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Immunomodulatory Role of Dietary *Chlorella vulgaris* against *Aeromonas hydrophila* Infection in the Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

The current study investigated the protective role of *Chlorella vulgaris* against *Aeromonas hydrophila* infection in Nile tilapia. A total of 135 *Orechromus niloticus* fish were divided into three groups in tri-replicates. The first control group was fed a basal diet, the second group (Ch 3%) fed a diet enriched with 3% *C. vulgaris*, and the last group (Ch 6%) fed a 6% *C. vulgaris* diet. Along with the feeding trial, the haematological, biochemical, immunological, and histopathological parameters were comparatively evaluated, and after 6 weeks, the challenge against *A. hydrophila* was conducted. The erythrocyte count and Hb level remained unchanged, while the leukogram profile, serum lysozyme activity, and respiratory burst activity showed significant upregulation in *Chlorella* fed groups compared with the control group. In addition, the levels of hepato-renal biomarkers were significantly unchanged compared to the control group. Furthermore, the lower *Chlorella* diet showed prominent effects on maintaining these biomarkers. The survival rate was estimated after the challenge with *A. hydrophila*. *Chlorella*-treated fish groups, Ch6% and Ch3%, exhibited a better survival rate of 100% and 93.33%, respectively, compared with the control (6.67%). In conclusion, this study recommended incorporating *C. vulgaris* into tilapia diets to improve their immunity and disease resistance power.

INTRODUCTION

The Nile tilapia (*Oreochromis niloticus*) is the most important farming species dating back to the ancient Egyptian eras, establishing Egypt as one of the leading countries in tilapia aquaculture and production worldwide, after China and Indonesia, followed by Thailand, Bangladesh, and the Philippines (FAO, 2020).

The fish culture systems have become more intensive in order to raise fish production. Aquaculture, like any other animal-producing industry, faces complex constraints. Among these challenges, infectious diseases account for the lion's share, causing billions of dollars losses yearly (Assefa and Abunna, 2018), particularly bacterial

pathogens that cause high fish mortalities, resulting in massive economic losses (Hassan *et al*., 2020). The genus Aeromonas leads the infectious bacterial diseases triggering outbreaks in freshwater fish, moreover Aeromonas hydrophila is the predominant pathogen implicated in tilapia outbreaks, causing food insecurity and economic loss worldwide (Aboyadak *et al*., 2015).

Antibiotic consumption is another constraint in this sector (El-Habashi *et al*., 2019). Antibiotics not only endanger fish through drug residues, but they also endanger humans and the environment through antibiotic-resistant pathogens (Desbois *et al*., 2021). Therefore, microalgae such as chlorella vulgais with its antimicrobial activity (Pratt *et al*., 1944; Natrah *et al*., 2014) have emerged as a top priority in the search for other eco-friendly control alternatives to mitigate chemical medication in fish farming. Numerous beneficial effects of microalgae as Chlorella supplementation have been reported, including promote growth in carp (*Labeo rohita*) and tilapia (*Oreochromis niloticus*), stimulate immunity in rainbow trout (*Oncorhynchus mykiss*), and enhance reproductive performance in yellow tail cichlid (*Pseudotropheus acei*) (Alagawany *et al*., 2021).

Chlorella vulgaris is a commercially important species with a good bio-molecular composition and a high economic potential that is extensively used in aquaculture (Ahmad *et al*., 2020). Because of its pigment and protein content, it is currently used as a healthy food supplement with valuable content, as well as for medicinal purposes. Several studies have looked into the Chlorella effect as a growth promoter and its role in protecting against water toxicity from harmful chemical materials (Zahran *et al*., 2019; Mahmoud *et al*., 2020; Abdelhamid *et al*., 2020), but little is known about the antibacterial activity and immune enhancement to prevent and control bacterial fish pathogens in tilapia aquaculture.

Ultimately, if we are to ensure the sustainability of aquaculture, we must conduct subsequent research aimed at the development of natural, harmless medications against various pathogenic bacteria. Therefore, the aim of this study was to investigate the role of Chlorella vulgaris in fish immune response and resistance to pathogenic *A. hydrophila* using bacteriological, haematological, immunological, and histopathological techniques.

MATERIALS AND METHODS

Chlorella vulgaris

A dried powder of *Chlorella vulgaris* was purchased from the Institute of National Research Centre, Cairo, Egypt.

Bactericidal assay

The antimicrobial properties of *Chlorella vulgaris* were tested against pathogenic *A. hydrophila* by disc diffusion method as described by Hudzicki (2009). One mL of fresh bacterial culture (at a concentration equivalent to 0.5 McFarland) was pipetted in the centre of sterile Petri dish. Molten cooled Muller Hinton agar (MHA) was then poured into the Petri dish containing the inoculum and mixed well. Upon solidification, sterile

paper disks (5 mm in diameter), that inoculated with 10 µL of algal suspension on the agar surface. Then, the plates were incubated at 28°C for 18h. Antimicrobial activity was detected by measuring the zone of clearance (Inhibitory zone) appeared after the incubation period.

Diet preparation

Three dietary regimes were prepared: a control (basal feed), a basal feed with 3% and a basal feed with 6% C. vulgaris, respectively. The Chlorella supplemented diets were prepared according to Toften and Jobling (1997). A dried C. vulgaris powder mixed with sterile distilled water before combining with the basal diet obtained from T. Skretting AS (Egypt). The diets were made by spraying a Chlorella water suspension onto the surface of the feed pellets and drying them in a cool, dry place.

Table 1. The experimental diets' ingredient (air dried basis, %).

Ingredients	**Control feed (Ch 0%)**	***Chlorella* 1 (Ch 3%)**	***Chlorella* 2 (Ch 6%)**
Rice bran	7.85	7.85	7.85
Soybean meal	52.9	52.9	52.9
Soybean oil	4.00	4.00	4.00
Wheat bran	5.00	5.00	5.00
Ground corn	29.1	26.1	23.1
C. vulgaris	0	3	6
Mono-calcium phosphate	1.00	1.00	1.00
Food salt	0.07	0.07	0.07
Mineral & Vitamins Premix	0.08	0.08	0.08
Chemical analysis (%)			
Crude protein	30	30	30
Crude fats	6.1	6.1	6.1
Crude fibres	4.8	4.8	4.8

Fish maintenance and experimental design

The Nile tilapia (*Oreochromis niloticus*) used in the experiment (average body weight of 20-30 g) were obtained from the Suez Canal University's Fish Farming and Technology Institute and kept in well-aerated aquarium tanks with 30% of water changed daily. Prior to the experiment, the fish were acclimated for 3 weeks and fed a control basal diet twice daily ad libitum. During this time, no disease signs or deaths were recorded (Aly *et al*., 2021). One hundred thirty-five Nile tilapia were distributed into three equal groups, with triplicate tanks assigned to each dietary treatment (Fig. 1). During the experiment, water was changed daily at an 50 % rate, as well as the water temperature was kept at 22°C. For 6 weeks, fish were fed ad libitum twice daily at a rate of 3% body weight. To reduce ammonia levels and maintain water quality, faeces and other waste materials were syphoned off daily.

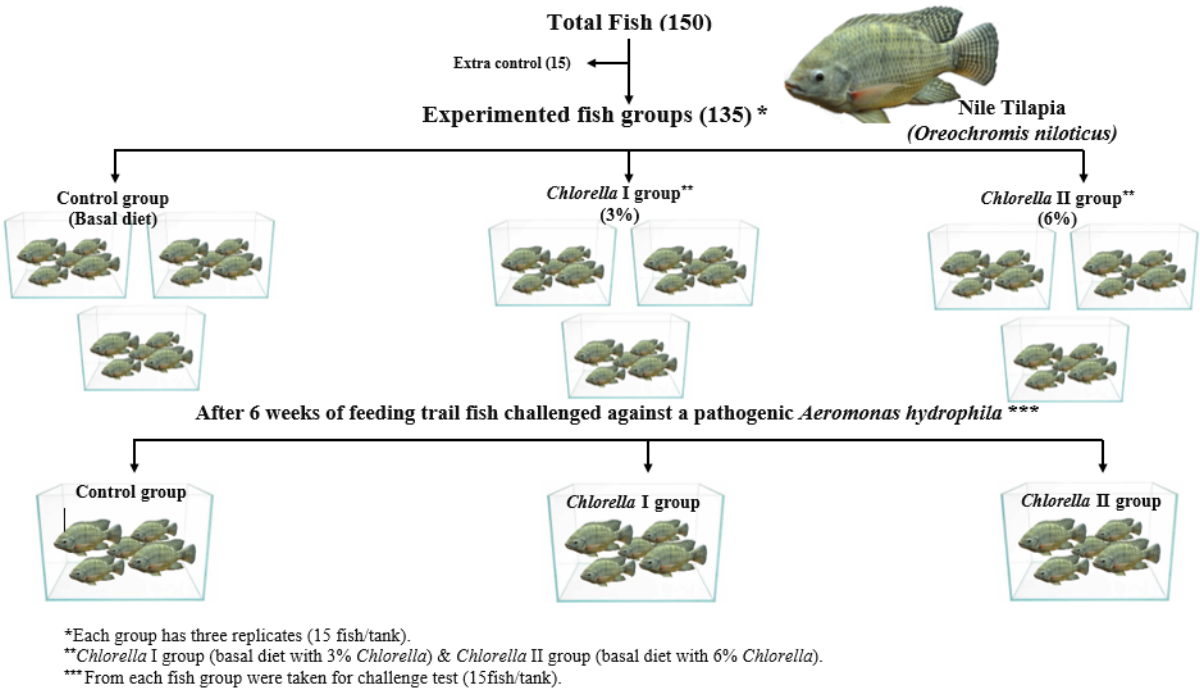
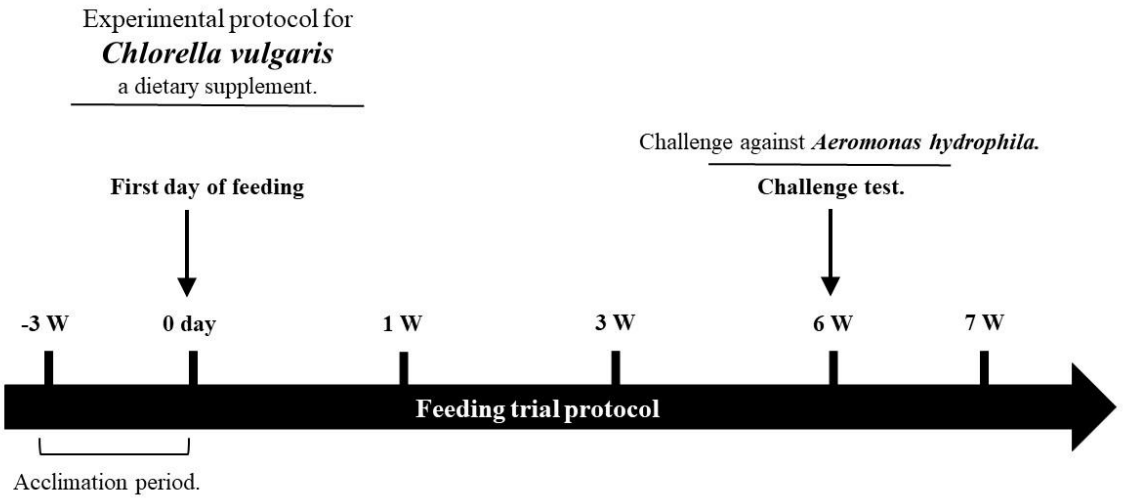


Fig. 1. The experimental design. The diagram reveals the experimental fish trails along the workflow.

Sample collection

The experimented fish from each aquarium (9 fish/group) were sampled at 1 and 3 weeks of feeding experiment and at 10 days post challenged with pathogenic *A. hydrophila* strain (Fig. 2).

Fish were anaesthetized with clove oil solution (75 mgL^{-1}) via immersion before collecting blood and serum samples, then euthanized for histopathological tissue samples (Tancredo *et al*., 2015). Caudal vein puncture was used to obtain blood samples, which were immediately transferred into 1.5 mL sterile Eppendorf tubes using 3 mL/CC sterile syringes (23-gauge needle X 11/4m). A portion of the blood was collected in 20 µL/mL blood dipotassium salt of EDTA Eppendorf tubes for haematological and respiratory burst activity. While blood samples for biochemical and other immunological parameters were collected in sterile centrifuge tubes without anticoagulant, allowed to clot at 4^{o}C for 4 h, then centrifuged at 3000 *xg* for 10 min, clear serum samples were carefully decanted and stored at –20^{o}C until analysis.



Blood, serum and tissue samples were taken at (1, 3 weeks and 10 days after challenge) for hematological, immunology and histopathogical analysis.

Fig. 2. A simplified figure shows the experimental feeding protocol and sample collection in Nile tilapia groups.
Blood, serum, and tissue samples were collected at 1, 3, and 10 days post challenge. After 6 weeks, the experimental fish were exposed to a pathogenic strain of *A. hydrophila*.

Haematological examination

Blood samples were collected from nine fish per treatment (three fish/ aquaria) then total erythrocytes (10^6/µL–1), total leukocytes (10^3/µL–1), and haemoglobin concentration (g/dL) were done following standard procedure (Houston, 1990).

Serum biochemical parameters:

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, as well as urea and creatinine levels were determined colorimetrically according to Reitman and Frankel (1957).

Determination of immunological parameters

Serum lysozyme activity

Lysozyme activity was performed as described by (Ellis, 1990), based on the lysis of *Micrococcus lysodeikticus* (Sigma Chemical Co). A 0.25 mL of serum mixed with 0.75 mL suspension of *M. lysodeikticus* (0.2 mg/mL in 0.05 M PBS, pH 6.2). At 25°C, the mixture reacted for 5 min, then the optical density was measured at 1 min intervals for 5 min at 540 nm (5010, Photometer, BM Co. Germany). The serum lysozyme activity was calculated using a calibration curve constructed using dilutions of lyophilized chicken egg-white lysozyme (Sigma, Germany) ranging from 2.0 to 20.0 µg/mL

Respiratory burst activity (RBA)

A 50 µL of blood was added to microliter plate wells then 50 µL of 0.2% nitroblue tetrazolium (NBT) solution was added to each well and after that incubated at room temperature for 30 min. A 50 µL of the NBT blood cell suspension was added to 1 mL N, N-dimethyl formamide (Sigma) and centrifuged at 3000 *xg* for 5 min. The supernatant was measured in a spectrophotometer at optical density (OD) of 620 nm (GENG *et al*., 2012; Aly *et al*., 2021).

Challenge test

A pathogenic *Aeromonas hydrophila* strain was isolated previously from Nile tilapia and kindly obtained from the Aquaculture Diseases Control department, Fish farming and Technology Institute, Suez Canal University, Egypt. The lethal dose (LD_{50}) of *A. hydrophila* for Nile tilapia was determined; whereas, fish were intraperitoneally (*IP*) injected with different doses of 24-h aged bacteria and fish mortality was observed for 5 days post infection (Yonar *et al*., 2019). The LD_{50} which resulted in 50% fish mortality was 3×10^8 CFU/mL that was used for the bacterial challenge.

At the end of the feeding treatment fifteen fish from each group were distributed in separated tanks (Fig. 1) and then injected intraperitoneally with 0.5 mL/fish of the virulent *A. hydrophila* strain at a dose of 3×10^8 CFU/mL (Vasudeva Rao *et al*., 2006). Fish were kept under observation for 10 days to record any mortality. The survival rates and the protection (expressed as relative percent survival, RPS) of treated Nile tilapia were evaluated according to the following formula described by Amend (1981).

RPS = 1–[mortalities of experimented fish / mortalities of control fish] X100.

Histopathological examination

Tissue specimens from visceral organs (liver, kidney, and intestine) of experimentally treated fish, and also from challenged fish groups, were collected and immediately fixed in neutral buffered 10% formalin for histological analysis as described by Bancroft and Gamble (2008).

Ethical statement

This study was carried out following strict guidelines for the use of laboratory animals. All experimental techniques followed Suez Canal University's Animal Care and Use Guidelines and were endorsed by the local Administrative Panel on the Laboratory Animal Care Committee.

Statistical analysis

The collecting values were analysed with one-way analysis of variance, ANOVA (SPSS 25.0, SPSS Inc., USA)(IBM, 2017) followed by Duncan's test. The data are expressed as means ± standard errors (SE). The P value < 0.05 was considered as statistically significant.

RESULTS

Haematological examination

At the 1st week, RBCs count and Hb concentrations within *Chlorella* fed fish unsignificantly changed compared with the control fish ($P < 0.05$). While at 3rd week, RBCs count remain in normal values unlike the Hb level that significantly increase in Ch 6% group in compare with the control group. Meanwhile, after infection RBCs counts and Hb level significantly decreased in the control group compared with the Chlorella treated groups ($P < 0.05$) that still in the normal levels.

Additionally, the total leukocytic count (TLC) was significantly elevated in all *Chlorella* supplemented groups compared with the control group along the feeding period. Moreover, after challenge TLC was remaining significantly raised unlike the control group (Table 2).

Table 2. Total RBCS count (10^6/µL), Hb content (g/dL), and total leukocytes count (10^3/µL) among Nile tilapia experimented groups in relation to control group along the experiment (Mean ± SE).

Groups	Before challenge						After challenge		
	1st week			3rd week					
	RBC_S (10^6/µL)	**Hb (g/dL)**	**TLC (10^3/µL)**	**RBC_S (10^6/µL)**	**Hb content (g/dL)**	**TLC (10^3/µL)**	**RBC_S (10^6/µL)**	**Hb (g/dL)**	**TLC (10^3/µL)**
Control	1.1±0.2[a]	5.53±0.7[a]	37.28±3.7[b]	1.07±0.2[a]	4.05±0.3[b]	38.80±5.9[b]	0.82±0.1[b]	3.85±0.3[c]	35.35±0.9[b]
Ch. 3%	1.21±0.2[a]	6.23±0.7[a]	61.9±3.7[a]	1.33±0.2[a]	5.06±0.4[ab]	65.47±3.6[a]	1.54±0.2[a]	7.30±0.4[a]	57.90±0.7[a]
Ch. 6%	1.01±0.1[a]	4.47±0.4[a]	61.29±4.7[a]	1.50±0.1[a]	6.56±0.7[a]	76.26±5.2[a]	1.27±0.1[a]	6.15±0.3[b]	58.45±0.7[a]

Different small letters in each column showed significant differences between groups ($P < 0.05$).

Biochemical parameters

Liver function parameters

Serum ALT and AST activities at first week of feeding experiment unsignificantly altered within experimented groups. After 3 weeks, ALT activity slightly upregulated in Chlorella fed groups compared with the control group ($P < 0.05$) and AST activity statistically unchanged among groups. Meanwhile after infection a significant elevation in serum aminotransferases activities was noticed in the control group dissimilar to the Chlorella supplemented groups (Table 3).

Table 3. Serum ALT (U/L) & AST (U/L) activities in experimented Nile tilapia groups in relation to control group along the experiment (Mean±SE).

Groups	Before challenge				After challenge	
	1st week		3rd week			
	ALT (U/L)	AST (U/L)	ALT (U/L)	AST (U/L)	ALT (U/L)	AST (U/L)
Control	14.65±0.9[a]	40.21±1.6[a]	13.87±0.7[b]	38.62±2.1[a]	22.28±0.2[a]	66.38±0.5[a]
Ch. 3%	14.53±1.4[a]	40.95±2.1[a]	19.06±1.6[a]	45.24±5.7[a]	17.10±1.1[c]	47.78±2.9[b]
Ch. 6%	13.44±1.3[a]	32.95±3.6[a]	19.71±0.6[a]	47.78±2.9[a]	19.95±0.7[b]	50.66±0.4[b]

Different small letters in each column showed significant differences between groups (P <0.05).

Renal function parameters

The results showed no significant difference among kidney biomarkers, such as serum creatinine and urea at 1st week of feeding; while, after 3 weeks the creatinine level significantly increased in the control group unlike the Ch.3% group and the urea level remain unchanged. Meanwhile, after challenge creatinine level within Ch.6% group exhibited significant elevation compared with the other groups, moreover the urea level slightly raised in Ch.3% group in compare to the control group (Table 4).

Table 1. Renal biomarkers, creatinine (mg/dL) & urea (mg/dL) levels among experimented Nile tilapia groups in relation to control group along the experiment (Mean ±SE).

Groups	Before challenge				After challenge	
	1st week		3rd week			
	Creatinine (mg/dL)	Urea (mg/dL)	Creatinine (mg/dL)	Urea (mg/dL)	Creatinine (mg/dL)	Urea (mg/dL)
Control	0.21±0.0[a]	15.31±0.5[a]	0.33±0[a]	14.48±0[a]	0.24±0[b]	14.55±0[b]
Ch. 3%	0.20±0.0[a]	16.65±0.6[a]	0.24±0[b]	15.30±0.6[a]	0.24±0[b]	14.80±0[a]
Ch. 6%	0.20±0.0[a]	16.82±0.4[a]	0.26±0[ab]	14.02±0.8[a]	0.28±0[a]	14.75±0.1[ab]

Different small letters in each column showed significant differences between groups (P <0.05).

Immune parameters

Chlorella supplement to the Nile tilapia diet revealed significant higher serum lysozyme activity as well as respiratory burst activity than the control non-supplemented group ($p < 0.05$) (Fig. 3-4).

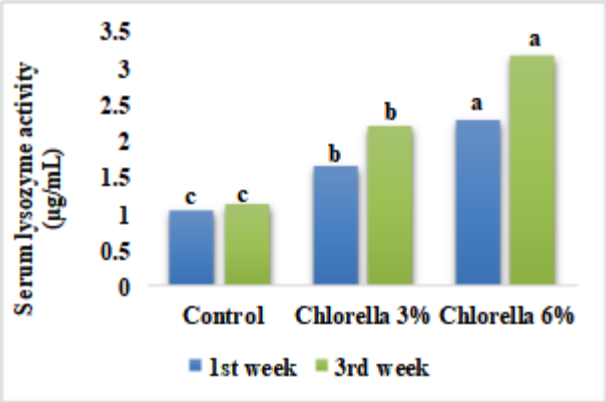


Fig.3. Serum lysozyme activity in experimented Nile tilapia fed Chlorella diet along the experiment. Data is expressed as the mean ± SE. Values with a different letter are significantly different between groups ($P < 0.05$).

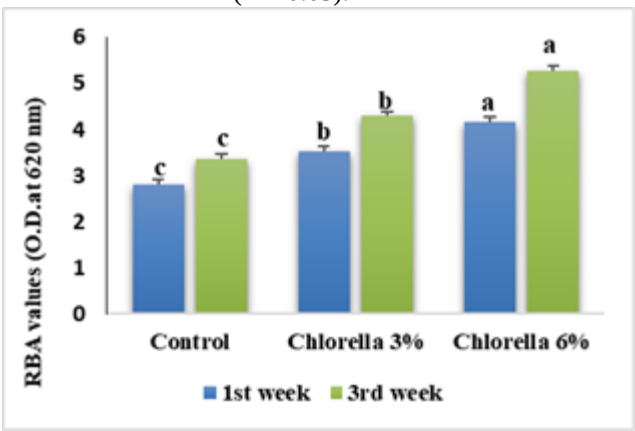


Fig. 4. Respiratory burst activity (RBA) level in experimented Nile tilapia fed Chlorella diet for 3 weeks. Value is expressed as the mean ± SE. Data with a different letters are significantly different between groups ($P < 0.05$).

Challenge test

Survival rate, mortality percent, and relative percent survival (RPS), induced by prepared *Chlorella* supplemented diets on experimented Nile tilapia groups after challenges against pathogenic *A. hydrophila* were summarized in Table (5).

After challenge, fish feed with 3% *Chlorella* supplemented regime showed better survival rate than control with RPS of 93.33% & 6.67%, respectively. Additionally, fish feed with 6% *Chlorella* supplemented diet displayed SR of 100% compared with the control group (Fig. 5).

Table 2. The mortality and the relative percentage survival experimented Nile Tilapia (*O. niloticus*) challenged against pathogenic *A. hydrophila*.

Fish Groups	Level of supplemented Chlorella (%/ B. WT)	No. of challenged fish	Mortality		SR (%)	RPS (%)
			No.	%		
Control	0%	15	11	73.33	6.67	-
Chlorella I (3%)	3%	15	1	6.67	93.33	90.9
Chlorella II (6%)	6%	15	0	0	100	100

RPS = relative percentage survival.
SR= Survival rate.

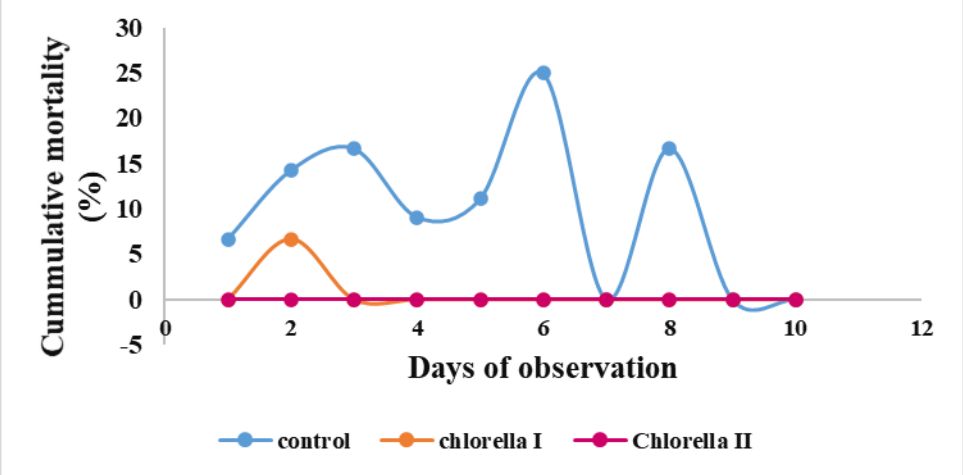


Fig. 5. The effects of the experimental treatments on the mortality percentages among *O. niloticus* challenged against *A. hydrophila* (3×10^8 CFU/mL) after 6 weeks of feeding.

Histopathological examination of experimented Nile tilapia

At first week of the feeding experiment, the visceral organs of the control group showing normal tissue architecture and cellular details with no remarkable histopathological alterations (Fig. 6). In Ch 3% group, the liver and kidney showed mild vacuolar degeneration. The intestine displayed mucosal degeneration in the lining epithelium with mononuclear cells infiltration in the lamina propria. While, in Ch 6% group, the liver showed congestion, and kidney showed slight oedema with activated melano-macrophages (MMC), while the intestine showed focal infiltration of mononuclear cells in the lamina propria.

At third week of the feeding experiment (Fig. 7), Ch 3% group, showed vacuolar degeneration in the hepatic and renal parenchymal with mild mucinous degeneration in the lining epithelium of intestine with massive mononuclear cells infiltrated in the lamina propria. Moreover, Ch 6% group, the liver showed congestion in the hepatic in addition, pancreatic vessels & vacuolar degeneration, as well as coagulative necrosis of some hepatocytes. The kidney revealed advanced vacuolar degeneration and focal coagulative necrosis of renal epithelium with atrophy of glomerular tuft. The intestine displayed

advanced mucinous degeneration with coagulative necrosis of the lining epithelium with marked oedema in the lamina propria and submucosa.

After challenge with the pathogenic *A. hydrophila* strain, control infected group showed vacuolar degeneration and coagulative necrosis in the parenchymal cells of various visceral organs (Fig. 8). Ch 3% group showed marked congestion in the hepatopancreatic vessels with mild parenchymal oedema. The kidney revealed degeneration in the renal epithelium and focal aggregation of MMCs. The intestinal lining epithelium showed mild mucinous degeneration with massive mononuclear cells infiltrated in the lamina propria. Additionally, 6% Chlorella supplemented group, the liver showed mild parenchymal oedema and focal aggregation of MMCs. The kidney showed vacuolar degeneration in the renal epithelium with focal aggregation of MMCs and proliferation of hematopoietic tissue. The intestine displayed advanced mucinous degeneration with coagulative necrosis of the lining epithelium.

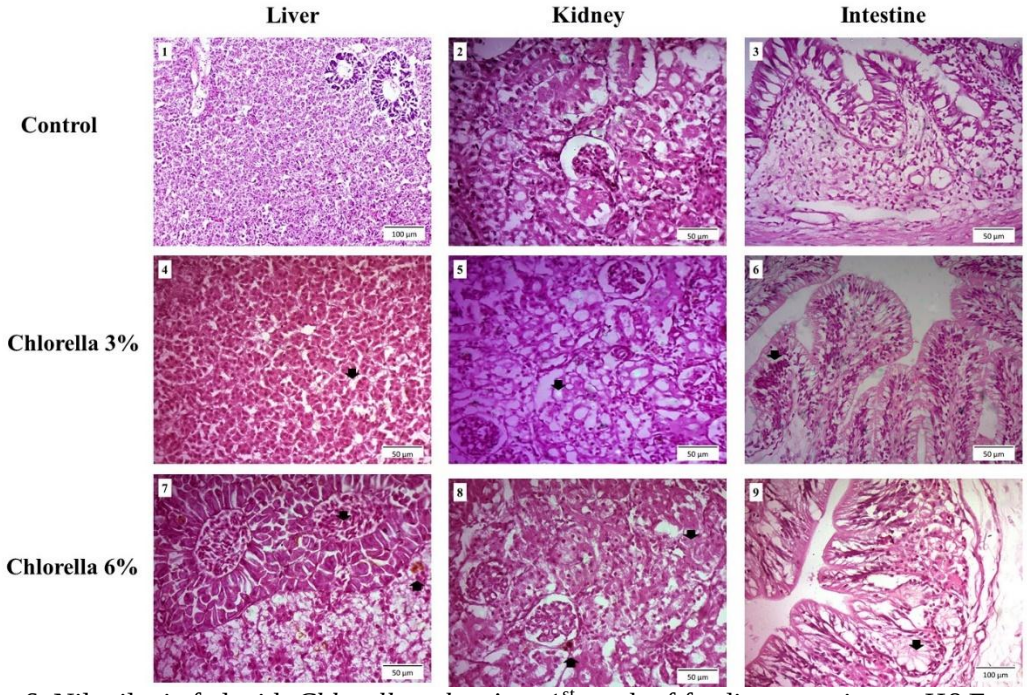


Figure 6. Nile tilapia fed with *Chlorella vulgaris* at 1st week of feeding experiment. H&E stain.

Liver 1 normal hepatocytes, kidney 2 normal renal parenchyma, intestine 3 normal structure, liver 4 mild parenchymal oedema, kidney 5 vacuolar degeneration, intestine 6 mononuclear cells infiltration in the lamina propria, liver 7 displaying congestion with focal melano-macrophages (MMC), kidney 8 tubular nephrosis and some MMCs, & intestine 9 mucinous degeneration with focal infiltration of mononuclear cells in the lamina propria.

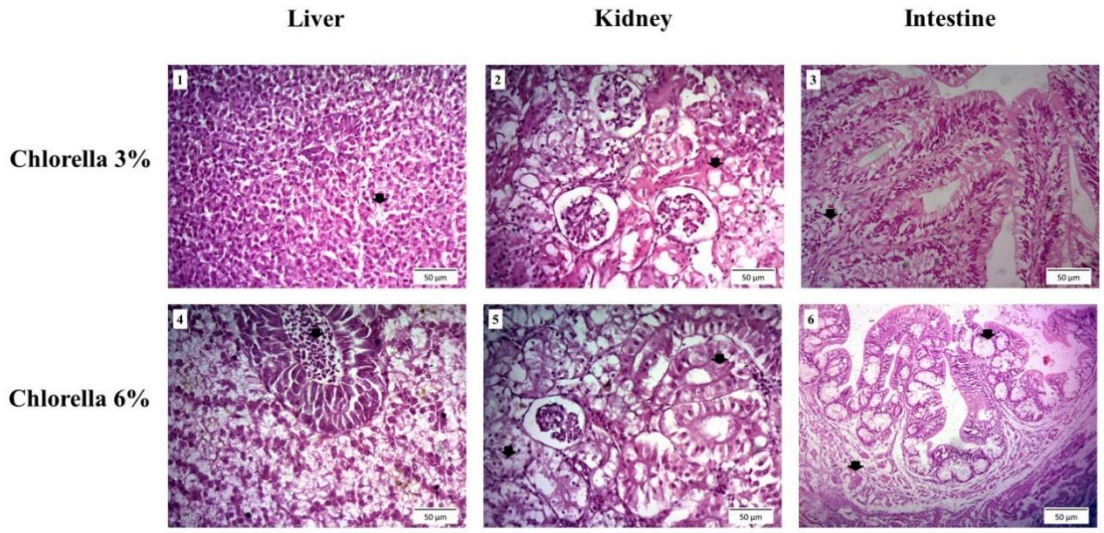


Figure 7. Nile tilapia fed with *Chlorella vulgaris* at 3rd week of feeding experiment. H&E stain.

Liver 1 vacuolar degeneration of the hepatocytes and the pancreatic acinar cells, kidney 2 revealed vacuolar degeneration in the renal epithelium, intestine 3 massive mononuclear cells infiltrated in the lamina propria, liver 4 congestion in the hepatic and pancreatic vessels, kidney 5 vacuolar degeneration and focal coagulative necrosis of renal epithelium with atrophy of glomerular tuft, & intestine 6 mucinous degeneration with coagulative necrosis of the lining epithelium with marked oedema in the lamina propria and submucosa.

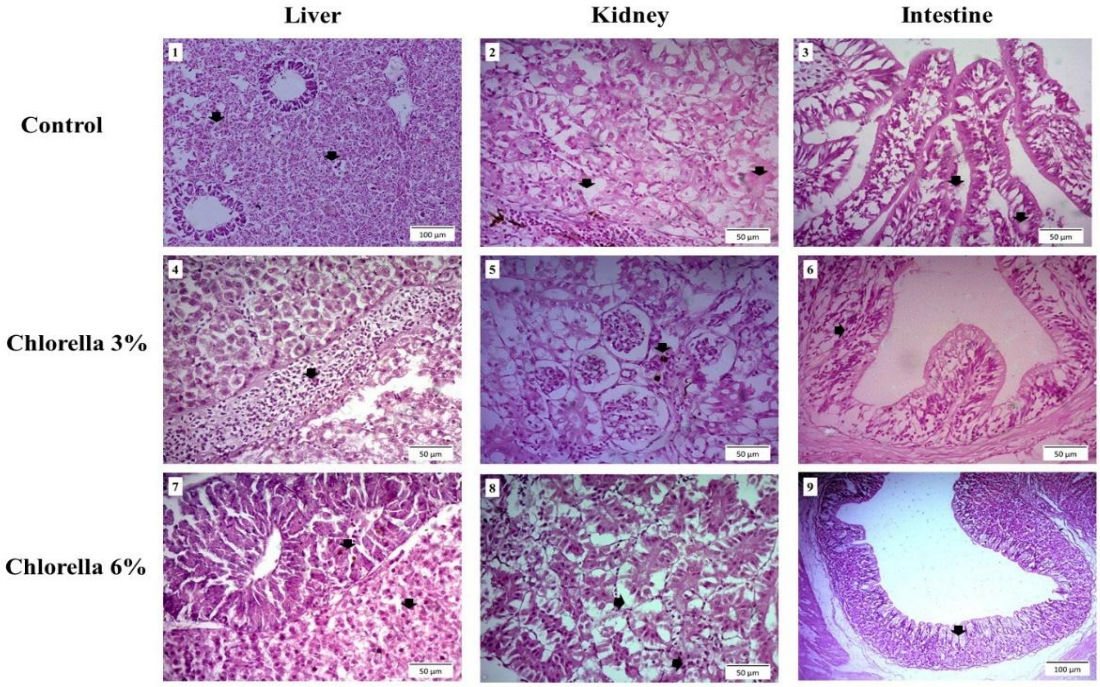


Figure 8. Experimented Nile tilapia fed with *Chlorella vulgaris* after challenged with pathogenic *A. hydrophila* strain. H&E stain.

Visceral organs 1, 2, &3 vacuolar degeneration and coagulative necrosis in the parenchymal cells, liver 4 marked congestion in the hepato-pancreatic vessels, kidney 5 focal aggregation of MMCs, intestine 6 mild mucinous degeneration with massive mononuclear cells infiltrated in the lamina propria, liver 7 mild parenchymal oedema and focal aggregation of MMCs, kidney 8 vacuolar degeneration in the renal epithelium with focal aggregation of MMCs and proliferation of hematopoietic tissue, & intestine 9 advanced mucinous degeneration with coagulative necrosis of the lining epithelium.

DISCUSSION

The aquaculture industry leads in food production systems with a high-quality protein diet that fulfils a great role in food security for future generations worldwide (FAO, 2020). To achieve sustainability and support a growing market for cultured fish, the demand for aquaculture diets with high-quality nutirants sources, directs the attention of fish nutritionists to search for alternative, cheaper, and sustainable ingredients (Pakravan *et al*., 2018) that boost fish health and augmentedtheir disease resistance. The global attention focused on green microalga, especially *Chlorella* species, as an important member of the aquatic food chain (Maliwat *et al*., 2021) that is broadly applied as a probiotic nutritional supplement in aquaculture (Abdelhamid *et al*., 2020).

The popularity of microalgae as aquaculture feed is increasing day by day and to recognize the efficacy of including microalgae in the diet of farmed fish, we evaluated the effects of partial addition of a microalga, *C. vulgaris*, on haematological, immunological, hepatic, and renal responses, as well as survival rate in experimented Nile tilapia.

The haematological examination revealed that the total RBCs count not significantly changed when compared to the control group in experimented Nile tilapia along feeding trial. Our results were in consistent with the findings obtained by Abdelhamid *et al*. (2020) who noticed no significant differences in RBCs count, Hb concentration, and PCV value among all tilapia experimental groups when examining the antitoxic effect of *Chlorella* on diazinon intoxication. Similarly, in Korean rockfish, Sebastes schlegeli, the blood parameters did not improve with dietary inclusion of Chlorella spp. at 4% concentration (Bai *et al*., 2001). Meanwhile, after infection RBCs significantly decreased in control group compared with the Chlorella treated groups. These findings could be attributed to *C. vulgaris* high pigment content, which includes carotenoids Markou and Nerantzis (2013), which scavenge free radicals (Gammone *et al*., 2015) and protect host cells from oxidative damage caused by pathogenic bacteria. Thus, fish with a high level of carotenoids are more resistant to bacterial and fungal infections.

In this study, the experimented Nile tilapia fed with *C. Vulgaris* inclusion diets displayed a significant elevation in the total leukocytic count when compared to the control group. Our results were in accordance with those of Dawood *et al*. (2020) who observed a rise in TLC and differential leukocytic counts following feeding with a C. Vulgaris diet. Also, similar results previously reported (Mahmoud *et al*., 2020; Abdelhamid *et al*., 2020). A probable explanation is that the cell wall of C. vulgaris contains lipopolysaccharide (LPS)-like molecule similar to the true bacterial lipopolysaccharide (Armstrong *et al*., 2002), as well as the wall rich in glucans, both stimulate leukocytes population trigger the elevation of TLC in Koi carp, *Cyprinus carpio* (Chen *et al*., 2015). Consequently, this data validates the immunostimulatory effects of *C. Vulgaris* especially on the cellular, non-specific arm of the immune system. Hence, recently, several microalgae including *C. vulgaris* are commercialised were used as

nutritional supplements for both terrestrial and aquatic animals, which defined as novel foods in the novel food regulation NFR (van der Spiegel *et al*., 2013).

The serum ALT and AST activities, liver biomarkers, revealed no significant changes in the experimented groups during the first week of feeding, whereas after 3 weeks the *Chlorella* groups showed an slight rise in the ALT levels when compared to the control group. Moreover, throughout the feeding experiment, the serum AST level was maintained in its normal pattern in the *Chlorella* treated groups compared to the control group that cocstant with previous studies (Khani *et al*., 2017; Zahran *et al*., 2019; Abdelhamid *et al*., 2020). In addition, after infection, the ALT activity significantly increased in the control group compared with the other *Chlorella* treatment groups. Also, the lower *Chlorella* level, Ch 3% group, showed significantly lowered ALT activity in comparison to other groups. As well as AST levels in the control group recorded the highest value and the *Chlorella* fed groups displayed significant lowered values. These findings might be attributed to *C. vulgaris* with lower doses has a hepatoprotective properties and antioxidant activity (Goiris *et al*., 2012), which protect the hepatocytes cell wall form oxidative damage of the free radicals produced during infection to prevent leakage of their enzymes into the blood during bacterial infection. Furthermore, Radhakrishnan *et al*., (2014) noticed high values of vitamin C and E concentrations in the hepato-pancreas and muscle tissues of *M. rosenbergi* fed a diet supplemented with *C. vulgaris* that have antioxidant activity to protect the cells integrity.

Regarding the renal biomarkers, tilapia groups feed chlorella enriched diets showed normal values near to the control group especially the low chlorella diet that validates protective efficacy of chlorella on fish kidney. In the same way, Zahran *et al*., (2019) found that diet supplemented with 5% &10 % *C. vulgaris* powder, improve kidney bioactivity and alteration of renal damage in Nile tilapia feed, which was caused by sodium arsenate toxicity. These results might attributed to the high concentration on carotenoids, particularly astaxanthin, in *C. vulgaris* (Pakravan *et al*., 2018), which protect the renal parenchyma from oxidation by scavenging free radicals and suppressing oxidative stress damage. Additionally, *Chlorella* species found to contain a potent antibacterial compound called chlorellin (Mostafa, 2012) that fight the bacterial infection and prevent they pathogenic effect on the visceral organs.

The innate immune response is a foremost defence of fish. Our findings revealed that *Chlorella* fed groups showed significantly higher level of serum lysozyme activity throughout the experimental periods. In addition, the respiratory burst activity among the *Chlorella*-supplemented groups increased significantly over the time compared with the control non-supplemented group. Additionally, Ch 6% group showed significant higher respiratory activity values compared with the Ch 3% group and control group throughout the experimental periods. In our study, the dietary supplementation of *Chlorella* enhanced the innate immune responses that supported with previous studies (Zahran and Risha, 2014; El-Habashi *et al*., 2019). These findings might attributed to not only the

stimulatory effect of *Chlorella* to increase number and activity of phagocytic cells specially macrophages that enhance innate response via macrophage activation (Liu *et al*., 2006), but also its rich in natural antioxidants such as chlorophyll, polyphenol, vitamins, sulphur-containing compounds that have the capacity to scavenge free radicals.

The challenge test results with pathogenic *A. hydrophila* strain revealed that Nile tilapia fed with *Chlorella* diet showed varying degrees of protection. Fish fed with a 3% *Chlorella* supplemented regime showed better survival rate than control, with RPS of 93.33% and 6.67%, respectively. Additionally, fish fed with a 6% *Chlorella* supplemented diet had a 100% survival rate when compared to the non-supplemented control group. These findings are in constant with those of Lim *et al*., (2018) who declared that the carotenoid enhances survival, growth performance, stress tolerance, physiology, disease resistance, and immunity in various animal species. Moreover, *C. vulgaris* contains a beneficial phytonutrient called *Chlorella* growth factor (CGF), which is rich in nucleic acid-related substances such as peptides, proteins, amino acids, vitamins and vital sugars. Furthermore, Dinev *et al*., (2021) informed that green microalgae like *C. vulgaris* are capable of producing a diverse range of active substances with antimicrobial, immunostimulant, cytotoxic, and antioxidant activity that improve health and increase disease resistance.

The histopathological results of applying *Chlorella* to the Nile tilapia diet were in constant with the previous research (Zahran *et al*., 2019; El-Habashi *et al*., 2019). This result stated that the low *Chlorella* supplementation diet not only has mild tissue alteration, but also has moderate activation in melanomacrophage centres that indorsed to activate the non-specific immune system which affirmed the protective and therapeutic roles of *Chlorella.*

CONCLUSION

In conclusion, the current study indicated that the microalga *C. vulgaris* could be incorporated in the diet of the Nile tilapia. Diet contained low levels (3%) of *C. vulgaris* is significantly safe with no adverse effect on the performance of fish, aside from increasing disease resistance power. Moreover, adding *C. vulgaris* in the diet in low concentrations had no minimum effects on hepato-renal function that improves survival and growth. From an economic standpoint, the inclusion of fish diet with *C. vulgaris* is recommended. The results of this study provide important information regarding the potential application of *C. vulgaris* as a valuable immunostimulant for *O. niloticus* as it improves innate immunity. However, it must be noted that these results were obtained under experimental conditions and more research is needed to verify the approbriate concentration and support our results in real tilapia culture systems.

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