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ORIGINAL ARTICLE

Effects of Dunaliella microalgae *(Dunaliellasalina)* on different levels of complement C_3, C_4 and antioxidant capacity in rainbow trout *(Oncorhynchusmykiss)*

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ABSTRACT

Main purpose of this research was to study the effects of Dunaliella microalgae on physiological and antioxidant capacity of the rainbow trout. It was planned to measure the amount of complement C_3, C_4 and proxidases enzyme of the fish. Rainbow trouts were separated in five equal groups and were fed with diets containing 0.,5,7,9 and 11 grams of pure dried Dunaliella in each kilogram of food respectively. Blood samples were taken from 25 random collected fish, at the end of first and third months of culture and were send to the laboratory to measure complement and proxidase factors. Result indicated that the amount of complement C_3, C_4 and proxidases enzyme in the blood plasma of those fish fed with Dunaliella algae, with the increased levels of Dunaliella in food, and increased weight, levels of C_3, C_4 and proxidases were also increased.Treatment 4 had highest effect on increased weight of fish and was significantly different with other treatments. In term of fish length , the longest length was observed in treatment 4 , which had significant different with treatment 1 ($p<0/05$). Obtained results indicated that Dunaliellaalgaecontaing β-carotene, has favourable effects on immune system of rainbow trout. Thus Dunaliella algae can be used as a nutrient in rainbow trout diet, improving fish weight and length gain while having physiological positive properties on the fish.

Key words: Dunaliella microalgae *(Dunaliellasalina)*,Rainbow trout, complement , proxidase

Introduction

Dunaliella is a unicellular, naked biflagellate green algae, and without cellular membrane .It'stipic species is *Dunaliellasalina.* Dunaliella first discovered in 1838 in the Atlantic coast of france by dunal [8], after it's identified by teodoresco in 1905, and it was named dunal. He was the first one who find out that the pigment responsible for the red colouration displayed by Dunaliellasalina [24]. The algae*Dunaliellasalina* has been on of the most studied members of chlorophycea by 27 species.

The genusDunaliella has, the unicellular green algae which is responsible for most of the primary production in hypersalinenviroments worldwide.

β -Caroten on of the naturallypigments with the highly. Prized antioxidantproperties [9], and by reason of antioxidant and anticancer properties, Dunaliella was used to production nutritional supplement and pharmaceuticals [27].

Feeding by supplement of β-caroten from *D. salina,* by reason of enhancement β-caroten due to complement activity increased, and on the other hand, serum lysozyme activity increased, and the result indicated that body immune levels increased [1]. In the other trial Japanse parrotfish *(Oplegnathusfasciatus)* and spotted parrotfish *(Oplegnathuspunctatus)* larvae were fed with β -

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Carotensupplement rotifers, that results showed survival rates of β -Carotensupplemented groups of both Japanese and spotted parrotfish higher than the control [23].

In a research effects of Dunaliella microalgae on growth, survival of change, skin and flesh colour, and also antioxidant capacity factors in rainbow trout by Wang *et al* [28], results showed that β-caroten due to pigments of skin and flesh, weight, survival and antioxidant activity (superoxidas and proxidases) increased. Also the effects of *Dunuliellasalina* dry powder on growth, immune function and disease resistance were determined in black tiger shrimp *penaeusmonodon,* showed higher weight gain and resistance to white spot syndrom virus infection and also demonstrated significantly higher stress resistance. The colour intensity of shrimp, was correlated with the level of *D. Salina* in the diet.

In recent years, the use of *Dunaliellasalina* paste as a sole food source or in combination with other marin organisms such as Rotifer and Artemia is now a standard, in aquarium and hatcherie and it was recognized, that beside of weight gain, due to enhancement the colouration, improved lipid content of the marine microorganisms [28].

In this research, two basic and appliable aims including information collection and knowledge earning about Dunaliella algae effects on immunology and physiology of rainbow trout fish and marketing acceptance of it were studied. Since Dunaliella has anticancer and antioxidant effects, it has been thought that weather It's use could be resulted in increasing fish potential in immunology and changes in immunological complements and proxidase indicators.

Methods and materials

The experiment was accomplished in Emamzadeh Ali trout hatchery, 75 kilometers north east of Tehran, east side of laar River, during August and September 2009. 450 young France race trouts with average weight of 100 grams were selected and separated in five equal groups and were placed in 5 small cement canals (65 × 100 × 37 centimeters). Water income was 1.5 liter per second for each canal. For 14 days,thesewere fed with common hatchery diet. These were fed for 90 days with 5 special processed diets, containing 0.0, 5.0 , 7.0 , 9.0 and 11.0 grams of *Dunaliellasalina,* respectively. They were fed twice daily at 10 and 16 hours, 1.8 percent of their body weight [3]. Hatchery diet which was then mixed with Dunaliella algae and starch, was provided from skretting co. from Italy (table 1).

Duanliellaalgae was collected from hose-e-soltan lake near ghom and was cultured under salinity and light stress situations in biotechnology laboratory of the scientific and Industrial research organization.

Increased salinity from 15 to 30 percent was effective in increasing β-caroten in the algae. Cultured Dunaliella was separated by centrifuging with MAB separator, model 204, with 8400 RPM. Separated Dunaliella was dried and powdered, containing 30 percent salt. Water in the hatchery was supplied from spring, with a rather costant temperature (14±1^{q}c), oxygen concentration of 9.5 mg/l and pH value of 7.5.

Sampling took place in a 3 months period. At the start of the experiment. 5 samples were taken from each canal by chance and after 3 months of culture, similar sampling was done in all of the canals. Samples were anaesthetized with the extract of clove flower (1:5000). After drying, blood was taken from peduncle area [11]. Blood of each fish was transfered to a small laboratory tube and weretransfered inice box to Hemmat laboratory. They were transfered to a centrifuge with 4600 PRM and after 10 minutes, their plasma was separated and were Kept in -80^{q}c.

Indicators were measured by using special trade Kits, Nos. 88001-2 and on the basis of immunoturbidiometric [25] and Eliza method [6] with Roche cobasmira apparatus and accuracy of 0.1. Quantity of results were analysed using One –Way-Anova statistical method, and to appoint if there are meaningful differences between obtained average results, Duncan test in level of $P<0.05$ was tested. Kelmogrofsmirno test was used to analyse the quality of the results and leven test was used to control rivalness of the results using SPSS soft ware[31].

Results

Produced results from measuring C_3 and C_4 complements and proxidase for beginning and end of 90 days experiment in average and standard deviation are shown in table2 . It shows that average C_3 in the witness sample 1 was lower than witness samples, 1.04 and 1.36 respectively. Highest amount of C_3 was measured 2.1 mg/dec L in treatments and lowest was 1.68 mg/dec L in treatments. Average amount of C_4 in witness 1 was less than witness 2, 0.57 and 1.64 mg/dec L respectively. Highest amount of C_4 was measured in treatment 2 (2.08 mg/dec L) and lowest amount of proxidase was measured in treamtment 4 (1.22 mol/l). Laboratory analysis has been done in 5 repetitions and are shown in table 2.

Obtained results indicated that levels of the amount of complement 3 in the plasma of fishes received Dunaliellaalgae were not similar and had meaningful differences ($P<0.05$) with witness groups. C_3 was increased in all treatments received Dunaliellaalgae, with highest increase in treatment 2. Its amount in the blood plasma of the witness 2 group, had meaningful differences with other groups.

Results obtained from using One - Way –ANOVA test and comparsion of the results using duncan's test, is shown as mean + SE in figure 1. Alternate letters show meaningful differences, obtained by using Duncan method.

Changes of the levels of C_4 complement in blood plasma of those received Dunaliella algae in Their diets, in comparsion with the group of witness 1, showed a meaningful increase ($P<0.05$). It was also increased in the blood plasma of witness 2 group, compared with the witness 1 group. Amount of this complement was increase in all of the treatments. With highest one for treatment 2. Treatment 1 and 4 were placed after that respectively.

Level of proxidase activity in plasma fishes which received Dunaliella algae in Their diets, compared with thewitness 2, had significant differences ($P<0.05$).Increase inproxidase levels not equal in all was increase in all treatment but it was highest in treatment 4, particulary in the end of experiment with high statistical difference. Results obtained by using One- Way- ANOVA test and comparing obtained averages by Duncan test, are shown in fig-3. Alternate letters show meaningful differences.

Biometric results

Increase in average weight and length of the trouts during 90 days duration of culture are shown in table 3. Increased weight in witness 1 group (102.6 grams) was lower than witness 2 (460 grams). Highest weight increase belonged to treatment 4 (550 grams) and lower one belonged to treatment 1 (450 grams). Average length in witness 1 group with 20.36 centimeter was lower than witness 2. With 2.84 centimeters. Highest length increase belonged to treatment 4 (30.74 cm) and lowest one belonged to treatment 1 (29.2 cm)

Paying attention to Obtained datas from weight increase and Obtained results from Duncan test, it perfectly shows that with increase of Dunaliellaalgein the diet, growth increasedalmost similar in all treatments and witnesses which had no statistical differences ($p<0.05$).

Results obtained by using One- Way- ANOVA test and comparing obtained averages by Duncan test, are shown in fig-4. Alternate letters show meaningful differences.

Increased length was also obtained related to the amount of Dunaliella algae in the diet. Highest increase was obtained in treatment 4 and then treatment 3. There was a meaningful difference between all treatments and witness one. No meaningful difference was observed between all treatments ($P<0.05$).

Results obtained by using One- Way- ANOVA test and comparing obtained averages by Duncan test, are shown in fig-5. Alternate letters show meaningful differences.

Discussion

Dunaliella is microalgal –derived water, that containing β-caroten pigmentandacid-soluble immunomodulatorysubstances such as phycocyanin, poly saccharide, Fe and Zn [21]. Serum complement activity was high in the blood plasma of those fish feed with Dunaliella algae, because feeding by Dunaliella, due to enhancement of complement activity by β-caroten and the result indicated that body immune level increased.

The alternative serum complement as a component of the non-specific defense mechanism is most important in fish and higher complement levels in the blood plasma could indicate better fish health [29]. Increased the serum complement activity of rainbow trout maybe, by reason of that β-caroten activate a novel receptor, perhaps belonging to the retinoid X receptor (R×R) family of receptors with ligand binding affinity for retinoids and carotenoids [30].

Furthermore, retinoic acid response elements (RARE) are present in the promoter region of the complement factor H gene and retinoic acid increased the H gene mRNA and protein levels in cells [14]. The above avidence indicates that R×R activation and binding to a RARE in the promoter region of target genes is a putative mechanism through which carotenoids or their metabolites could affect the synthesis of complement components.

Notwithstanding the low serum levels of β-caroten obtained in this study, a significant effects on some immune indices was observed and it is speculated that its conversion to vitamin A or its more potent derivatives such as the retinoids in the intestine before absorption into the lymph and portal blood [10]. In a research using food complement including vitamin A, by Thompson *et al* [26] was shown that increased level ofvitamin Ain Atlantic salmon *(Salmosalar*) diet, resulted inincreasing effects of complements and lysosyme.onthe other hand, high level of vitamin A inthe diet will increase the effect of antiprotease and also increase phagocytosis and antibacterial effects [26].

Successful responses were already observed at the lower carotenoid dose. Theserum carotenoid levels required for immunomudulation in fish. This trial showed, that carotenoid supplement, protected non-specific and specific immune system in fish, and innunomodulatingdefence mechanismsinrainbow trout. In a research effects of marine algae *Dunaliellasalina*inc luding β-caroten and Red yeast *phafiarhodozyma* including astaxanthin on the non-specific defense mechanisms of rainbow trout ,by Amar *et al* [1] was indicated that carotenoid

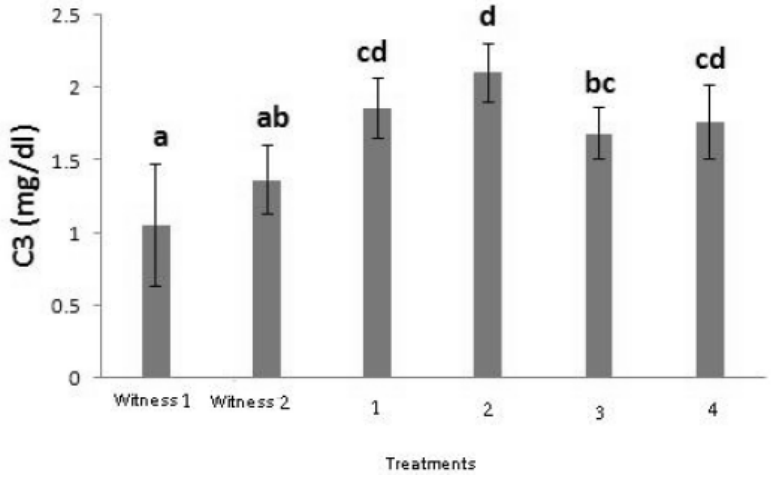

Fig. 1: Average changes of the amounts of complement C_3 and standard deviation of witness groups and treatments in the rainbow trout fed with diets containing different amounts of Dunaliellaaglae

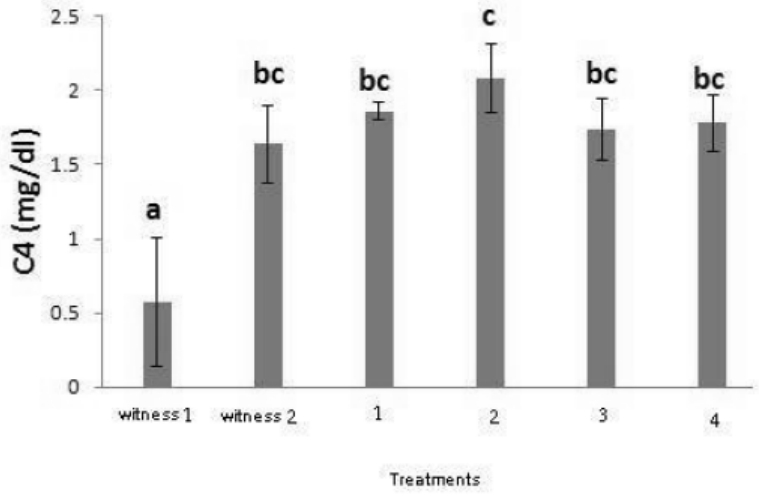

Fig. 2: Average changes of the amount of C_4 complement and standard deviations of witnesses and treatment groups in the rainbow trout fed with diets containing different amounts of Dunaliella algae.

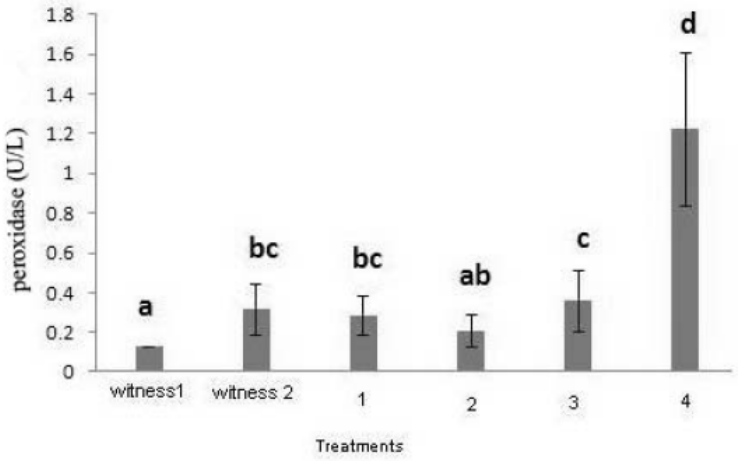

Fig. 3: Average changes of the amount of proxidase and standard deviations for witness and treatment groups for rainbow trout fed with diets containing different amounts of Dunaliella algae.

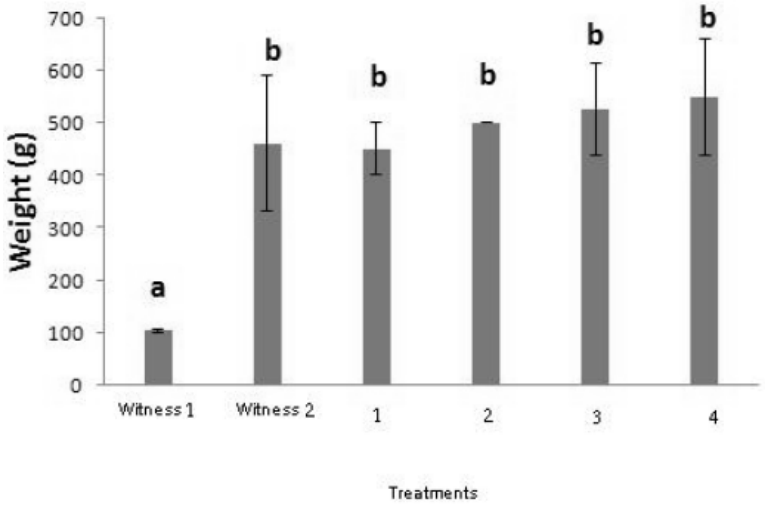

Fig. 4: Average weights and standard deviations of witnesses and treatments of rainbowtrout fed diets containing different amounts of Dunaliellaalgae.

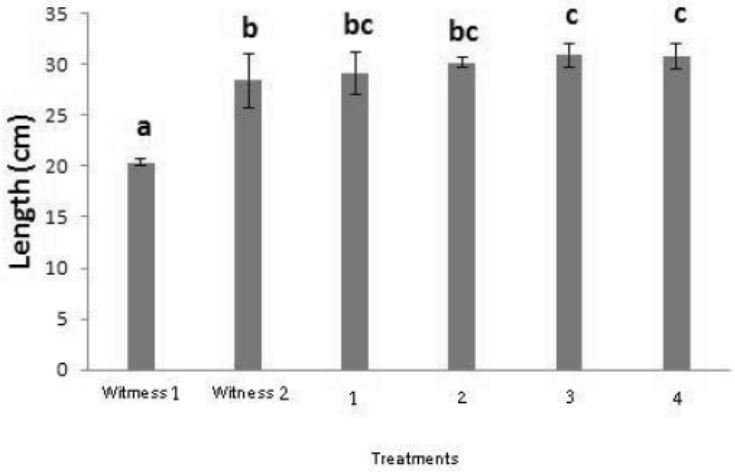

Fig. 5: Average length and standard deviations of witnesses and treatments of rainbowtrout fed diets containing different amounts of Dunaliella algae obtained results from condition factor indicated that, fish growth was similar in all treatment and no significant difference was observed comparing with witness groups ($P<0.05$).

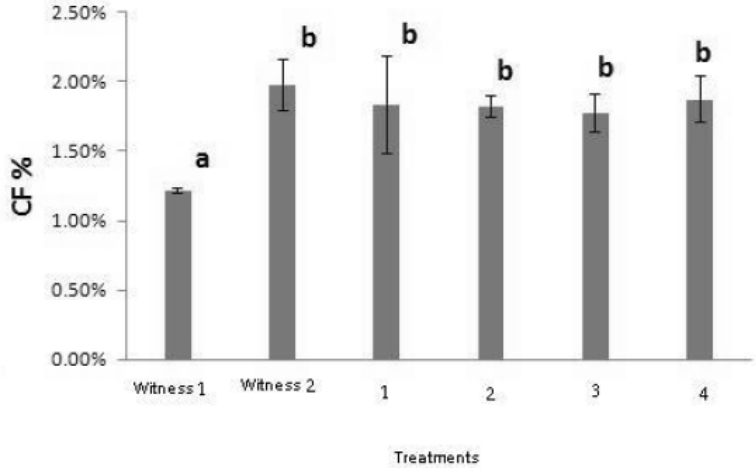

Fig. 6: Average condition factor and standard deviations of witnesses and treatments of rainbowtrout fed diets containing different amounts of Dunaliella algae.

Table 1: Composition of hatchery basic diet

Compositions	Percent
Crude protein	43
Crude fat	12
Fiber	2.8
Ash	6.8
Phosphate	0.9

Table 2: Average differences of C_3 and C_4 and proxidase in rainbow trout fed with diets containing different amounts of Dunaliella algae from start (witness 1) up to 3 months of culturing (witness 2 and 4 treatments)

Time	Treatments	C_3 in mg/dec L		C_4 in mg/dec L		Proxidase in mol/l	
		Average	S.D.	Average	S.D.	Average	S.D.
Start	Witness 1	1.04	0.42	0.57	0.42	0.12	0.001
	Witness 2	1.36	0.24	1.64	0.26	0.31	0.128
After 3 mouths	Treatment 1	1.86	0.2	1.86	0.05	0.28	0.097
	Treatment 2	2.1	0.2	2.08	0.22	0.2	0.0841
	Treatment 3	1.68	0.17	1.74	0.2	0.35	0.155
	Treatment 4	1.76	0.25	1.78	0.19	1.22	0.384

Table 3: Average weight and length differences and condition factor for trouts fed with diets containing Dunaliella algae at the beginning and end of experiment.

Measuring time	Treatments	Averages		Condition factor rate
		Weight in grams	Length in cm	Average
Beginning	Witness 1	102.6	20.6	1.22
	Witness 2	460	28.4	1.97
after 3 mouths	Treatment 1	450	29.2	1.83
	Treatment 2	500	30.2	1.82
	Treatment 3	526	30.9	1.77
	Treatment 4	550	30.74	1.87

pigments resulted serum complement and lysosyme activity increased and consequently total lococyte and phagocytosisincreased [1].

Complement components C_3, C_4 belong to the alpha-2 macroglobulin super family of thoidester containing proteins [7]. Many of multiple forms of C_3 are found in teleost fish, that are the products of different genes [22].

Functional studies shown that these. C_3 isoforms demonstrate different binding efficiencies to several complement activating surfaces that teleost fish have developed a novel strategy to augment the innate recognition and destruction of microbes.

C_4 plays an integral role in the activation of the classical and lectin pathways like factor B and C2, C_4 also within the MHC III region [12]. C_4 is a β-1 protein, that leds to the activation of C1s into 4a and C_4b. C_4b C2a interaction on each other and forms convert C_3 in classic pathway. C_4 molecules been cloned in several teleost species although the functional characterization of the C_4 protein has only been carried out in rainbow trout so far. Trout C_4 has been cloned and its primer sequence was found to possess the catalytic his residue within the PNPV/H motif, only with a single gene was found to encode trout C_4 [4].

Two C_4 proteins bound only in the increased, and if both molecules equally restored the classical pathway – mediated hemolytic activity of trout serum depleted of C_3 and C_4. Reconstitution of this hemolytic activity was dependent on the presence of both trout $C_{3\text{-}1}$ and $C_{4\text{-}1,}$ as well as on the presence of IgM bound to the target cells [4]. This studied was recognized, that increase amount of C_4 by reason of hemolytic activity, and amount of immunoglobulinsIg M bound to the target cell. Could at amount of this index effectively. Complement in fact is immune system high phase proteins. And their density almost changes after that death of tissue and necrosis cell. On the other hand ,change of level complement a response immune high phase, is sort of systemic action, that could related to temperamental immune respons and allergy [20].

Proxidase activity with increase of Dunaliella algaeand β-caroten increased. β-carotenis provitamin A and O2-suppressing ,by reason of could effectiveiy on immune system due to antioxidant properties and retinoid way . Increased amount of β-caroten suppresses rather than enhances O2- , which is consistent with the antioxidant function of β-caroten. Phagocytic cells produce O2- by the one-electron reduction of molecular O2, when plasma or membrane is stimulated and when carotenoids are present in these cells, O2- production is suppressed right at the early stages of the stimulation and carotenoids has possess free radical scavenging properties and therefore act as antioxidants [15].

β -caroten in rainbow trout diet decreased the susceptibility of the liver to lipid proxidation and strengthened the protective ability of the liver against oxidative stress, that ultimately enhanced bacterial killing and prevented damage to the cell membrane [16]. Studies on the mechanisms of phagocytic revealed that βcaroten enhances phagosome-lysosome fusion, a phenomenon associated with fluidity of the lysosomal membrane [18].

Druing phagocytic activation, there is a dynamic interaction between the subcellular membranes and the plasma membrane, ultimately resulting in the reorganization of the cellular membrane system [2]. In fact antioxidants protection of the phagocytosis cellularmembrane against free radicals. β -caroten is provitamin A and has antioxidant properties.

In addition, it has been suggestion that anti-infction effects of β-caroten have a close relationto antioxidant properties, because of increase resistant to infectious disease by pathogenic viruses and bacteria [26].

The antioxidant capacity that TAS expresses includes enzymatic and non-enzymatic antioxidant activities. The higher TAS value, the higher antioxidant capacity it has. Result this studied base of research nakano *et al* [16,17], was showen that β-caroten significantly decreased levels of lipid proxidase in the liver in rainbow trout [16,17] and fishes were feel with carotenoid supplement significantly altred the total lipid profile and hepatic mucopolysaccharide content [19]. In a research Lygren *et al* [13], was shown that in diet with high levels of Fat-soluble antioxidants such as astaxanthin and vitamin E, there was a reduced need for endogenous antioxidant enzymes, such as superoxidasdesmotaz in protection againts H2O2 and O2 respectively [13]. In the other trial accomplished in tiger prawn*penaeusmonodon*astaxanthin –fed ,ones had lower suproxidase dismutase than the control[5].

Paying attention to the obtain results, complement andproxidas level increase, indicated effects this algae on protect of immune system, increase resistant equally disease and the result survival increase in culture term of fish.

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