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Chlorella vulgaris Enhances the Efficacy of Florfenicol in the Treatment of *Aeromonas hydrophila* infection in the Nile tilapia

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ABSTRACT

To assess the impact of *Chlorella vulgaris* on the performance of the Nile tilapia, fish specimens with body weight 53.26 g ± 0.64 were fed on diets supplemented with 0, 5, 10, 15 and 20g of *C. vulgaris* for 60 days at a daily rate of 3% of body weight. The growth performance, feed conversion ratio, final weight, weight gain and daily weight gain were enhanced with supplemented levels up to 15g of *C. vulgaris*; while higher addition insignificantly differed with the other groups. Supplemented fish were well-nourished, and their health status was improved. Blood parameters {RBCs, WBCs, hemoglobin (Hg) and hematocrit (PCV)} were significantly higher than the control. Serum total protein and liver enzymes were significantly increased in supplemented fish; however, the levels of the liver enzymes revealed no adverse impact on liver health. *C. vulgaris* could stimulate the activities of antioxidants (catalase and total antioxidant capacity) and pro-inflammatory cytokines (interleukin-1β and tumor necrosis factor-α), improving the immune status. Fish that received *C. vulgaris*- supplemented diets had a higher survival rate against *Aeromonas hydrophila* infection, while the relative protection level (RPL) of florfenicol treatment was significantly improved compared to the control group. It was obvious from the obtained results that the Nile tilapia could be fed up to 15g of *C. vulgaris* /kg fish feed without compromising the health status; it also enhanced the response of fish to florfenicol treatment raising the relative protection level to 42.86%.

INTRODUCTION

Aquaculture is considered as one of the fastest-growing agricultural activities, and it is distributed in many countries **(FAO, 2020)**. The feed ingredients cost have been duplicated many times; thus, there is a need to decrease the cost and keep the quality. Therefore, natural additives have become

a well-choice to improve the growth performance and fish health while feed preserve its quality **(NRC, 1993; Sherif *et al.*, 2022a)**.

Infectious diseases result in high losses in the aquatic sector counted by billions of dollars threatening world economy and aquaculture industry, *Aeromonas hydrophila* is one the ubiquitous and virulent bacteria in freshwater fishes worldwide **(Hossain *et al.*, 2014; Peterman & Posadas, 2019; Abdelsalam *et al.*, 2022; Sherif & AbuLeila, 2022; Sherif & Kassab, 2023)**.

Antibiotics are still the keystone in bacterial treatment in aquaculture, raising the probability of emerging the multidrug-resistant bacteria, especially with the haphazard uses by fish farmers **(Patil *et al.*, 2016; Stratev & Odeyemi, 2016; Sherif *et al.*, 2021a)**. Consequently, the European Union has outlawed their applications in fish production **(Heuer *et al.*, 2009)**. To face such hazards and minimize antibiotics uses, new approaches were developed, non-chemical compounds such as acidifiers (organic acid salts), essential oils in addition to probiotics have been used to improve growth performance and health status at a low cost **(Koh, 2008; Hoseinifar *et al.*, 2016; Hoelzer *et al.*, 2018; Sherif *et al.*, 2022a, b)**.

Microalgae are single-cell organisms presented in the base of the food chain of the aquatic animals as a protein source (50%–70%); for fish, they are efficiently consumed and could replace fish meal and soybean protein in aqua-feeds **(Han *et al.*, 2019; Zhang *et al.*, 2019; Sherif *et al.*, 2022c)**. They have a growth promoting effect **(Kousoulaki *et al.*, 2016)**. Many studies reported that incorporation of *C. vulgaris* is safe and it promotes the growth of some fishes such as rainbow trout (*Oncorhynchus mykiss*) **(Gouveia *et al.*, 1998)**, gilthead seabream (*Sparus aurata*) **(Gouveia *et al.*, 2002)**, sterlet (*Acipenser ruthenus*) **(Sergejevova & Masojidek, 2012)**, the Nile tilapia (*Oreochromis niloticus*) **(Teuling *et al.*, 2017)** and zebrafish (*Danio rerio*) **(Carneiro *et al.*, 2020)**.

The *C. vulgaris* has natural bioactive ingredients for growth factors such as available protein, vitamins, minerals, fiber, antioxidants, and feeding attractants as well as unknown growth promoting factors **(Nakagawa *et al.*, 2000)**. The *C. vulgaris* could enhance the antioxidant status of fish through their contents of phytochemicals having antioxidant activities, including carotenoids, chlorophylls, tocopherols, ubiquinone, flavonoids and polyphenols **(Coulombier *et al.*, 2021)**. Moreover, *C. vulgais* possesses antimicrobial activity **(Natrah *et al.*, 2014)** that could mitigate chemical medication in aquaculture.

The goal of this study was to assess the effect of dietary *C. vulgaris* on the Nile tilapia via determining the growth performance, hematological and serum biochemical markers as well as resistance against bacterial infection.

MATERIALS AND METHODS

1. Fish collection and acclimatization

Apparently healthy (showed no clinical symptoms) fingerlings of the Nile tilapia *Oreochromis niloticus* were obtained from a private fish farm located at the village of Tolompate 7 in Kafrelsheikh Governorate. Fish count was 450 with an average body weight of 53.26 ± 0.64g. Fish specimens were transported alive (2 h) to the wet-laboratory of Animal Health Research Institute (AHRI) according to methods of **Sherif *et al.* (2022d)** and **Eldessouki *et al.* (2023)**. Fish were stocked and acclimatized in the fiberglass tank (3 m^3 volume) for two weeks while feeding on a basal diet provided without any feed additives. Fish were randomly distributed into 15 glass aquaria (90×60×40 cm), filled with dechlorinated tap water (30 fish/aquarium). During the acclimatization and experimental period, water temperature and dissolved oxygen levels were maintained at 25±1 °C and 5.9±0.5, respectively. To maintain constant and suitable water parameters, the water at the bottom of aquaria was daily drained, and 30% of aquaria water was replaced with clean dechlorinated water.

2. The experimental design

The experimental Nile tilapia specimens were distributed into five groups G1–5 in triplicate (Table 1), fish feed was formulated and given twice a day (at 8:00 a.m. and 5:00 p.m.) at a rate of 3% b.w. The amount of food was modified every week according to the National Research Council **(NRC, 2011)**. After 60 days, blood and serum samples were collected, and fish were challenged against *A. hydrophila* then treated with antibiotic of choice.

Table 1. The distribution of fish in the experimental groups

Group	Supplementation *C. vulgaris*	No. of fish	Time
G 1	Control (basal diet)	30/replicate	8 weeks
G 2	5 g/kg of fish feed	30/replicate	8 weeks
G 3	10 g/kg of fish feed	30/replicate	8 weeks
G 4	15 g/kg of fish feed	30/replicate	8 weeks
G 5	20 g/kg of fish feed	30/replicate	8 weeks

In Table (1), the algal *C. vulgaris* was added to the diet by mixing it with gelatin (Canal, Egypt) at a concentration of 5% w/w. A dried powder of *C. vulgaris* was obtained from the Institute of National Research Center, Cairo, Egypt. The ingredients (Table 2) used in fish feed formulation were calculated and weighed then thoroughly mixed,

moistened with warm water (400 ml/kg) and then cold pressed and extruded to produce 2 mm pellets, which were dried using an air convection oven at 45°C. After drying and cooling, the formulated feeds were chemically analyzed (Table 2) before storing in airtight bags.

Table 2. Fish feed ingredients and chemical analysis

Ingredient	%	Chemical composition	%
Corn	36.65	**Moisture**	10.35
Soya (44%)	37	**CP**	31.1
Fish meal	9	**Ether extract**	3.57
Wheat bran	1.5	**Ash**	6.06
Corn gluten	11	**Crude fiber**	4.22
Soya oil	4	**NFE**[3]	44.85
Carboxy methyl cellulose	0.2	**Calcium/phosphorus**	0.74/0.6
Salt	0.2	**DE Kcal/kg**	3200.5
DL. Methionine	0.15		
Mineral premix[1]	0.15		
Vitamin premix[2]	0.15		

1. Mineral premix: each one kg contain Manganese 60g, Copper 4 g, Zinc 50g, Iodine 1g, iron 80g, Cobalt 0.1g, Selenium 0.1g, Calcium carbonate (CaCO3) carrier to 1000g.

2. Vitamin premix: each one Kg contains vitamin A 12000000 IU, vitamin D3 2200000 IU, vitamin E 10 g, vitamin K3 2 g, vitamin B1 1 g, vitamin B2 5 g, vitamin B6 1.5 g, vitamin B12 0.01 g, vitamin C 250 g, Niacin 30 g, Biotin 0.050 g, Folic acid 1 g and Pantothenic acid 10 g and carrier to 1000 g.

3. NFE= Nitrogen free extract.

4. DE Kcal/kg = Digestible energy (DE) was calculated using formula based on chemical composition of feed stuffs nutrients according to **NRC (2011)**.

3. Growth performance

Fish samples were weekly counted and weighed to assess growth performance, weight gain (WG) and feed conversion ratio (FCR) that were calculated using the following formulas:

Weight gain (WG) = final body weight (g) – initial body weight (g)

Feed conversion ratio (FCR) = feed intake (g) / weight gain (g)

4. Hematological and serum biochemical parameters

After 60 days, five fish were randomly collected from each aquarium to drain blood samples, which were divided into two portions:

The first blood portions (whole blood samples) were collected and mixed with the anticoagulant 10% ethylene diamine tetra acetate (EDTA); they were used to determine

the red blood counts (RBCs), total count of white blood cells (WBCs) hemoglobin (Hb), and hematocrit (PCV) following the standard methods of **Rawling *et al.* (2009)**.

The second blood portions (serum samples) were allowed to coagulate at 4°C and centrifuged at 3000 rpm for 10 minutes to collect serum, which was preserved at −20°C. The levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined according to the method of **Reitman and Frankel (1957)**. Serum total protein (TP) and albumin (ALB) were measured following the methods of **Henry (1964)** and **Wotton and Freeman (1982)**, respectively. However, according to **Coles (1974)**, globulin (GLO) was measured by subtracting albumin from total protein.

5. Antioxidant enzyme activity and cytokines in the experimental fish

After 60 days, for fish fed on *C. vulgaris*-diets, the total antioxidant capacity TAC and catalase CAT (EC 1.11.1.6) activities were measured in the hepatic tissues using ELISA. Serum interleukin 1β (IL-1 β), interleukin 10 (IL-10) and tumor necrosis factor TNF-α were measured by ELISA using a solid phase sandwich ELISA test kit obtained from My BioSource Co., San Diego, California, USA. The procedures were performed according to the manufacturer's protocol.

7. Challenge test

After 60 days, twenty fish from each aquarium were intraperitoneally injected with 0.1ml pathogenic *A. hydrophila* according to the method of **Schaperclaus *et al.* (1992)**. In addition, pure saline solution (0.65 %) was injected in a similar way into 5 fish as negative controls **(Boijink *et al.*, 2001)**. Bacterial strain *A. hydrophila* was previously isolated and identified as AHRAS2, the accession numbers in GenBank were under MW092007 and the LD_{50} of 2.4×10^5 CFU/ml, in addition it was highly susceptible to florfenicol **(Sherif & AbuLeila, 2022)**. After 3 days of bacterial injection, only ten fish were treated with florfenicol 10mg/ kg b.w. for ten days in separate aquarium. For two weeks, infected fish were observed for clinical symptoms, postmortem lesions and daily mortalities. Pathogenic bacterial strains were re-isolated from the liver, kidney and the gut of deceased fish. The injected fish were observed for 14 days to record the mortality rate (MR) as follows:

MR % = no. of deaths in a specific period x 100 / total population during that period

Meanwhile, the relative protection level (RPL) was verified among the challenged fish according to **Ruangroupan *et al.* (1986)** as follows:

RPL (%) = {1–(% mortality in treated group/% mortality in the control group)}*×100

8. Statistical analysis

After 60 days of feeding trial, the obtained data were analyzed to assess the impacts of *C. vulgaris* supplementation on the performance of the Nile tilapia. To analyze the significance of differences, the analysis of variance (ANOVA) was determined using

Duncan's test **(Duncan, 1955)** among the means of the different treatment groups at a significance level of 0.05 using SPSS **(SPSS, 2004)**.

9. Biosafety considerations

This study followed the biosafety measures outlined on the pathogen safety data sheets entitled infectious substances *A. hydrophila*, Pathogen Regulation Directorate **(Public Health Agency of Canada, 2010)**.

RESULTS

1. Growth performance

In Table (3), the experimental Nile tilapia specimens received dietary-*C. vulgaris* at degraded levels of 0, 5, 10, 15 and 20g/ kg fish feed. Growth parameters were significantly higher for FW, WG and DWG (95.7 g, 45.4 g, and 0.79), respectively, in group (G4), which was fed on 15g of *C. vulgaris*-diets, compared to the control and the other groups. No further improvements were recorded in growth performance with higher level of *C. vulgaris* (G5). The value of FCR was gradually decreased with increasing the supplementation level from G2 up to G4, ranging between 2.37 and 1.18 *vulgaris*, whereas G5 was 1.35.

Table 3. The growth performance parameters of different experimental groups

Group	Growth performance parameters					
	IW (g)	**FW (g)**	**FI (g)**	**WG (g)**	**DWG (g)**	**FCR**
G1	$53^{A}\pm0.58$	$78.2^{C}\pm1.1$	$69.56^{AB}\pm4.42$	$25.2^{C}\pm0.7$	$0.42^{C}\pm0.01$	$2.77^{A}\pm0.2$
G2	$54.7^{A}\pm2.03$	$87.07^{B}\pm1.38$	$76.9^{A}\pm4.58$	$32.4^{B}\pm1.25$	$0.54^{B}\pm0.02$	$2.37^{A}\pm0.05$
G3	$54.3^{A}\pm1.2$	$90.73^{AB}\pm1.34$	$58.8^{BC}\pm6.6$	$36.4^{B}\pm0.53$	$0.61^{B}\pm0.01$	$1.62^{B}\pm0.18$
G4	$50.3^{A}\pm0.6$	$95.7^{A}\pm2.03$	$53.5^{CD}\pm2.08$	$45.4^{A}\pm1.78$	$0.76^{A}\pm0.03$	$1.18^{C}\pm0.04$
G5	$54^{A}\pm2.6$	$86^{B}\pm3.79$	$43.05^{D}\pm2.7$	$32^{B}\pm2.6$	$0.53^{B}\pm0.04$	$1.35^{BC}\pm0.03$

IW: initial weight; FW: final weight; FI: feed intake; WG: weight gain; DWG: daily weight gain; FCR: food conversion rate.

2. Blood and serum analyses

In Table (4), blood indices of RBCs, Hg, PCV and WBCs were improved with *C. vulgaris* supplementation, and G4 scored significantly higher values with 3.38×10^{6}, 10.6 g/dl, 37.6%, and 6.03×10^{3}, respectively, compared to the other groups. Serum analyses for TP, ALB, GLO, ALT and AST showed enhanced health status after feeding on *C. vulgaris* supplemented-feed. Higher TP was recorded in G3 and G4, with values of 5.59 and 5.48 g/dl, respectively. Additionally, GLO had the same trend, whereas no significant

alterations were recorded in serum ALB. Liver enzymes (ALT and AST) were slightly increased in all *C. vulgaris*- supplemented groups, compared to the control, while creatinine significantly raised with high supplementations levels in G4 and G5, compared to the other groups.

Table 4. Blood and serum analyses of the experimental Nile tilapia

Group	Blood parameters					Serum parameters				
	RBCs $\times10^6$	**Hg** g/dl	**PCV** %	**WBCs** $\times10^3$	**TP** g/dl	**ALB** g/dl	**GLO** g/dl	**ALT** IU/l	**AST** IU/l	**Creat** mg/dl
G1	2.73^{C} ±0.12	8.58^{C} ±0.38	30.39^{C} ±1.34	4.21^{E} ±0.08	4.78^{C} ±0.07	2.56^{A} ±0.02	2.21^{C} ±0.06	23^{B} ±0.6	31.97^{D} ±1.3	0.94^{B} ±0.01
G2	3.2^{AB} 0.05±	10.02^{AB} ±0.16	35.5^{AB} ±0.157	4.66^{D} ±0.1	5.23^{B} ±0.04	2.6^{A} ±0.01	2.63^{B} ±0.06	26.17^{A} ±0.7	33.63^{CD} ±0.09	1.06^{AB} ±0.05
G3	3.02^{B} ±0.08	9.49^{B} ±0.26	33.6^{B} ±0.94	5.68^{B} ±0.07	5.59^{A} ±0.05	2.56^{A} ±0.02	3.03^{A} ±0.04	26.7^{A} ±0.5	34.87^{C} ±0.4	1.02^{AB} ±0.04
G4	3.38^{A} ±0.04	10.6^{A} ±0.14	37.6^{A} ±0.5	6.03^{A} ±0.09	5.48^{AB} ±0.1	2.42^{A} ±0.02	3.05^{A} ±0.11	27.53^{A} ±0.47	40.73^{A} ±0.6	1.21^{A} ±0.15
G5	3.03^{B} ±0.04	9.5^{B} ±0.11	33.64^{B} ±0.4	5.24^{C} ±0.09	5.21^{B} ±0.12	2.53^{A} ±0.04	2.68^{B} ±0.08	27.5^{A} ±0.6	38.23^{B} ±0.8	1.27^{A} ±0.06

RBCs: red blood cells; WBCs: white blood cells; Hg: hemoglobin; PCV: packed cell volume; TP: total protein; ALB: albumin; GLO: globulin; ALT: alanine amino transferase; AST: aspartate transaminase; Creat: creatinine.

3. Antioxidants enzymes and cytokines determination

Hepatic antioxidant CAT and TAC levels had significantly improved with *C. vulgaris* supplementation till the 15g, while supplementation with 20g (G5) was insignificant for G2 and G3 (Fig. 1). The serum cytokine IL-10 was insignificantly different with *C. vulgaris* supplementation except in G4 (3.3), while IL-1β and TNF-α were gradually elevated till the 15g supplementation for G5 (20g/ kg fish feed supplementation), showing insignificant difference in G1-3 (Fig. 2).

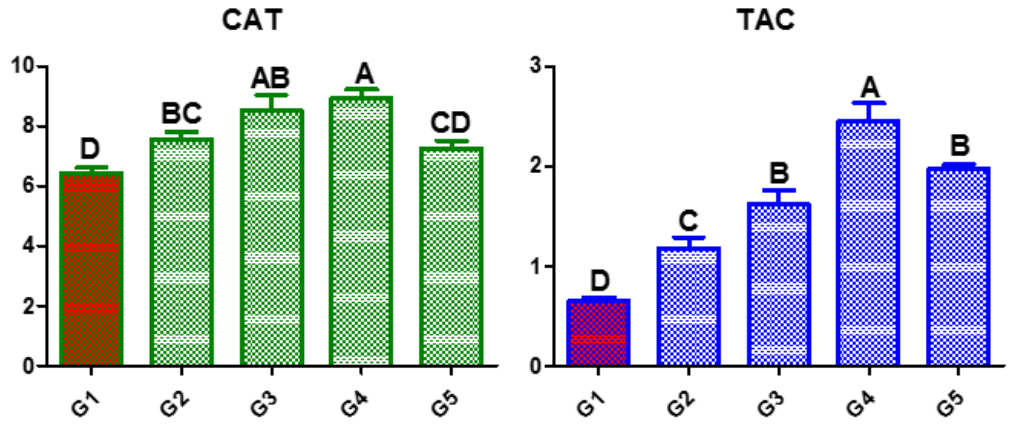

Fig. 1. Antioxidant enzymes in the hepatic tissues of the experimental Nile tilapia

CAT: catalase; TAC: total antioxidant capacity

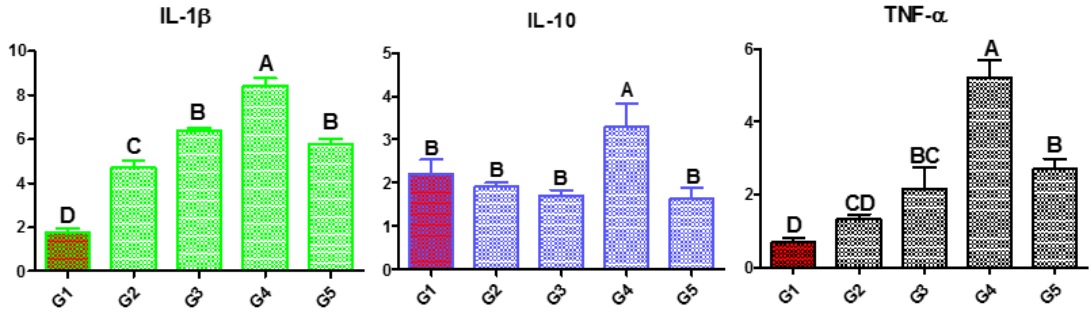

Fig. 2. Serum cytokines levels of the experimental Nile tilapia

IL: interleukin; TNF: tumor necrosis factor

4. Bacterial challenge and antibiotic treatment

Fish were challenged against *A. hydrophila* LD_{50} and treated with FLO, and the response to treatment was measured via RPL% and bacterial re-isolation. Over all, fish fed on *C. vulgaris* -supplemented diets had significantly low MR% and FLO, exhibiting significantly higher RPL%, compared to the control group. The higher RLP% (42.86%) was achieved by fish in G3 and G4, which fed *C. vulgaris* at a rate of 10 and 15g/ kg of fish feed, respectively (Table 5).

Table 5. Experimental Nile tilapia challenged against *A. hydrophila* and treated with florfenicol

Group	Fish no. (Un/FLO)	Un-treated		FLO-treated		RPL%
		Dead	%	Dead	%	
G1	20 (10/10)	7	70	5	50	28.57
G2	20 (10/10)	6	60	5	50	28.57
G3	20 (10/10)	6	60	4	40	42.86
G4	20 (10/10)	4	40	4	40	42.86
G5	20 (10/10)	6	60	5	50	28.57

Note: Un= untreated fish, FLO= fish treated with florfenicol, RPL%: relative protection level.

DISCUSSION

In our results, *C. vulgaris*-diets enhanced the growth performance of the Nile tilapia as they showed significant higher FW, WG and DWG for mainly those fed on 15g/ kg fish feed. The FCR was gradually improved with *C. vulgaris* addition. Similarly, **Chen *et al.* (2022)** reported that, upon feeding juvenile rainbow trout with a dietary *C. sorokiniana* (5%) for ninety days, FI and WG were significantly increased by 19.3% and 17.3%, respectively. Additionally, the Nile tilapia recorded an enhanced growth performance when fish meal was partially exchanged with chlorella spp. at rates of 10%, 25%, 50% and 75% **(Badwy *et al.*, 2008)**, and a replacement up to 1.2% in Gibel carps-diet with Chlorella had improved the growth parameters **(Xu *et al.*, 2014)**. Accordingly, WG, DWG, and FCR were promoted in common carp fingerlings, which were fed on 7.5 g of *C. vulgaris* /kg fish feed for 105 days **(Abdulrahman *et al.*, 2018)**; while, **Abdel-Tawwab *et al.* (2022)** observed a dose-dependent increase in FI and WG of the Nile tilapia fingerlings (16.3 g b.w.) after seventy days of *C. vulgaris*. Meanwhile, the survival rate SR% was not affected by supplementation compared to the control. These improvements achieved by dietary *C. vulgaris*-supplementation could be due to high contents of proteins, lipids, polysaccharides, vitamins such as vitamin A, K and B-complex vitamins (vitamin B1, B3, B2, B12 and B6), some minerals (such as Cu, Ca, Mn, Mg, Zn and Fe), pigments (chlorophyll a, b and carotenoids) among other bioactive compounds in addition to β-glucan, which could stimulate immunity and growth performance of fish **(Mohan *et al.*, 2019; Prabakaran *et al.*, 2019).**

In the current findings, the supplementation of *C. vulgaris* was insignificantly different with lower supplementation levels. Moreover, the excessive supplementation of chlorella meal led to high dietary carbohydrate (28.85%) (crude fiber-free), which was

not efficiently utilized by fish **(Xi *et al.*, 2022)**. In addition, the high dietary starch levels (24 to 36%) could cause significant adverse effects on largemouth bass **(Zhang *et al.*, 2019).**

Upon using *C. vulgaris*-diets supplementation, blood indices of RBCs, WBCs, Hg, and PCV revealed that fish were well-nourished and immune-promoted. Similarly, the Nile tilapia which received a dietary- *C. vulgaris* had significant raise in the WBCs, compared to the control fish **(Aly *et al*., (2022)**. This finding could be due to the presence of lipopolysaccharide (LPS)-like molecule in the cell wall of *C. vulgaris* **(Armstrong *et al*., 2002)** in addition to the richness of the cell wall in glucans, which stimulates the elevation of WBCs of *Cyprinus carpio* **(Chen *et al*., 2015)**. Whereas, no significant differences were detected in the blood parameters tested for the Korean rockfish *(Sebastes schlegeli)* at a level of 4% *C. Vulgaris* **(Bai *et al*., 2001)**. Additionally, **Abdelhamid *et al*. (2020)** claimed that dietary *C. Vulgaris* did not significantly affect blood parameters of the Nile tilapia. These differences confirm the safety of *C. vulgaris* supplementation, and could be due to differences of addition level and experimental periods.

Serum TP, ALB and GLO showed elevated values indicating that dietary-*C. vulgaris* (5-20 g/kg fish feed) could promote the immune status of the Nile tilapia and keep the liver healthy. Similarly, **Abdel-Tawwab *et al.* (2022)** observed that the Nile tilapia that received 10– 20g of *C. vulgaris*/ kg in their diets showed marked elevation of serum TP and GLO. It is well-known that the elevation of TP, ALB and GLO levels is an indicator for the enhancements of the immune responses **(Harikrishnan *et al.*, 2021)**.

To assess the extent of liver dysfunction, some enzymes such as AST and ALT are considered as a reliable diagnostic tool; they are crucial metabolic enzymes playing a fundamental role in nitrogen metabolism, oxidation of amino acids and gluconeogenesis **(Murray *et al.*, 2003; Sherif *et al.*, 2021b, c)**. The Nile tilapia received *C. vulgaris*-diets for eight weeks, their liver was not impacted since the liver enzymes of ALT and AST differed slightly, compared to the control group; no significant alteration was detected among the levels of supplementation. In addition, the creatinine level had the same pattern of the liver enzymes. These alterations resulted from feeding and metabolism that could be overcome by high values of vitamin C and E concentrations that were determined in the hepato-pancreas and muscle tissues of *M. rosenbergi,* which fed on dietary-*C. vulgaris,* that possesses antioxidant properties to maintain the integrity of the hepatic cells **(Radhakrishnan *et al*., 2014)**. Furthermore, lower levels of *C. vulgaris* supplementations had antioxidant and hepatoprotective properties **(Goiris *et al*., 2012)**. Close to our findings, **Mahmoud *et al.* (2020)** postulated that, dietary-*C. vulgaris* kept the levels of ALT and AST in the serum of the Nile tilapia. Whereas, supplementing the diets with *C. vulgaris* insignificantly affected the activities of ALT, AST and ALP, compared to the control diet for *Cyprinus carpio* **(Khani *et al*., 2017)** and the Nile tilapia serum **(Abdel-Tawwab *et al.*, 2022)**. Accordingly, the Nile tilapia fish that received a dietary- *C. vulgaris* at a rate of 5% and10% recorded an improvement in their kidney

bioactivity against renal damage, showing renal protective property **(Zahran *et al.*, 2020)**.

Dietary-*C. vulgaris* showed antioxidant properties as serum antioxidant CAT and TAC levels had significantly improved in the Nile tilapia, regardless of the supplementation levels. These findings could be attributed to high pigment content, which includes carotenoids in *C. vulgaris* **(Markou & Nerantzis, 2013)**, which could scavenge the generated free radicals **(Gammone *et al.*, 2015)**, protecting the host cells from oxidative damage. Similarly, microalga upregulated CAT and GPX levels in Korean rockfish *(Sebastes schlegeli)* fed diets supplemented with 0.5% chlorella powder **(Bai *et al.*, 2001)**, the Nile tilapia fed on 5% *C. vulgaris* **(Zahran *et al.*, 2020)** and gibel carp *(Carassius auratus gibelio)* fed diets containing 0.8%–2.0% **(Chen *et al.*, 2022)**. Previous studies have proved the effectiveness of *Chlorella* in counteracting the oxidative stress for the Nile tilapia **(Abdelhamid *et al.*, 2020)**.

Here, the immune related IL-1β, IL-10, and TNF-α were gradually and effectively elevated in serum for fish fed up to 15g *C. vulgaris* /kg. Accordingly, the gene expression of these cytokines mRNA of splenic and hepatic tissues were significantly elevated by feeding on dietary-*C. vulgaris* for the Nile tilapia. Additionally, the transcription of hepatic growth hormone, insulin-like growth factor 1, IL-1β and TNF-α genes were upregulated in fish receiving *C. vulgaris* at 15 – 20 g/kg diet, compared to the control fish **(Galal *et al.*, 2018; Mahmoud *et al.*, 2020)**. On *C. vulgaris*-supplementation, the Nile tilapia had low MR%, and RPL% of FLO was significantly improved. In this regard, dietary- *C. vulgaris* has antibacterial activities against *A. hydrophila* in *Staphylococcus aureus* **(Dineshkumar *et al.*, 2017)**, *M. rosenbergii* post larvae **(Maliwat *et al.*, 2017)** and *A. salmonicida* in juvenile rainbow trout *(Oncorhynchus mykiss)* **(Chen *et al.*, 2022)**. These findings could be due to the fact that *C. vulgaris* produces a diverse range of active substances with antimicrobial, immunostimulant, cytotoxic and antioxidant activity that improve health and increase disease resistance **(Dinev *et al.*, 2021)**. Additionally, microalgae secrete bioactive compounds that prevent the growth of pathogenic bacteria **(Guedes *et al.*, 2015; Ibrahim *et al.*, 2015)**. Among these compounds, hot-water-soluble polysaccharides (**Hsu *et al.*, 2010)**, chlorellin **(Mostafa, 2012)**, water-soluble α-glucans **(Tabarsa *et al.*, 2015)** and D-Lactic acid **(Lee *et al.*, 2020)** are considered.

CONCLUSION

From previous results, the health status of the Nile tilapia was improved after being fed with the dietary *C. vulgaris* at levels up to 20g/ kg fish feed, while at the level 15g/ kg fish feed, fish showed higher blood indices indicating well-nourished status. No impacts were detected on fish liver since liver enzymes and serum protein were within the normal values, compared to the control. Antioxidant enzymes and proinflammatory cytokines were raised in fish that received dietary *C. vulgaris*. Fish under study could resist *A.*

hydrophila infection with a healthy liver which could withstand the stress of florfenicol treatment. Feeding on *C. vulgaris* at rates of 10 and 15g/ kg fish feed plus florfenicol provided a 42.86% relative protection level. Thus, the addition of *C. vulgaris* to fish feed at a level of 15g/ kg is recommended.

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