Iranian Journal of Fisheries Sciences 19(3) 1130-1139 2020
DOI: 10.22092/ijfs.2019.119600.0

Effect of dietary supplementation of *Chlorella vulgaris* on several physiological parameters of grey mullet, *Mugil cephalus*

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Received: July 2017 Accepted: April 2018

Abstract

The present study was investigated on effect of optimum dietary level of *Chlorella vulgaris* powder (CP) as a feeding supplement on various blood biochemical criterion (Cholesterol (CHO), triglyceride (TG), total protein (TP), glucose (GLU), and lysozyme) and digestive enzymatic activities (amylase, lipase and protease) of the grey mullet (*Mugil cephalus* L). Four experimental regimens were supplemented with CP at 0, 5, 10 and 15 g kg^{-1} diet (CP0, CP5, CP10 and CP15). Number of twelve pools (60-L) with three duplicates for analysis groups (n=10 per pool with initial weight average 14.95±2.01 g) and the control group were studied. Upon 60 days of the feeding trial, fish fed CP5 diet had lower serum CHO and TG levels than fish fed CP0, CP10 and CP15 diets ($p<0.05$). No considerable difference were found in GLU when comparing fish fed CP5 and CP10 diet ($p>0.05$). Most serum total protein and amylase, protease, lipase and lysozyme activities were observed in fish fed CP5. Also, fish fed CP10 and CP15 diets had higher digestive enzymatic activities, serum total protein and lysozyme activities than fish fed CP0 ($p<0.05$). The outcomes proved the inclusion of 5g chlorella powder dietary supplementation in the commercial regimen may improve the blood chemical responses and the activity of digestive enzyme in grey mullet.

Keyword: *Mugil cephalus*, *Chlorella vulgaris*, Additive, Digestive enzymes, Blood biochemical parameters

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Introduction

Breeding larval fish successfully is the most vital stage for many species during their production cycle. *Mugil cephalus* has a prosperous market value in Europe, East and South Asia (Yelghi *et al*., 2012). In Iran, is considered an essential aquaculture species. Consumer demand caused the progression of intensive aquaculture of this species in Asian countries. The issue in breeding larval fish is relevant to the supply of food (Akbary *et al*., 2011). Hence, an easily accessible, acceptable and digestible regimen containing rich nutritional value should be considered as a diet with larval fish (Girri *et al*., 2002.; Akbary *et al*., 2011).

Plants are considered as natural sources of safe and cheap chemicals. Plant-based production is known to enhance activities such as anti stress, growth boost, appetite incitement and immunostimulation in aquaculture practices (Citarasu *et al*., 2002; Sivaram *et al*., 2004).

Chlorella sp. as one of the most prevalent microalgae utilized in aquaculture, have been reported to improve lipid metabolism, digestive enzymatic activities, growth, feed utilization in Korean rockfish, *Sebastes schlegeli* (Bai *et al*., 2001), juvenile Japanese flounder, *Paralichthys olivaceus* (Kim *et al*., 2002), Gibel carp, *Carassius auratus gibelio* (Xu *et al*., 2014; Shi *et al*., 2016), olive flounder, *Paralichthys olivaceus* (Rahmaninejad and Lee, 2017).

For example, Xu *et al*. (2014) observed that growth performance, total protein, lysozyme and activity of digestive enzyme in 8 and 1.2% of the group with chlorella were greater than in the control group. Rahmaninejad and Lee (2017) proved that adding 10-15% chlorella meal may increase growth enforcement and lipid metabolism in olive flounder. Bai *et al*. (2001) showed that diet supplemented with chlorella powder at 5% can improve growth and feed utilization in juvenile Korean rockfish. Khani *et al*. (2017) showed that koi carps (*Cyprinus carpio*) fed with 5% *Chlorella vulgaris* diet increased level of serum protein and activity of digestive enzymes compared with the control group. Also serum cholesterol and triglyceride level of fish fed 5% chlorella were less in comparison with the fish in the group in question.

Freshwater algae such as *Chlorella* and *Spirulina* appear to be protein rich source. However, the main source of the long-chain polyunsaturated fatty acids is the marine microalgae which are vital for human health and the health of aquaculture animals. *Chlorella vulgaris* appears to be an appropriate choice as a substitute feed ingredient in aquaculture due to its richness in protein, polysaccharides, vitamins and microelements (Xu *et al.*, 2014). Even though the algal feeds were not utilized as effectively as the fishmeal feed, but *Chlorella* was accepted appropriately. As example Shi *et al*. (2016) showed that Chlorella meal could totally replace dish meal in diet of crucian carp, *C.auratus*.

Up to today, no experiment has been carried out to examine the influence of dietary *Chlorella* powder on various serum biochemical criterion and digestive enzymatic activities of grey mullet. This research was therefore designed to examine the effect of optimum dietary level of *Chlorella vulgaris* powder (CP) as a feeding supplement on some blood biochemical parameters (Cholesterol (CHO), triglyceride (TG), total protein (TP), glucose (GLU) and lysozyme) and digestive enzymatic activities (amylase, lipase and protease) of the grey mullet.

Materials and Methods

Empirical diets and Feeding circumstances

Chlorella vulgaris powder were purchased from the Yakhteh Nano-chemistry Co in Tehran, Four regimens were arranged that included *Chlorella* powder (CP) supplementation at inclusion level of 0, 5, 10 and 15 g kg^{-1} diet, evaluated proximate compositions are shown in Table 1. Upon combining with ingredients, 10% oil fish and 30% refined water were included and mixed well. The resulting dough was pelletized with chopper machine (National, Japan). The experimental diets were freeze-dried, sieved into the considered particle size (1 mm) and then kept at a temperature of −4 until use (Choi *et al*., 2015).

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Table 1: Ingredients (g kg^{-1}) and proximate composition (%) of the experimental diets.

	Diets			
Ingredients (g kg^{-1})	**CP0**	**CP5**	**CP10**	**CP15**
Chlorella vulgaris (g kg^{-1})	0	5	10	15
Fish meal	427	427	427	427
Soybean meal	192.5	192.5	192.5	192.5
Wheat flour	93	93	93	93
Dried yeast	37.5	37.5	37.5	37.5
Fish oil	55	55	55	55
Soy oil	27.5	27.5	27.5	27.5
Choline chloride	2	2	2	2
Bi calcium phosphate	3.7	3.7	3.7	3.7
Lecithin	28.15	28.15	28.15	28.15
Premix[a]	9.4	9.4	9.4	9.4
Proximate composition(%)				
Crude protein	51.6	51	50.6	51.6
Crude lipid	11.9	11	11.4	11.2
Crude ash	12.1	12	11.8	12.6
Dry matter	92.2	92.1	92	92

[a]Premix (mg kg $^{-1}$) KI, 250; $MnSO_4 \cdot H_2O$, 2800; $ZnSO_4 \cdot H_2O$, 2350; vitamin K, 225; biotin 3500, (2%)niacin, 4850; calcium pantothenate, 11,000; folic acid, 2000; vitamin B1, 1500; vitamin B_2, 2000; vitamin B_6, 2000; and vitamin C, 50,000.

This feeding test was carried out at Fisheries Research Center, Chabahar, Iran. one hundred- twenty grey mullet with an introductory mean weight of 14.95 g were dispersed randomly into twelve pools (60 L) at a stocking density of 10 fish/pool (triplicates per analysis) and hand fed adequately twice (09:00 and 17:00) every day for 60 days and feed intake was recorded on daily basis. The measurements for dissolved oxygen, ammonia nitrogen concentration and pH were approximately 7.01± 0.87 mg L^{-1}, 0.11± 0.04 mg L^{-1} and 7.8±0.4 correspondingly. The photoperiod was regulated as a 12:12 h (dark/light) cycle.

Biochemical analysis

At the end of experiment, nine fish from each test were anesthetized (with clove oil at 5 mg L^{-1}) and samples of blood were extracted upon excising caudal peduncle and then poured into un-heparinized sterile tubes 1–1.5 ml for the serum biochemistry testing,(Shaluei *et al*., 2012; Akbary *et al*., 2016).

Serum glucose levels was measured based on an approach By Trinder (1969). Serum total protein was measured by utilizing the approach by Wootton (1964). Triglyceride and cholesterol level measurement took place based on the method explained by Sankar (2011). Biochemical estimation of blood glucose, protein, cholesterol and triglyceride were determined by means of standard analyses kits (Pars Azmon, Iran) using automatic analyzer (Furuno, CA-270, Japan).

The turbidimetric assay for lysozyme took place (Parry *et al*., 1965) with negligible modification (Ellis, 1990). To summarize, substrate for the assay of lysozyme took place by utilizing 0.03% of lyophilized cells of *Micrococcus lysodeikticus* (Sigma, ATCC No. 4698) in 0.05 mM sodium phosphate buffer (pH 6.2). Fish plasma (25 microliters) was included in 175 µl bacterial suspensions in equivalent wells of a microtitre plate. The concoction was incubated at room temperature and 600 nm of absorbance was determined after 15 s via an ELISA plate reader (Argus, PerkinElmer, France). A unit of lysozyme movement was determined as a plasma-decrease amount of lysozyme in absorbance of 0.001 ml^{-1} min^{-1}.

Digestive enzyme activity

For preparation of enzyme extracts, three fish were taken from each pool randomly and sacrificed. The digestive tracts were cautiously dissected out, completely washed using sterile distilled water for them to be weighed and separately homogenized with cooled buffer phosphate (0.65 %, pH 7, 1: 10 w/v). The supernatant, extracted by centrifugation (3000 g for 20 min at 4˚C) (Centrifuge EBA21, Hettich, Germany), was utilized for enzyme assays. Amylase levels of activity was assessed by 3, 5- dinitrosalicylic acid (DNS) method (King, 1965). 0.1 ml tissue homogenate, 2 ml phosphate buffer (0.1 M, pH 7) and 0.1 ml of 1 % (w/v) starch solution was mixed and incubated at 30˚C for 35 min. Then adding 2 ml DNS reagent stopped the reaction. After 5 min in boiling water, the reaction mixture was cooled, diluted with distilled water and recorded the absorbance at 540 nm. Activity levels of protease was determined by the casein digestion method of king (1965). 0.1 ml tissue homogenated, 0.05 M tris phosphate buffer (pH 7.8), 0.01 N NaOH and 2.5 ml of 1% (w/v) consisted the reaction mixture. The concoction was incubated at 30˚C for 10 min and stopped by 2.5 ml, 10% trichloroacetic acid (TCA) and filtered. The reagent blank consisted just tissue homogenate before stopping the reaction and without incubation. The absorbance was recorded at 320 nm. Activity levels of unit amylase was measured as the weight (mg) of resulting maltose for a duration of 10 minutes at 30˚C. Activity levels of unit protease was presented as the volume of tyrosine liberated for the duration of 15 minutes under the assay circumstances. Activity levels of lipase was assessed by King (1965) method. Olive oil emulsion, phosphate buffer (pH 7.8, 0.1 M), tissue homogenate and refined water consisted the reaction concoction. The reaction concoction was incubated at 30˚C for 24 h and added two drops phenolphthalein indicator and 95% alcohol for titration against 0.05 N NaOH until the appearance of permanent pink color. Activity levels of unit lipase presented as the amount of 0.025 N NaoH needed to neutralize the fatty acids liberated for the period of 18 h of incubation at pH 6.9 and temperature 30˚C. Digestive enzymes were measured as enzyme unit per gram tissue.

Statistical analysis

Every calculation was carried out three times. Data (means±standard Error) was assessed by utilizing one- way variance analysis (ANOVA). Every groups was assumed to be considerably varying if $p<0.05$ .When a significant value of F value resulted from ANOVA the differences among every group was examined by utilizing the Duncan multiple comparisons test. Every statistic was carried out using SPSS 16.

Results

Influences of Chlorella powder (CP) on the blood biochemical criterion of grey mullet

The blood criterion of fish fed with the empirical regimens were presented in Table 2. Data analysis in Table 2 assumed that the dietary chlorella mostly influenced the parameters relative in protein/ lipid metabolism and grey mullet immunity. The serum total protein content and lysozyme activity were considerably ($p<0.05$) enhanced for fish on CP diet compared to the control group. Most total protein content was measured in those fish fed CP5 diet. There was considerable ($p<0.05$) decline in cholesterol, glucose and triglycerides levels within those fish fed CP diet over the control (Table 1). The lowest cholesterol, glucose and triglycerides levels of serum were observed in fish fed CP5.

Table 2: Serum biochemical parameters of *Mugil cephalus* fed CP diets at different levels

	CP diet (g kg^{-1} feed)			
Parameter	**0**	**5**	**10**	**15**
Total protein (g dl^{-1})	4.42±0.43^{d}	6.58± 0.33^{a}	5.45±0.21^{b}	4.97±0.18^{c}
Glucose (mg dl^{-1})	55±1.52^{a}	31±2.18^{c}	35.33±4.01^{bc}	37±3.10^{b}
Triglyceride (mg dl^{-1})	227.33±11.76^{a}	172±10.05^{d}	191±12.64^{c}	209.28±14.75^{b}
Cholesterol (mg dl^{-1})	104.79±10.76^{a}	75.33±13.7^{c}	95±11.73^{b}	100±11.15^{b}
Lysozyme	149±15.53^{c}	275.07±18.45^{a}	199.67±10.88^{b}	185±12.88^{b}

CP diet, *Chlorella powder* diet. Values (mean± SE of three replication. In each row not sharing a common superscript are significantly different (P<0.05).

Effect of Chlorella powder (CP) on the digestive enzymatic activities of grey mullet

Three digestive enzymes activity levels including amylase, lipase and protease in intestine were examined, and the results are shown in Table 3. These three enzymes were significantly increased for fish fed CP diet compared with control. The highest digestive enzymatic activities were recorded in those fish fed CP5 diet. However there was no significant changes in the enzymes' activity levels among fish on the CP10 diet and CP15 diet (p>0.05).

Table 3: Activity levels of digestive enzymes (unit mg^{-1} protein) of *Mugil cephalus* fed CP diets at different levels.

Specific activity of enzyme (unit mg^{-1} protein)	CP diet (g kg^{-1} feed) 0	5	10	15
Amylase	26.3±8.92^{c}	45.66±11.45^{a}	34.33±9.32^{b}	36±12.08^{b}
Protease	356.67±23.07^{c}	520±11.73^{a}	450±15^{b}	432±14.93^{b}
Lipase	211±10.21^{b}	514±11.58^{a}	406.67±17.67^{b}	378.67±16.90^{b}

Cp diet, *Chlorella powder* diet. Values (mean±SE of three replication). In each row without a common superscript are considerably different (P<0.05)

Discussion

Based on this research, various Chlorella powder content were included in the staple regimen of grey mullet and the influences of Chlorella on the blood biochemical criterion and digestive enzyme were noticed.

Chlorella powder (5-15 g) in diets may considerably improve the blood criterion of grey mullet. In this research, considerably lower serum total cholesterol levels were noticed in all levels of CP compared to those on the control diet. In compliance to the results obtained, Rahimnejad and Lee (2017) reported the significant decrease of serum cholesterol level in olive flounder, *Paralichthys olivaceus* fed regimens with 5-15 % *C. vulgaris*. Moreover, Xu *et al*. (2014) established the considerable reduction of serum cholesterol level in Gibel carp fed regimens that included 1.6-2% *Chlorella* powder. Identical inclination of a reduction in blood cholesterol levels also been found in olive flounder fed diets with 2-4% *C. ellpsoidea* (Kim *et al*., 2002) Koi carp (*Cyprinus carpio*) fed diet containing 5% *C.vulgaris* (Khani *et al*., 2017), grey mullet fed diet containing 15g kg^{-1} *Spriulina platensis* (Akbary and sondak zehi, 2016) suggesting that *Chlorella* supplementation may simulate hormonal control of lipid metabolism (Xu *et al*., 2014). The results of indicated that Chlorella as a wide group of photosynthetic organisms can be as an good additive for fish diets. Chlorella consists of vitamins, minerals, bioactive substances, immunostimulants in the form polysaccharides, lipid, and vital amino acids involved in many physiological activities (Khani *et al*., 2017). In this research, considerably higher serum total protein levels and lysozyme activity were observed at every CP level compared to those on the control diet. Similar tendency of increasing the serum total protein and lysozyme activity also been achieved in olive flounder that are fed according to diets with 2-4% *C. ellpsoidea* (Kim *et al*., 2002), koi carp fed diet containing 5% *C. vulgaris* (Khani *et al*., 2017), grey mullet fed regimen consisting of 15 g kg^{-1} *S. platensis* (Akbary and sondak zehi, 2016) and in Gibel carp fed 1.6-2% *Chlorella* powder (Xu *et al*., 2014).

Protein and lysozyme are vital when it comes to the immune system (Kumar *et al*., 2012; Xu *et al*., 2014). Our results implied that the addition of *Chlorella* may enhance the immune response of grey mullet. Enhanced levels of serum protein is a main factor for indicating the liver function improvement and the immune function of the fish (Akbary and Sondak zehi, 2016). Lysozyme is a vital defense molecule of the elemental immune system, which in turn, is vital in mediating protection when faced with microbial invasion (Kumar *et al*., 2012). Increasing lysozyme and total protein by dietary Chlorella implied that the Chlorella may include bioactive substances relevant to the regulation of fish immune response.

In this study, significantly lower triglyceride and glucose levels were observed at every CP level compared to those on the control diet, implying that the chlorella may have a role in the metabolism of carbohydrate (Akbary and Sondakzehi, 2016). Conversely, Xu *et al*. (2014) and Khani *et al*. (2017) showed that Chlorella powder could reduce blood cholesterol which is not true for the glucose of Gibel carp. This was also observed by Güroy *et al*. (2011) suggesting that its influence also variably depend on dietary *Chlorella* species and its concentrations (Kim *et al*., 2002).

Research results indicated that using different levels of *C.vulgaris* powder play a positive role on the activity of digestive enzymes. Digestive enzyme activity analysis is a simple and reliable method that may be utilized as a barometer of digestive actions and nutritional state of fish (Abolfathi *et al*., 2012). *M. cephalus* is a stomatch- less fish. The intestine is where digestion takes place and numerous intestinal enzymes are included in digestive and absorptive actions, such as amylase, protease, lipase (Das and Tripathi, 1991). Identical positive recordings were determined by Xu *et al*. (2014) and Khani *et al*. (2017) with increasing the digestive enzyme in the hepatopancreas and intestine in Gibel carp, *Carassius auratus gibelio* and koi carp respectively, fed with *Chlorella*, suggesting the Chlorella may improve the diet processing rate by enhancing digestive enzyme activity. Also, Shi *et al*. (2016) showed that dietary *Chlorella* meal replacement may considerably enhance amylase activities not including that of trypsin and lipase in intestine of crucian carp, *Carassius auratus*. Enhanced activities of amylase may promote carbohydrate utilization in diets. Vizcaino *et al*. (2014) recorded algae inclusion could considerably enhance trypsin activity but not that of trypsin of sea bream, *Sparus aurata*. Similarly Radhakrishnan *et al*. (2015) showed that *Macrobrachium rosenbergii* fed *C. vulgaris* up to 50% level significantly enhanced digestive enzymatic activities. In this study, the highest digestive enzyme activities were observed in fish fed with CP5, while the three enzyme activity levels in Gibel carp fed with 2 % *Chlorella* group were the higher than those of other groups. Suggesting the ideal levels of dietary microalga on digestive enzymatic activities was fluctuating even in conspecific (Xu *et al*., 2014). Reports on applicable concentration of dietary microalga that advantageously influence fish digestive enzymes may be needed for use in aquafeed.

In conclusion, this research showed that supplementation of 5 g *C. vulgaris* powder in each kg diet, as a feeding supplement, enhanced biochemical parameters and digestive enzymatic activities in grey mullet.

Acknowledgment

We express our gratitude to the personnel of the Fisheries Researches Center, Chabahar, Iran. We also appreciate greatly the valued efforts of Dr N Tayari Sadaf laboratory expert, Chabahar, Iran.

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