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Research Article

The effect of *Chlorella vulgaris* (Chlorophyta, Volvocales) microalga on some hematological and immune system parameters of Koi carp (*Cyprinus carpio*)

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Abstract: This study aimed to examine the effects of *Chlorella vulgaris* (CV) on the hematological and immune parameters of Koi carp (*Cyprinus carpio*). An experiment was designed with five treatments, including diets with 0, 2, 5, 7 and 10% of *C. vulgaris* each with three replicates for 8 weeks. At the end of experiment, the hematological and, immune system parameters were measured and analyzed. The results revealed that supplementation of *C. vulgaris* in diets resulted in higher percentage of haemoglobuling (Hb), haematocrit (Hct), red blood cells (RBC) and white blood cells (WBC). In addition, a significant increase in the levels of IgM, lysozyme and C4 complement levels were found in fish fed CV at different levels showing a positive effect by this microalgea on the immune status of Koi carp. Based on the results, supplementation of CV particularly at 5% of its dry powder in diet of Koi carp can play an important role in the stimulation of fish immune system.

Keywords: Algae, Aquaculture, Fish, Immunity, Blood.

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Introduction

Koi carp, *Cyprinus carpio*, is a valuable ornamental fish due to its coloration, patterning, and scalation (Jha & Barat 2005). Like other freshwater fishes, its farming can be along with incidence of some infectious and non-infectious diseases such as koi carp herpesviral disease. In traditional methods, antibiotics and chemical disinfectants are applied, but they might be led emergence of bacterial resistance (Esiobu et al. 2002) and environmental concerns (Bachère 2000, 2003). Hence, application of the immunostimulants can be considered as an environment friendly approach to control and prevent diseases in aquaculture (Peddie et al. 2002; Merrifield et al. 2010; Nayak 2010; Ringo et al. 2010). Immunostimulants promote the innate immune system, thereby inhibiting infectious diseases. Several immunostimulants such as chitin

(Sakai et al. 1992; Esteban et al. 2001), lactoferrin (Sakai et al. 1993), dimerized lysozyme (Siwicki et al. 1998), CpG oligodeoxy nucleotides (Tassakka & Sakai 2002, 2003), nisin (Villamil et al. 2003) and recombinant transferrin (Stafford et al. 2004) have been used in aquaculture with significant increase of the resistance against diseases in cultured fishes.

Recently, the immunostimulating properties of microalgae have attracted the interest of researchers. For example, 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 could enhance gilthead seabream (*Sparus aurata*) defense activity.

Chlorella vulgaris (CV) is a fresh water and unicellular algae comprising a nutrient-dense super food containing 60% protein, 18 amino acids, and various vitamins and minerals. One of its particular features as phytonutrient is *Chlorella* growth factor (CGF). CGF is concentrated in the nuclei of the algae, and is consisted of the nucleic acid associated with substances i.e. peptides, proteins, amino acids, vitamins and sugars. It is of particular interest due to detoxification property that related to the peptide glutathione function (Nick 2003). In addition, more than 20 vitamins and minerals are found in *Chlorella*, such as iron, calcium, potassium, magnesium, phosphorous, pro-vitamin A, vitamins C, B1, B2, B2, B5, B6, B12, E, biotin, inositol, folic acid, plus vitamins C, E and K (Nick 2003).

Chlorella vulgaris is also used for medical treatment (Justo et al. 2001; Morris et al. 2009), due to having immune-modulating and anti-cancer properties, protection properties against haemato-poiesis, and age-related diseases (Safi et al. 2014). However, the effects of *Chlorella* on the immunity of aquacultural important species remain limited. Since little information is available on the immune-stimulatory effects of CV in fish, this study was aimed to evaluate some hematological and immune parameters of Koi carp fed with different concentrations of CV.

Materials and Methods

Koi carp weighing 11±0.1g (average total length 29.8±0.05mm) were obtained from a local ornamental fish farm and transferred to the laboratory. Fish were reared in fifteen 250 L tanks with a water-recirculated system and acclimated for two weeks prior to the experiment. After acclimation period, a total of 225 fish weighing 30±0.11g were randomly introduced into 15 experimental 250 L tanks (15 fish per tank) each treatment in three replicates (Tables 1, 2).

The control group was fed the formulated diet without CV, and the four treatments were fed the diets supplemented with 2% (C2), 5% (C5), 7% (C7), and 10% (C10) of dry powder of CV. The experiment was carried out for 8 weeks and fish were fed three times (8:30, 12:30 and 18:00 h) a day at 3% of body weight. During the experiment, one-third of the water was exchanged every other day, and water temperature, dissolved oxygen, NH_3, H_2S and pH were 20°C, > 5mg L^{-1}, < 0.05mg L^{-1}, < 0.1mg L^{-1} and 6.8-7.2, respectively.

At the end of 8 weeks of experimental period, fish were starved for 24 hrs and anesthetized with MS-222 (Sigma, USA) and blood samples were collected from caudal vein of 5 fish per tank (15 fish per trail). The blood samples were collected into both non-heparinized and EDTA tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) to obtain the serum and whole blood, respectively. Then, the blood from non-heparinized tubes were allowed to clot at room temperature for 4 hrs, and after centrifugation (3000×g, 10 min, 4°C), the serum was removed and frozen at -80°C until analysis.

Hemathological parameters, including white blood cells (WBC), red blood cells (RBC), hemoglobin (Hb) and hematocrite (Hct) were measured based on Ameri Mahabadi (2008), Klontz (1972) and Housteon (1990). Immune parameters consisting of C4, total immunoglobulin and lysozyme were measured according to the procedures described by Feldman et al. (2000), Pannall & Zilva (1984) and Sahoo et al. (2008).

Table 1. Composition and proximate analyses of four experimental diets supplemented with *Chlorella vulgaris* powder at 2, 5, 7 and 10%.

Ingredients (g kg^{-1})	**D0**	**D2**	**D5**	**D7**	**D10**
Fish meal	150.0	130.0	100.0	80.0	50.0
Chlorella vulgaris	0	20.0	50.0	70.0	100.0
Soy bean meal	170.0	170.0	170.0	170.0	170.0
Soy bean full-fat	80.0	80.0	80.0	80.0	80.0
Solvent-extracted cotton seed meal	110.0	110.0	110.0	110.0	110.0
Wheat shorts	250.0	250.0	250.0	250.0	250.0
Wheat flour	150.0	150.0	150.0	150.0	150.0
Attapulgite meal	40.0	40.0	40.0	40.0	40.0
Vitamin/minerals premix[a]	10.0	10.0	10.0	10.0	10.0
Soy bean oil	20.0	20.0	20.0	20.0	20.0
%dry matter					
Crude protein	37.59	37.40	37.21	37.34	37.12
Crude lipid	6.90	6.56	6.44	6.12	6.10
Ash	11.49	10.88	10.20	10.01	9.44
Moisture	8.82	8.45	8.46	8.62	8.70

*Becosules capsules (Each capsule contains); Thiamine mononitrat (IP): 10mg; Riboflavin (IP): 10mg; Pyridoxine hydrochloride (IP): 3mg Vitamin B12 (as tablets 1:100) (IP): 15mcg; Niacinamide (IP): 100mg; Calcium pantothenate (IP): 50mg Folic acid (IP): 1.5mg; Biotin USP (IP): 100mcg; Ascorbic acid (IP): 150mg.

Table 2. Proximate composition of *Chlorella vulgaris* (g kg^{-1}) used in this study.

Measurement factors	g kg^{-1}
Crude protein	428.20
Carbohydrate	213.32
Lipid	147.00
Ash	111.86
Moisture	99.30
Gross energy (kcal kg^{-1})	3187.71

Data were presented as mean ± standard error (SE) and analyzed using one-way analysis of variance (ANOVA). Data percentage were arcsine transformed prior to analysis. Duncan's multiple range tests were analyzed among the groups. The significant level was set as $P<0.05$. All statistical analyses were done using the SPSS (version 18.0).

Results

RBC was significantly higher in all treatments with the significantly higher levels obtained for C7 and C10 treatments ($P<0.05$). Hb and Hct in all treatments were significantly increased with the highest level observed in C5 trail ($P<0.05$). WBC also showed an increase in all CV trails with higher values seen in C5 and C7 treatments. No significant difference was seen in levels of WBC between C5 and C7 and also between C2 and control group ($P<0.05$) (Table 3). Differential leucocyte counts are presented in Table 4. The population size of neutrophil and lymphytes showed the highest levels in C5 group ($P<0.05$) (Table 4).

The value of IgM was significantly increased in fish fed with CV ($P<0.05$) and the highest value was observed in C5. Lysozyme activity was higher in all treatments than control one ($P<0.05$) with the highest values measured in C5 and C7. This activity of lyszoyme in C4, C5 and C7 groups were significantly higher than C2, C10 and control group ($P<0.05$) (Table 5).

Discussion

It is estimated that about 30% of microalgal production is used for animal feed due to the increasing demand for food with natural composition instead of synthesized ingredients (Safi et al. 2014). In this regards, *C. vulgaris* contains important amount of carotenoids and other nutrients (considered as nutrient-dense super food) and after feeding it to fish, it showed interesting pigmentation potential for fish flesh, together with enhancing health and increasing its life expectancy (Gouveia et

Table 3. Effect of *Chlorella vulgaris* administration on the hematological parameters of Koi carp.

Factor	Control	C2	C5	C7	C10
HB (g/dl)	5.77±0.25[e]	6.4± 0.27[d]	7.97±0.15[a]	7.37±0.15[b]	7.03±0.05[c]
WBC (×10^3)	16.66±0.58[b]	18.33±0.52[b]	26.00±1.00[a]	23.66±2.51[a]	19.33±0.52[b]
PCV (%)	30.33±0.58[e]	34±1.00[d]	41.33±1.53[a]	38.00 ±1[b]	36.00±1.00[c]
RBC (×10^3)	3.10±1.00[b]	3.47±0.15[ab]	3.53±0.3[ab]	3.73±0.2[a]	3.77±0.27[a]

The same lowercase letter are not significantly different (P>0.05)

Table 4. Effect of different amounts of *Chlorella vulgaris* administration on the level of the serum Igm, lysozyme and C4 of Koi carp.

Factors	Control	C2	C5	C7	C10
Igm	13.67±1.15[d]	17.00±1[c]	27.33±1.53[a]	23.33 ± 0.58[b]	18.67±1.53[c]
Lysozyme	24.33±2.08[c]	28.00±1[b]	35.33±1.53[a]	35.00±1.00[a]	29.33±1.53[b]
C4	21.67±0.58[c]	21.67±1.53[c]	33.33±2.52[a]	25.00±1.00[b]	23.00±1.00[bc]

The same lowercase letter are not significantly different (P>0.05)

Table 5. Number of WBC in Koi carp after adding *Chlorella vulgaris* to the diets.

Factors	Control	C2	C5	C7	C10
Thrombocytes	0.33±0.57[a]	0.00[a]	0.67±0.58[a]	1.00±1.00[a]	0.33±0.58[a]
Monocytes	2.67±0.58[a]	2.33±0.58[ab]	1.33±0.58[b]	2.33±0.58[ab]	2.67±0.58[a]
Basophils	3.00±1.00[ab]	4.67±0.58[a]	2.33±1.15[b]	4.67±1.53[a]	4.00±1.00ab
Neutrophil	55.00±1.00[c]	49±1.00[d]	77.00±4.36[a]	61.00±1.00[b]	51.33±1.53[cd]
Lymphytes	38.00±1.00[c]	18.33±2.08[e]	44.00±1.00[a]	31.00±1.00[d]	41.67±0.58[b]

The same lowercase letter are not significantly different (P>0.05)

al. 1999, 2002).

Haemotological parameters are influenced by species, age, sexual maturity, health condition, nutritional quality of the target fish and the environmental conditions (Bielek & Strauss 1993). Based on the results, the application of CV in feed of Koi carp showed proliferation of all measured haemotological parameters. Supplementation of CV in koi carp feed was led a rising in RBC level. RBC in teleost is related to the oxygen requirement (Zanjani et al. 1967) and in the present study, its increase can be considered as positive effect made by CV. Among the immune cell parameters, RBC count is a frequently used parameter to evaluate possible undesired collateral effects (anaemia) provoked by immunostimulant administered in supplemented feed (Morera et al. 2011). WBCs are the immune-competent cells of immune system which play critical roles to both infectious and non-infectious diseases (Magandottir 2006). An enhancement in the WBC population size was seen in fish fed CV at different concentrations. Such enhancement is in part be due to the positive effects of some ingredients of CV e.g. vitamins and glucans available in the cell wall of CV. Therefore, inclusion of CV in the fish feed can provide a stimulatory role for the fish immune status i.e. increasing in phagocytosis capacity and cell-mediated immune responses resulting in an increasing in the fish resistance to the diseases and disorders (Feldman et al. 2000, Montero-Rocha 2005; Sakai 1999; Parra et al. 2015). This is supported by a significant increase in the levels of IgM, lysozyme and C4 in the fish fed CV. It has been shown that *Chlorella* could be involved in the regulation of animal adaptive and innate immunity. For instance, Cerezuela et al. (2012) found that microalgaes could increase the expression of major histocompatibility complex class I (MHC I) of gilthead seabream resulting in the stimulation of cytotoxic cells. Therefore, an improving in these immune parameters clearly suggest a positive role of CV administration (up to 10% of the diet) on the mucosal immunity of fish (Magnadottir 2006; Dunkelberger & Song 2010).

In the present study, Hb and Hct had higher levels in fish fed *C. vulgaris*. Physiologically, Hb and Hct are crucial to the survival of fish, being directly related to the oxygen binding capacity of blood (Bielek & Strauss 1993). Obviously under such conditions the animal metabolism is improved resulting in a better growth performance (Khani et al. In Press).

In conclusion, the results of this study show that supplementation of CV particularly at 5% dry powder in diet of Koi carp can play an important role in the stimulation of fish immune status. Such stimulation can bring fish in a position to be more resistant to both unsuitable environmental conditions and outbreaks of infectious diseases. Obviously this better immunophysiological condition will lead to improving in a more production. Therefore, use of 5% CV in feed can be recommended in the diet of this economically important commercial fish species.

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مقاله پژوهشی

اثر تغذیه‌ای ریزجلبک کلرلا ولگاریس، *Chlorella vulgaris* (Chlorophyta, Volvocales) بر روی برخی پارامترهای خونی و ایمنی ماهی کوی (*Cyprinus carpio*)

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چکیده: این مطالعه با هدف بررسی اثرات اثر پودر جلبک کلرلا ولگاریس(*Chlorella vulgaris*) بر روی برخی از پارامترهای خونی و ایمنی ماهی کوی (*Cyprinus carpio*) به اجرا درآمد. برای این منظور یک طرح کاملاً تصادفی شامل سطوح ۲، ۵، ۷، ۱۰ درصد کلرلا ولگاریس در قالب ۵ تیمار با سه تکرار برای ۸ هفته طراحی شد. در پایان آزمایش، پارامترهای خونی و ایمنی ماهی کوی اندازه‌گیری و تحلیل شدند. نتایج نشان داد که افزودن کلرلا ولگاریس به جیره غذایی باعث افزایش درصد هماتوکریت و غلظت هموگلوبین، تعداد گلبول سفید و گلبول‌های قرمز شد. به علاوه یک افزایش معنی‌دار در سطوح برخی پارامترهای ایمنی لیزوزیم، ایمنوگلبوین Igm و عامل کمپلمان C4 یافت شد که نشان‌دهنده اثر مثبت این ریز جلبک بر پارامترهای ایمنی ماهی کوی می‌باشد. براساس نتایج، افزودن کلرلا ولگاریس به ویژه به میزان ۵ درصد پودر جلبک خشک شده به جیره ماهی کوی می‌تواند یک نقش مهمی در تحریک سیستم ایمنی آن داشته باشد.

کلمات‌کلیدی: جلبک، آبزی‌پروری، ماهی، ایمنی، خون.