Egyptian Journal for Aquaculture
P-ISSN: 2090-7877
E-ISSN: 2636-3984
www.eja.journals.ekb.eg/
Nada *et al.,* 2022; 12 (2):01-17
DOI: 10.21608/eja.2022.92950.1061

Effect of *Chlorella vulgaris* on the Immunity and Pathogenicity of *Aeromonas sobria* in Nile tilapia

Nada O. Abbas[1]; Abdelfattah M. Ali[1]; Waleed F. Khalil[1]; Refaat M. Ali El-Gamal[2] and Heba A. Tolba[2]

[1]Pharmacology Department, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt.
[2]Central Laboratory for Aquaculture Research (CLAR) Agriculture Research Center (ARC), Abbassa, Egypt.

Received: March 29, 2022; Accepted: April 10, 2022 published: 2022 Vol.12 (2):01-16

ABSTRACT

Chlorella vulgaris is focused for its potential as immunostimulants against the infectious diseases of fish. The present study was planned to demonstrate the immunostimulant effect of *Chlorella vulgaris* and evaluate its effects on the resistance of Nile tilapia (*Oreochromis niloticus*) challenged with *Aeromonas sobria*. One hundred and twenty of Nile Tilapia were divided into four groups supplemented with diet incorporated with *Chlorella vulgaris* at levels of zero, 0.5, 1 and 1.5 gm/kg standard diet for 60 days. After the experimental period, results indicated that fish supplemented with *Chlorella vulgaris* produced significant increase in total white blood cell (WBC), lymphocyte & granulocyte counts, Phagocytic index with significant increase in serum lysozyme activity, total serum protein, albumin level, and globulin levels. In addition, *Chlorella vulgaris* produced 100% & 83% of protection in Nile tilapia infected with *Aeromonas sobria* when supplemented at levels of 1 & 1.5 gm/kg, respectively.

This study concluded that *Chlorella vulgaris* can be used as an immunostimulants in fish.

Key words: Immunostimulant, immunity, leucocyte, phagocytic index, lysozyme.

INTRODUCTION

Intensive production in aquaculture becomes troublesome, particularly with poor environmental conditions and poor husbandry practices, worldwide including Egypt. These conditions can be stressful and compromise the immune response and increase susceptibility to infection and disease (FAO,

2010; World Fish Centre, 2010; Zhang *et al* 2014).

Strategies for combating diseases in aquaculture include antibacterial, vaccination program and strengthening the innate immune responses of fish (Galindo-Villegas and Hosokawa 2004). Nowadays, Immunostimulants from natural origin was encouraged by Health Organizations. Of these, several plants are confirmed as immunostimulants that often act through targeting complement activation, phagocytosis and cytokines secretion (Tafalla *et al*., 2013).

Algae are green eukaryotic, normally found in the ponds, where it grows freely and can easily be harvested and re-grown. microalgae as Chlorella are a wide group of photosynthetic heterotrophic organisms consisting of vital amino acids, protein, minerals, vitamins, chlorophylls, carotenoid pigments, antioxidants, rich in n-3 long-chain polyunsaturated fatty acids (LC-PUFA) and other bioactive components involved in many physiological activities. (Yamaguchi, 1996; Kwak *et al.*, 2012, Takeuchi *et al.* 2002, Ortiz *et al.*, 2006 and Dawczynski *et al*., 2007 and Xu *et al*., 2014).

In aquaculture, algae are valuable and have been utilized as live feeds for larval or juvenile crustaceans and finfish and as a feed for zooplankton in aquaculture. It is also used as growth enhancers and immunostimulants (Muhammad et al., 2020), biofertilizers, bio-fuels, and the development of pharmaceuticals (Becker, 2004).

Chlorella vulgaris contains 10% minerals and vitamins, 5% fiber, 20% carbohydrates, 20% fat, and 45% protein. It has been reported that inclusion of *C. vulgaris* at different levels (20-80 g/kg) improved growth performance, anti-oxidant enzyme activity, lipid metabolism and resistance of M. rosenbergii post-larvae against *Aeromonas hydrophila* infection (Radhakrishnan *et al*., 2015, Rahimnejad *et al.*, 2016, Maliwat *et al.*, 2016 & Essam *et al.*, 2020). It is also known with its antibacterial, antiviral action and its ability to prevent toxin-caused oxidative stress and cellular damage (Rahimnejad *et al*., 2016).

So, this study was designed to evaluate the effect of *Chlorella vulgaris* as immunostimulant and its protection against Pathogenicity of *Aeromonas sobria* in Nile tilapia.

Materials & Methods

Experimental fish and Experimental diet:

Feed collection and preparation

Chlorella vulgaris powder from National Research Center.

Feed preparationFish diets

A commercial diet contained 30% protein was prepared with addition different concentration of *Chlorella vulgaris* powder as additives.

Experimental fish

Apparently healthy Nile tilapia (*O. niloticus*; total n=120) obtained from a local commercial fish farm with average body weight of 20 ± 5 g transported alive to the laboratory of Fish Diseases Dept., Central Laboratory for Aquaculture Research , El-Abbassa, Egypt. They were randomly distributed in 12 glass aquaria filled with de-chlorinated tap-water supplied with adequate aeration and under water internal power filters for 2 weeks under observation for acclimatization before the start of the experimental diet. Thirty percent of the water was weekly exchanged to maintain good water quality. They fed a commercial diet containing 30% crude protein twice daily.

Table (1): Ingredients and chemical analysis of the experimental diets (on dry matter basis) containing different component of *Chlorella vulgaris*:

Ingredients	G1	G2	G3	G4
Chlorella vulgaris:	0.00	0.50	1.00	1.50
Fish meal (HFM)	6	6	6	6
Soybean meal (SBM)	43.8	43.8	43.8	43.8
Ground corn (CNM)	21.3	21.3	21.3	21.3
Wheat bran (WB)	19.4	19.4	19.4	19.4
Cod fish oil	2.65	2.65	2.65	2.65
Corn oil	1.35	1.35	1.35	1.35
Vitamin's premix	1.5	1.5	1.5	1.5
Minerals Premix	1.5	1.5	1.5	1.5
Starch	2.5	2.0	1.5	1.0
Chemical analysis (%)				
Dry matter	91.01	91.01	91.01	91.01
Crude protein	30.21	30.21	30.21	30.21
Crude fat	3.48	3.48	3.48	3.48
Ash	8.65	8.65	8.65	8.65
Fiber	5.10	5.10	5.10	5.10
NFE	52.56	52.56	52.56	52.56
GE(Kcal/100g)	419.06	419.06	419.06	419.06
P/E ratio	72.08	72.08	72.08	72.08

2.1. *Chlorella vulgaris:*

Product name: *Chlorella vulgaris* powder obtained from National Research Center (NRC), department of algae, Dokki, Egypt.

1.1 Microbial strains:

Candida albicans and *Aeromonas Sobria* was kindly supplied by fish health and management department, Central Laboratory for Aquaculture Research, Abbassa, Sharkia, Egypt.

2.3. Fish and Experimental design.

One hundred and twenty of Nile Tilapia of 116 ± 1.7 gm initial weight were divided into four groups (30 fish each), each group distributed into 3 glass aquaria (70×60×50 cm) with a density of 10 individuals per aquarium. The fishes were kept in the laboratory and grown under lab condition for 7 days. The experimental fish were randomly allocated into four groups (n = 30 fish/group), and each group contained three replicates (10 fish/replicate). The first group (control G1) was fed on the basal diet without supplements. The second (G2), third (G3) and fourth (G4) groups were fed on basal diets supplemented with 0.5, 1 and 1.5 gm/kg of *Chlorella vulgaris*, respectively for 60 days. The fishes were allowed to feed with the commercial feed (table 1) twice a day in a range of 3% / body weight. Each aquarium was equipped with an aerator and one submersible water pump for water recalculating. During the experiment, the average of water temperature was 28˚C and pH 6.5-7.5.

2.4 Blood sample collection:

Blood samples were collected from the caudal vein of each fish from all the experimental groups at zero, 30 and 60 days from the experimental period. The first set of blood samples were collected using sterile syringes without anticoagulant, held at room temperature (22 ◦C) for 6 h, and centrifuged at 1000 rpm for 5 minutes (Lied *et al.,* 1975) to separate the serum. The serum was then used for measuring the immunological and biochemical markers. The second set of blood samples were collected in the EDTA-coated tubes to evaluate for WBC count and differential leukocyte count.

2.5. Total and differential leukocyte count:

Total leucocyte was counted by the method of Anderson and Siwicki, (1995). The blood sample was drawn using 0.5 scale pipette (a special pipette for leukocyte analysis), and Then, turk's solution was drawn until it reached the scale of 11 of the pipettes. The first droplet was contained on the haemocytometer (Newbauer chamber, Germany) and covered with a coverslip. The counting was done and the cells were counted in a haemocytometer viewed under fluorescent microscope (40X; Optika, Japan).

Differential leukocyte count was measured based on Ameri Mahabadi (2008) & Klontz (1972).

2.10 Phagocytic index (PI):

The procedure followed the method of Smith and Rommel, 1977. Briefly, X 100 Heat-killed C. albicans was added to white blood cells suspension at concentration of 50 yeast per cell. The mixture was incubated at 37°C for 15 min. then pelleted by centrifugation. Smears were made from the pellets and stained by Leishman's stain. Intracellular C. albicans cells were counted inside oil immersion.

Phagocytic percentage and phagocytic index were calculated according to this formula:

$$\text{Phagocytic \%} = \frac{\text{Number of phagocytic cells engulfing any No. of labeled } \textit{C. albicans}}{\text{Total number of phagocytic cells}} \text{X } 100$$

$$\textbf{P.I.} = \frac{\text{Total number of } \textit{C. albicans} \text{ in 100 phagocytic cells}}{100}$$

2.6. Lysozyme activity

The lysozyme activity was measured using Fig electric colorimeter with attachment for turbidity measurement. A series of dilution was prepared by diluting the standard lysozyme from hen egg-white (Fluka, Switzerland) and mixed with Micrococcus lysodeikticus (ATCC No. 1698 Sigma) suspension for establishing the calibration curve. Ten ml of standard solution or serum were added to 200 ml of Micrococcus suspension (35 mg of Micrococcus dry powder/95 ml of 1/15 M phosphate buffer 5.0 ml of NaCl solution). The changes in the extinction were measured at 546 nm by measuring the extinction immediately after adding the solution which contained the lysozyme (start of the reaction) and after a 20 min incubation of the preparation under investigation at 40˚C (end of the reaction) using ELISA reader (Bio TEC, ELX800G, USA) (Schaperclaus *et al.* 1992).

2.8. Challenge test:

At the end of the feeding experiment, the fishes of each groups were collected and randomly stocked at density level of 10 fish per 100-L tanks induplicates. The challenge test was carried out using *A. hydrophyla* which was isolated previously from fish health and management department, Central laboratory for aquaculture research, Agriculture

research center, preliminary challenge experiment was performed to determine the LD50 (lethal dose) of the pathogenic bacteria. Then, fish were challenged with pathogenic *A. sobria*. Where was grown on nutrient broth for 24 hr at30°C in an incubator, then centrifuged at 3,000 g for 30 min to collect bacterial cells form pellets. Which, were re suspended in 1.0 ml of 0.1% peptone water and using a sub lethal dose as recorded by **Schäperclaus (1992)** , the dose of IP injected was 0.1 ml of 24hr broth from virulent *A. hydrophila* (5 × 105 CFU/ml) .The fish group was IP injected with 0.1 ml of saline solution and considered as a negative control and all fish groups were IP injected, then, kept under observation for 10 days to record any abnormal clinical signs and recorded the daily fish mortality. *Aeromonas sobria* was reinsulated from liver, kidneys and spleen of the moribund and recently dead fish.

The relative percent of fish survival (RPFS) was calculated at 10 days post Relative percent of survival (RPS):

$$\text{RPS} = 1 - \frac{\%\ \text{immunized mortality}}{\%\ \text{control mortality}} \times 100$$

2.13 Statistical analyses:

The obtained data were subjected to one-way ANOVA. Differences between means were tested at the 5% probability level using Duncan's new multiple range test. All statistical analyses were done using the SPSS program V.10 (SPSS, Richmond, USA) as described by Dytham (1999).

Results

Effect of *C. vulgaris* on total and differential leukocyte count:

Oral administration of *C. vulgaris* produced significant increase in lymphocyte and granulocyte in G3 and G4 after 30 days and 60 days of administration to healthy *O. niloticus*, when compared with control group, G1) (table 2). Significant change was noticed only after 60 days of *C. vulgaris* administration for total WBC without any significant changes were observed in monocyte count in all experimental times.

Table 2. Effect of *Chlorella vulgaris* on total and differential leukocyte count in *O. niloticus*

Parameters	Experimental Time (month)	Experimental groups			
		G1	G2	G3	G4
Total WBCs	**Zero**	35.37±0.29 a	34.7±0.95 a	35.37±0.29a	35.37±0.29 a
Lymphocytes		73.83±1.39 a	74.07±1.29a	73.70±1.72 a	74.10±1.98 a
Monocytes		8.20±0.12 a	8.07±0.29 a	8.27±0.12 a	7.90±0.26 a
Granulocytes		6.20±0.35 a	6.13±0.38 a	6.37±0.20 a	6.10±0.30 a
Total WBCs	**one**	35.03±0.62 a	36.03±0.29a	36.37±0.34 a	36.37±0.34a
Lymphocytes		73.63±0.03 b	73.73±0.0b	77.40±1.87 a	78.73±0.6 a
Monocytes		8.53±0.35 a	8.53±0.35 a	8.87±0.44 a	8.60±0.06 a
Granulocytes		6.27±0.15 b	6.60±0.06 a	6.60±0.06 a	6.60±0.06 a
Total WBCs	**two**	35.03±0.62 b	36.03±0.3b	38.20±0.12 a	38.20±0.02 a
Lymphocytes		83.50±0.06 a	83.83±0.38a	82.30±0.12 b	80.90±0.06 c
Monocytes		9.20±0.12ab	9.37±0.09ab	9.10±0.06 b	9.47±0.09 a
Granulocytes		7.40±0.12 c	7.53±0.07 c	8.60±0.06 b	9.60±0.12 a

Data represents the mean & standard errors of 30 fishes per group. The different letter in the same raw means that there were significant changes at $p<0.05$. The first group (control G1) was fed on the basal diet without supplements. The second (G2), third (G3) and fourth (G4) groups were fed on basal diets supplemented with 0.5, 1 and 1.5 gm/kg of *Chlorella vulgaris*, respectively for 60 days.

Effect of *C. vulgaris* on Phagocytic activity:

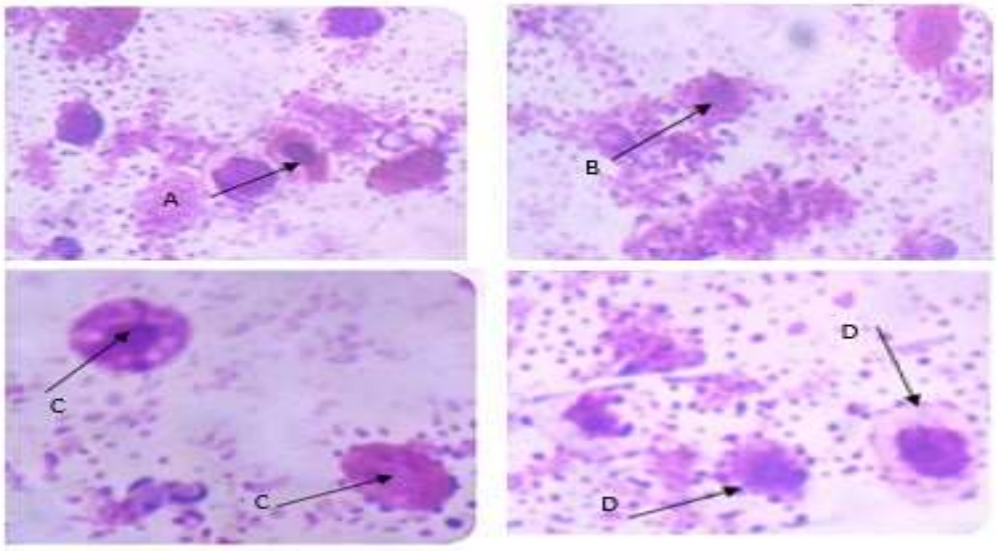
Oral administration of *C. vulgaris* produced significant increase in phagocytic activities represented by phagocytic percentage and phagocytic index in all treated groups after 30 and 60 days of administration, when compared with control group (table 3 and figure 1).

Table 3. Effect of *Chlorella vulgaris* on Phagocytic percentage and phagocytic index in *O. niloticus*

Parameters	Experimental Time (month)	Experimental groups			
		G1	G2	G3	G4
Phagocytic percentage	Zero	61.67±1.62 a	62.97±1.16 a	62.43±1.48 a	62.03±1.59 a
phagocytic index		3.30±0.15 a	3.20±0.153 a	3.267±0.186 a	3.13±0.145 a
Phagocytic percentage	one	60.00±0.06 c	63.73±0.32 b	62.00±0.12 b	74.00±1.10 a
phagocytic index		3.10±0.06 a	3.27±0.186 a	3.15±0.01 a	4.67±0.44 b
Phagocytic percentage	two	65.00±0.58 c	66.00±0.58 c	71.67±1.76 b	76.67±1.20 a
phagocytic index		3.50±0.06 c	3.54±0.058 c	4.53±0.338 b	5.47±0.34 a

Data represents the mean & standard errors of 30 fishes per group. The different letter in the same raw means that there were significant changes at $p<0.05$. The first group (control G1) was fed on the basal diet without supplements. The second (G2), third (G3) and fourth (G4) groups were fed on basal diets supplemented with 0.5, 1 and 1.5 gm/kg of *Chlorella vulgaris*, respectively for 60 days.

Figure 1: Yeast cells engulfment by phagocytic cells isolated from *O. niloticus* blood, fed on *Chlorella vulgaris* diet after two months



A: fish fed on standard diet free of c. vulgaris, B: fish fed on diet with c. vulgaris 0.5mg/Kg, C: fish fed on diet with c. vulgaris 1 mg/kg and D: fish fed on diet with c. vulgaris 1.5mg/kg.

Effect of *C. vulgaris* on Lysozyme activity:

Oral administration of *C. vulgaris* produced significant increase in Lysozyme activity in G3 and G4 after 30 days and 60 days of administration to healthy *O. niloticus*, when compared with control group, G1) (table 2 and figure 2).

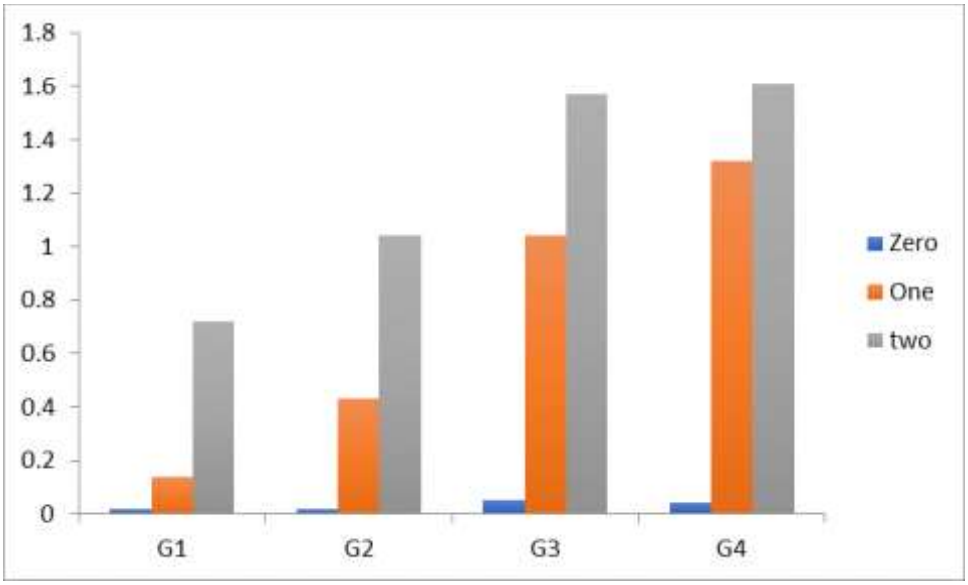
Table (2): Effect of *Chlorella vulgaris* on Serum lysozyme activity in *O. niloticus*

Experimental Time (month)	*G1*	*G2*	*G3*	*G4*
Zero	**0.02±0.001 e**	**0.02±0.001 e**	**0.05±0.001 e**	**0.04±0.02 e**
One	**0.14±0.03 e**	**0.43±0.23 de**	**1.04±0.29 bc**	**1.32±0.26 ab**
Two	**0.72±0.28 cd**	**1.04±0.09 bc**	**1.57±0.04 ab**	**1.61±0.25 a**

Data represents the mean & standard errors of 30 fishes per group. The different letter in the same raw means that there were significant changes at $p<0.05$. The first group (control G1) was fed on the basal diet without supplements. The second (G2), third (G3) and fourth (G4) groups were fed on

basal diets supplemented with 0.5, 1 and 1.5 gm/kg of *Chlorella vulgaris*, respectively for 60 day

Fig. 2: Effect of *Chlorella vulgaris* on Serum lysozyme activity in *O. niloticus*

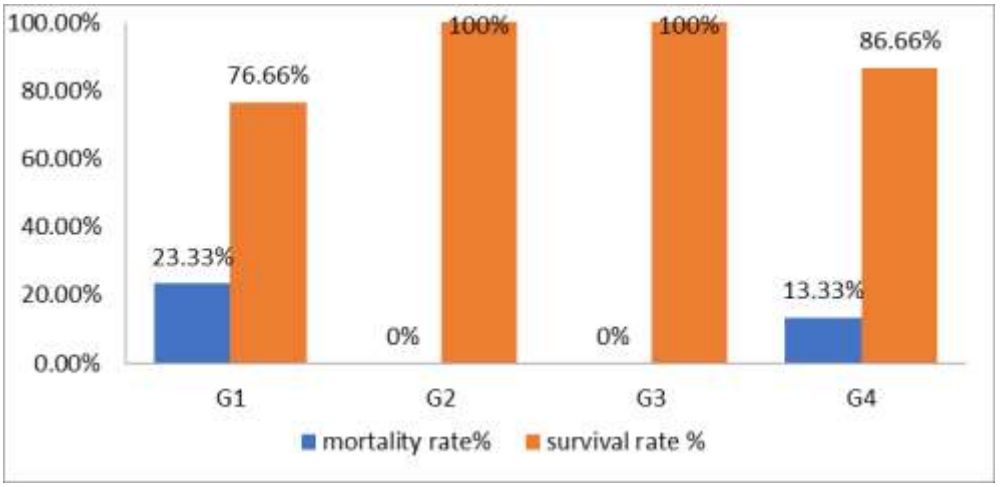


Data represents the mean & standard errors of 30 fishes per group. The different letter in the same column means that there were significant changes at $p<0.05$. The first group (control G1) was fed on the basal diet without supplements. The second (G2), third (G3) and fourth (G4) groups were fed on basal diets supplemented with 0.5, 1 and 1.5 gm/kg of *Chlorella vulgaris*, respectively for 60 days.

Effect of *C. vulgaris* on mortalities induced by *Aeromonas sobria* in *Oreochromis niloticus*:

Nile tilapia fed supplemented diet containing *C. vulgaris*, for two months showed high levels of protection against *A. Sobria*. The maximum Relative Level pf protection (RLP) (100 %) at a concentration levels of 0.5 gm & 1 gm compared to the control group. The protection was decreased to 83% when at 1.5 gm (figure 3).

Fig. 2. the effect of *Chlorella Vulgaris* on the mortality rate and relative level of protection (RLP) in *O. niloticus* challenged with *Aeromonas sobria*



Nile tilapia fed supplemented diet containing *C. vulgaris* at levels of zero (G1), 0.5 (G2), 1 (G3) and 1.5 (G3) for 60 days then challenged intraperitoneally with *Aeromonas sobria* and observed up to 8 days post-challenge. The first group (control G1) was fed on the basal diet without supplements. The second (G2), third (G3) and fourth (G4) groups were fed on basal diets supplemented with 0.5, 1 and 1.5 gm/kg of *Chlorella vulgaris*, respectively for 60 days.

Discussion:

Diseases epidemics associated with high death rates produces high economic fatalities to the aquaculture industry. Immunostimulants of synthetic or natural origin increase resistance to infectious diseases through enhancing non-specific immune mechanisms(Zhang *et al.* 2014).

Results of the present study showed that *C. vulgaris* produced immunostimulatory effects in *O. niloticus* through significant increase in total WBCs, lymphocytes and granulocytes. Different studies have been shown that microalgae such as *C. vulgaris* (Xu *et al.*, 2014), *Spirulina platensis* (Ibrahim et al., 2013), *Nannochloropsis oculata* (Yanuhar *et al.*, 2011) are effective agents in improving fish immune system. For instance, dietary supplementation of *Chlorella sp.* enhanced the innate immunity and antioxidant activity of gibel carp (Xu *et al*. 2014) when used at levels of

0.8−1.2%. In addition, it increased IgM, IgD, Interleukin-22 (IL-22) and chemokine levels when used at level of 0.4–1.2% of dietary supplementation as observed by Zhang *et al.* (2014). Moreover, dietary

supplementation of koi carp feed with *C. vulgaris* at 5% kg^{-1} enhances the fish hematological parameters with a significant increase in the levels of IgM and C4 complement of *Chlorella spp* (Khani *et al.*, 2017). They further observed that the microalga might be involved in regulating fish innate and adaptive immunity, immunostimulatory mediators and gene expression (Zhang *et al.* 2014); Khani *et al*., 2017). Such enhancement of WBCs may be 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, *Chlorella spp.* are the most widely accepted green microalgae used by many as a health food, for livestock and aquaculture feeds as well as in the drugs and cosmetics industries (Sharma *et al.* 2012).

Nowadays, many reports about the lysozyme activity have been used to evaluate health status in fish (innate immune system) when the fish live in different environmental conditions and is used to evaluate immunostimulants which were extracted from herbal plants (Tort *et al.* 2003; Dotta *et al*. 2014). The present findings also showed that *C. vulgaris* produced immunostimulatory effects in *O. niloticus* through significant increase in phagocytic percentage, phagocytic index and lysozyme activities in fish supplemented with *C. vulgaris*. It has been shown that *C. vulgaris* could be involved in the regulation of animal adaptive and innate immunity. For instance, Song (2010) found a positive role of *C. vulgaris* administration on the mucosal immunity of fish and could increase the expression of major histocompatibility complex class I of gilthead seabream (Cerezuela *et al.*, 2012) resulting in the stimulation of cytotoxic cells. Therefore, provide a stimulatory role for the fish immune status (Magnadottir 2006)

This research also found that the addition of *C. vulgaris* in fish feed was able to increase the resistance of *O. niloticus* to the *Aeromonus sobria* challenge pathogens because this material contains ingredients that can improve the immune system. According to Ke Ma *et al.,* (2020), *C. vulgaris* contains unique, diverse macro- and micro-nutrients, including omega-3 and omega-6 polyunsaturated fatty acids, proteins, polysaccharides, α-carotene, β-carotene, minerals, vitamins (C and E), pro-vitamins, chlorophyll and lutein (Panahi *et al*., 2016; Buono *et al.,* 2014; Miranda *et al.,* 2001).

Conclusions.

The present study found that supplementation of *C. vulgaris* into fish feed improved resistance of fish against *Aeromonus sobria* infection. The highest relative survival percent was achieved in fish fed with the addition

of *C. vulgaris* of 1 g/kg of feed while at higher doses, survival appeared to decrease.

References:

Amend, D.F. (1981): Potency Testing of Fish Vaccines. Fish Biologics: Serodiagnostics and Vaccines, 49, 447-454.

Ameri Mahabadi, A. (2008): Veterinary Hematological Laboratory Methods. Tehran university press. 126 p.

Anderson, D.P. and Siwicki, A.K. (1995): Basic haematology and serology for fish health programs. In: Shariff M, Subasinghe JRP (Eds.), Diseases in Asian Aquaculture *II*. Fish Health Section, Asian Fisheries Society, Manila, Philippines, pp. 185-202.

Becker, E.W. (2004): Microalgae in human and animal nutrition, in Handbook of Microalgae Culture. A. Richmond, ed. Blackwell, Oxford pp 312-215.

Buono, S., Langellotti, A.L., Martello, A., Rinna, F.and Fogliano, V. (2014): Functional ingredients from microalgae. Food Funct., 5:1669-1685.

Cerezuela, R.; Guardiola, F.A.; Meseguer, J. and Esteban, M.Á. (2012): Enrichment of gilthead seabream (*Sparus aurata L.*) diet with microalgae: effects on the immune system. Fish Physiology and Biochemistry 38: 1729-1739.

Dawczynski, C., Schubert, R. and Jahreis, G. **(2007):** Amino acids, fatty acids, and dietary fiber in edible seaweed products. Food Chem., 103, 891–899.

Dotta G, Andrade JIA, Gonçalves ELT, Brum A, Mattos JJ, Maraschin M, Martins ML (2014): Leukocyte phagocytosis and lysozyme activity in Nile tilapia fed supplemented diet with natural extracts of propolis and Aloe barbadensis. Fish & Shellfish Immunology 39:280–284

Dytham, C. (1999): Choosing and Using Statistics: A Biologist's Guide. Blackwell Science Ltd., London, UK. Pp. 147.

Essam, M.A., El -Sayed, B.M., Mahsoub, Y.H., El- Murr,A.I. and Neamet-Allah, A.N.F. (2020): Effect of *Chlorella vulgaris* enriched diet on growth performance, hemato-immunological responses, antioxidant and transcriptomics profiles disorders caused by deltamethrin toxicity in Nile tilapia (*Oreochromis niloticus*). Fish & Shellfish Immunology Volume 102 p 422-429.

FAO (2010): Aquaculture development. 4. Ecosystem approach to aquaculture. FAO Technical Guidelines for Responsible Fisheries. No. 5, Suppl. 4. Rome, FAO. 53p.

Galindo-Villegas, J. and Hosokawa, H. (2004): Immunostimulants: towards temporary prevention of diseases in marine fish. Internationale de Nutricion Acuı´cola, vols 16–19, pp 279–319.

Ibrahim, M.D., Mohamed, M.F. and Ibrahim, M.A. (2013): The role of Spirulina platensis (Arthrospira platensis) in growth and immunity of Nile tilapia (Oreochromis niloticus) and its resistance to bacterial infection. Journal of Agricultural Science, 5(6), 109-117.

Ke Ma, Qiuwen Bao, Yue Wu, Siwei Chen, Shuxin Zhao, Haizhen Wu and Jianhua Fan (2020): Evaluation of Microalgae as Immunostimulants and Recombinant Vaccines for Diseases Prevention and Control in Aquaculture. Frontiers in Bioengineering and Biotechnology. Vol. 8: article 590431.

Khani,M., Soltani M., Shamsaie, M., Foroudif, F. and Ghaenim, M. (2017): The effect of *Chlorella vulgaris* (Chlorophyta, Volvocales) microalga on some hematological and immune system parameters of Koi carp (*Cyprinus carpio*). Iran. J. Ichthyol. 4(1): 62–68.

Klontz, G. W. (1972): Haematological techniques and the immune response in rain bow trout. In; Disease of fish (Ed. Mawdeskley-Thomas, L.E.) Symposium of Zoological Society London No 30 New York and London Academic Press.

Kwak, J., Baek, S., Woo, Y., Han, J., Kim, B., Kim, O. and Lee, J. (2012): Beneficial immunostimulatory effect of short-term *Chlorella* supplementation: enhancement of Natural Killer cell activity and early inflammatory response (randomized, double-blinded, placebo-controlled trial). Nutrition Journal, 11(53): 1-8.

Lied, E., Gezrde Z. and Braskhan O.R. (1975): Simple and rapid technique for repeated blood sampling in rainbow trout .J. Fish Res. Board of Canada, 32 (5): 699-701.

Magnadottir, B. (2006): Innate immunity of fish (overview), Fish Shellfish Immunol. 20 137–151.

Maliwat, G.C., Velasquez, S., Robil, J.L., Chan, M., Traifalgar, R.F., Tayamen, M. and Ragaza, J.A. (2016): Growth and immune response of giant freshwater prawn *Macrobrachium rosenbergii* (De Man) post larvae fed diets containing *Chlorella vulgaris* (Beijerinck).Aquaculture Research, 48:1666-1676.

Miranda M.S. , S. Sato, J. Mancini-Filho (2001): Antioxidant activity of the microalga Chlorella vulgaris cultered on special conditions. Boll. Chim. Farm., 140, pp. 165-168.

Montero–Rocha, A.; Mcintosh, D.; Sanchez-Merino, R. and Flores, I., (2005): Immunostimulation of white shrimp Litopenaeus vannamei) following dietary administration of (Ergosan. Journal of Invertebrate Pathology 91: 188-194.

Muhammad, A. T., Shariff, M., Yusoff, F.M., Goh, Y.M. and Banerjee, S. (2020): Applications of microalga *Chlorella vulgaris* in aquaculture. Reviews in Aquaculture. 12, 328–346.

Ortiz, J., Romero, N., Robert, P., Araya, J., Lopez-Hernández, J., Bozzo, C., Navarretea, E., Osorioa, A. and Riosa, A. (2006): Dietary fiber, amino acid, fatty acid and tocopherol contents of the edible seaweeds Ulva lactuca and Durvillaea antarctica. Food Chem., 99, 98–104.

Panahi Y. , B. Darvishi, N. Jowzi, F. Beiraghdar, A. Sahebkar (2016): Chlorella vulgaris: a multifunctional dietary supplement with diverse medicinal properties. Curr. Pharmaceut. Des., 22, pp. 164-173.

Radhakrichnan, C., Caravana Bhavan, P., Seenivasan, C and Muralisankar, T. (2015): Effect of dietary replacement of fishmeal with *Chlorella Vulgaris* on growth performance energy utilization and digestive enzymes in *Macrobrachium rosenbergii* post larvae International Journal of Fisheries Aquaculture, 7: 62-70.

Rahimnejad, S., Bark, H.J. and Lee, S.M. (2016): Effects of dietary inclusion of *Chlorella Vulgaris* on growth, blood biochemical parameters, and antioxidant enzyme activity in olive flounder, *Paralichthys olivaceus*. Journal of the World Aquaculture Society, 48: 103-107.

Schaperclaus, W., Kulow, H. and Schreckenbach, K. (1992): Fish disease. Rotterdam, the Netherlands: A.A. Balkema. 101–105.

Sharma R, Singh GP, Sharma VK (2012): Effects of culture conditions on growth and biochemical profile of Chlorella. Journal of Plant Pathology & Microbiology 3: 1–6.

Smith, D.L. and Rommel, F. (1977): A rapid micromethod for the simultaneous determination of phagocytic-microbiocidal activity of human peripheral blood leukocytes in vitro. J Immunol Methods 17:241-247.

Tafalla C., Jarl Bøgwaldb , Roy A. and Dalmo b, (2013): Adjuvants and immunostimulants in fish vaccines: Current knowledge and future perspectives. Fish & Shellfish Immunology, 35(6):1740-50.

Takeuchi, R., Yun, S., and Russell, J. E. A. (2002): Antecedents and consequences of the perceived adjustment of Japanese expatriates in the USA. International Journal of Human Resource Management.

Tort L, Balasch JC, Mackenzie S (2003): Fish immune system. A crossroads between innate and adaptive responses. Imunologia 22:277– 286

World Fish Centre (2010): Barriers to Aquaculture Development as a Pathway to Poverty Alleviation and Food Security. Presentation by M. Beveridge, M. Phillips, P. Dugan and R. Brummett to the OECD Workshop, Paris, 12–16.

Xu, W., Gao, Qi, Zh., Qiu, Zh., Peng, M.J. and Shao, R., (2014): Effect of dietary chlorella on the growth performance and physiological parameters of gibel carp, Carassius auratus gibelio. Turkish Journal of Fisheries and Aquatic Sciences, 14, 53-57.

Yamaguchi, K. (1996): Recent advance in micro-algae bioscience in Japan, with special reference to utilization of biomass and metabolites: a review. Journal of Applied Phycology, 8(6): 487-490.

Yanhuar, U., Nurdiani, R. and Hertika, A.M.S. (2011): Potency of Nannochloropsis

oculata as antibacterial, antioxidant and antiviral on humpback grouper infected by Vibrion alginolyticus and Viral Nervous Necrotic. Journal of Food science and Engineering, 1, 323-330.

Zhang, Q., Giu, M., Xu, W., Gao, Z., Shao, R. and Qi, Z., (2014): Effects of dietary administration of *Chlorella* on the immune status of Gibel carp, *Carassius auratus* gibelio. Italian Journal of Animal Science, 13, 653–656.