Fish were reared in 18 tanks (diameter 70 cm, water volume 250 L) with a recirculating water system at 26°C with 12:12 light/dark photoperiod. Fish were acclimated with the commercial diets for at least two weeks prior to the experiments. All of the experiments were performed in compliance with the country’s regulations for the use of laboratory animals and were approved by the Institutional Review Board of the Yancheng Institute of Technology, China.

**Experimental diets**

The *Chlorella* powders were provided by Prof. Zhen Gao (College of Biotechnology and Pharmaceutical Engineering, Nanjing University of Technology, China). Six experimental diets were designed to contain *Chlorella* levels of 0, 0.4, 0.8, 1.2, 1.6 and 2.0% (*C*, *E1*, *E2*, *E3*, *E4*, *E5*), respectively. The diets were made according to our previous method (Xu et al., 2014). Briefly, dietary ingredients were ground into a fine powder though a 260 μm mesh and mixed with the dry powder of *Chlorella* though a SJF-30 mixer (Fishery Machinery and Instrument Research Institute, Chinese Academy of Fishery Sciences, Beijing, China). Then, the pellet feed (1 mm pellet diameter) were made using a SLP-80 feed mill (Fishery Machinery and Instrument Research Institute) at 70-85°C and dried for 18 h. The dry pellets were sealed in plastic bags and stored at 4°C until use. Table 1 shows the ingredients and proximate composition of the experimental diets.

**Experimental procedure and sampling**

Five hundred and forty fish were randomly divided into 18 tanks (30 fish per tank). The tanks were then randomly divided into six groups (three tanks per group), with one control group fed with *Chlorella*-free diets and five experimental groups fed with diets containing 0.4, 0.8, 1.2, 1.6 and 2.0% *Chlorella*, respectively. The initial body weight (IBW) of each tank was measured by mass weighing. The feeding trial lasted for 60 days. Fish were fed three times a day (at 6:30, 13:30 and 18:30 h) at a daily feeding rate of 8% of body weight. During
the experiments, one-third of the water volume in each tank was changed once a week. Water condition was as the following: water temperature 26°C, dissolved oxygen content >5 mg/L, NH<sub>3</sub><0.05 mg/L, H<sub>2</sub>S<0.1 mg/L and pH 6.8 to 7.8.

At the end of feeding trial, the fish were starved for 24 h, the batch weighed to obtain the final body weight (FBW), and then three fish from each tank were sampled. For sampling, the fish were anesthetised with 0.02% MS-222 (Shanghai Buxi Chemical Co. Ltd., Shanghai, China) and blood, head-kidney (HK) and liver were isolated from each fish under sterile conditions. The blood was allowed to clot for 1 h at room temperature, centrifuged for 15 min at 1000 rpm, and serum was extracted. Of tissues (HK and liver), 0.5 mg were added to 500 µL ice-cold phosphate buffered saline and homogenised with Biospec tissue-tearor (985370EUR-04; Biospec, Bartlesville, OK, USA), centrifuged for 20 min at 3000 rpm, and supernatants were collected. The serum and tissue supernatants were stored at -70°C until use for assay analysis. The total protein concentration of the serum and tissue supernatants were determined in triplicate by a Bradford assay.

Assessment of fish growth

The growth parameters including weight gain (WG) and specific growth rate (SGR) were calculated using the following formulae:

\[
\text{WG (g)} = \text{FBW} - \text{IBW}
\]
\[
\text{SGR (％)} = \left(\frac{\ln \text{FBW} - \ln \text{IBW}}{\text{number of feeding days}}\right) \times 100
\]

Enzyme-linked immunosorbent assay

The ELISA kits were used for detection of immunoglobulin (Ig) M and D (IgM and IgD), interleukin (IL)-22 and chemokine (C-C motif) ligand 5 (CCL-5) were purchased from Bogo Biotechnology Co. Ltd. (Shanghai, China). The 96 wells ELISA plates were pre-coated with the monoclonal antibody specific for the interested protein by the company, respectively. The ELISA assay was conducted according to the manufacture's instrument. Briefly, 50 µL of standard solution (standard group), distilled water (blank control group) and sample (sample group) were added to the well and then 100 µL enzyme were added to the standard group and sample group, respectively. The plates were sealed tightly with the plate sealing membrane and incubated in wet box at 37°C for 1 h. After being washed with diluted washing solution, 50 µL Substrate A and 50 µL Substrate B were added to each well for colour development. After having reacted in dark for 15 min at 37°C, 50 µL stop solution was added to each well and the optical density was measured at 450 nm. All measurements were performed in triplicate.

Statistical analysis

All data were expressed as mean ± standard deviation (SD) and subjected to one-way analysis of variance (ANOVA). Duncan's multiple range tests were used to determine the significant differences between the means. All the statistical analysis was performed using Microcal Origin 6.0 software.

Results and discussion

We first examined the effects of Chlorella on the growth performance of fish. The results showed that the Chlorella could significantly increase the growth of gibel carp, e.g. for fish fed with 0.8% Chlorella the body weight increased from 29.90±0.08 to 63.75±1.96 g with a WG of 33.85±1.96 g, which was higher than that of control group (P<0.05) (Figure 1). The SGR of fish in five groups fed with Chlorella were 1.16±0.03, 1.26±0.05, 1.26±0.01,
1.32±0.02 and 1.31±0.01, which were higher than that of control group (1.10±0.01) (P<0.05). These results were in accordance with previous studies (Takeuchi et al., 2002) and indicated that Chlorella could be used as an additive in fish diets (Xu et al., 2014).

Afterwards, we examined some immune proteins that reflect the fish immune status by using ELISA method. As shown in Figure 2, IgM level in blood was increased in the group supplemented with 0.4% Chlorella (P<0.05). No significant changes of IgM level were observed in the tissues from other groups (P>0.05). Similarly to the IgM level, the IgD was only increased in the blood in the 0.8% Chlorella group (P<0.05) (Figure 3). Compared with the control group, IL-22 level increased significantly in liver of 0.4% Chlorella group (P<0.05), and in the kidney of 0.4 and 1.2% Chlorella groups (P<0.05). There were no statistically significant differences in other groups (P>0.05) (Figure 4). Chemokine (C-C motif) ligand 5 level was found to increase in the group supplemented with 0.4 and 1.2% Chlorella, which was highly than that of control (P<0.05) (Figure 5).

The immune system represents a nodal point in the balance between animal health and disease (Barreda et al., 2014). Previous studies had found that Chlorella could be involved in the regulation of animal adaptive and innate immunity. Cerezuela et al. (2012) found that microalgae could increase the expression of major histocompatibility complex class I- (MHC-I) of gilthead seabream. Here, we found that the Chlorella could significantly increase the serum IgM and IgD levels of gibel carp. Increasing of IgD, one of the immunoglobins involved in mucosal defence (Saliâns et al., 2011), suggested that Chlorella might play some role in the mucosal immunity.

It has been found that the expression of many cytokines (e.g. IL-8, IL-1) involved in the regulation of fish immune responses, changed greatly when the fish were fed with diets added with microalgae (Díaz-Rosales et al., 2008). In the present work we found that another cytokine, IL-22, increased significantly in fish fed with Chlorella. In fish, IL-22 proved to be an immunoregulatory cytokine playing a key role in adaptive and innate immune systems (Dumoutier et al., 2000; Takatori et al., 2009). Some genes involved in the antibacterial, e.g. antimicrobial peptides IL-8, were regulated by this cytokine (Monte et al., 2011; Costa et al., 2013). The increase of IL-22 by Chlorella suggested that Chlorella might be involved in the regulation of fish anti-inflammatory and immunostimulatory functions. Chemokine (C-C motif) ligand 5 belongs to members of the...
chemokines family and has been found to play a crucial role in the coordination of innate immunity (Hsu et al., 2013). *Chlorella* could significantly increase CCL-5 levels in some tissues (P<0.05). These results indicate that *Chlorella* might involve in the regulation of fish innate immunity by enhancing some gene expressions.

**Conclusions**

In this paper, the effects of *Chlorella* on the immune parameters of gibel carp were studied by dietary administration of *Chlorella* for 60 days. *Chlorella* could increase the IgM, IgD, IL-22 and CCL-5 levels in some tissues. The results indicate that *Chlorella* could be involved in the regulation of fish innate and adaptive immunity and be used as an additive in fish diets.

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