Interaction of Low-level Dietary Supplementation of Chlorella vulgaris and Feeding Duration on Growth Hormone, Growth Performance and Biochemical Indices of Red Hybrid Tilapia

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Abstract

Chlorella is one of the most widely accepted Chlorophyta used by many as livestock and aquaculture feed. However, different studies on the overall performances of fish reported the unfavourable effect of high-level supplementations of *Chlorella vulgaris*. The current study determined the impact of low-level dietary supplementation of *C. vulgaris* alongside the different feeding durations and their interactions on the growth hormone, growth performances, biochemical indices, hepatic function, and some immunological parameters of red hybrid tilapia. The fingerlings were fed diets containing 0, 1%, 3%, and 5% of *C. vulgaris* powder kg^{-1} dry diet for 90 days. Growth hormone, growth performance, biochemical indices (total serum protein, albumin, globulin, glucose, aspartate aminotransferase, alanine aminotransferase), and some immunological (respiratory burst, lysozyme activities) parameters of the fish were examined after 30, 60 and 90 days of feeding. The results demonstrated that tilapia fed *C. vulgaris* supplemented diets showed increased levels of respiratory burst, lysozyme, albumin and total protein, growth hormone, and growth performances ($p < 0.05$), and the effects were duration dependent. Following the 90 days of feeding, there was no adverse effect on the hepatic function of the fish. Besides, low survivability was observed in the control group than in the group fed the experimental diets. The group fed the diet supplemented with 5% *C. vulgaris* had significantly higher ($p < 0.05$) activity at all the duration of feeding compared to other treatments. These results indicate that *C. vulgaris* enhanced growth performances, growth hormone concentration, biochemical indices, and some immunological parameters of red tilapia.

1. Introduction

Tilapia is among the widely cultured species of freshwater fish in the world, and accounts for more than 75% of the world production, projected to surpass the *Cyprinids* that have been in the lead (Hasan and Chakrabarti 2009). Several factors contributed to the increased interest in tilapia production, and a few among them are; their ability to rapidly and easily adapt to varying aquaculture conditions coupled with the high attractiveness and moderately stable market cost (Ng and Romano 2013). However, infectious diseases are known to be the major impediment to the development and sustainability of the aquaculture industry (Meena et al. 2013). In spite of the availability of several antibiotic agents that are readily used in treating infectious diseases in fish, there is increased concern over the use of antibiotic agents in aquaculture due to the threat of antibiotic residue accumulation and transfer of antibiotic resistant genes from animals of the lower food chain to humans (Meena et al. 2013). This development necessitates the withdrawal of many fish farmers from chemotherapeutic agents to plant and other natural products.

Microalgae are some of the natural products used in aquaculture to enhance growth, used as chemical messengers, serve as mediators of cellular activities and stimulate the innate immunity of fish hence, protecting fish against pathogens (Safı et al. 2014; Ahmad et al. 2020). Microalgae are used in the aquaculture industry as a whole food in shrimp farming and fed to fish at various stages of growth (Roy and Pal 2015). Owing to the high nutritional value, ability to confer protection against diseases, and good antioxidant activity of microalgae, its use as a supplement in aquaculture feed is increasing (Abdulsamad and Varghese 2017; Lim et al. 2021). It is also used as a source of nutrients and pigments in ornamental fish (Roy and Pal 2015; Batista et al. 2017).

Chlorella is one of the widely accepted green algae genera that is used by many as a health food for livestock and aquaculture feeds (Raji et al. 2018), as well as in the drug and cosmetics industries (Sharma et al. 2012; Abdelhamid et al. 2020). *Chlorella vulgaris* stores a beneficial phytonutrient called chlorella growth factor (CGF), which is rich in nucleic acid associated substances such as peptides, proteins, amino acids, vitamins and vital sugars (An et al. 2016). Different studies have reported dynamic effects of *C. vulgaris* at varying supplementation levels in aquaculture feeds. However, supplementation of *C. vulgaris* at higher inclusion levels results in suboptimal growth and other performances in fish (Shields & Lupatsch 2012; Maliwat et al. 2017; Ahmad et al. 2020; Talba et al. 2020). The conflicting reports in fishes fed *Chlorella* supplemented diets might be due to the variation in concentration of the *Chlorella* spp. in diets, the presence or absence of intact cell wall and the length of time the diet was fed to the fish. Therefore, the present study was designed to determine the impact of low-level whole *C. vulgaris* dietary supplementation and the different feeding durations on growth performance, growth hormone and serum-biochemical indices of red hybrid tilapia (*Oreochromis mossambicus* × *O. niloticus*).

2. Materials And Methods

2.1 Experimental fish and management

Two hundred and forty (240) healthy hybrid red tilapia (*Oreochromis* hybrid) fingerlings with an average body weight of 14.25 ± 0.01 g and average body length of 13.5 ± 0.49 cm were procured from a tilapia breeding farm at University Agriculture Park, Puchong, Malaysia. The fish were transported to the experiment site in polythene bags filled with dissolved oxygen. The fingerlings were acclimatised in a glass fibre tank (2000 L capacity), supplied with 800 L dechlorinated freshwater at 27 ± 0.45 C, pH 6.9 ± 0.46, dissolved oxygen (DO) 6.44 ± 0.56 mg L^{-1} (YSI 556 MPS multi-probe system, YSI inc. USA) under 12 hour photoperiod, continuous aeration and water recirculating system for two weeks. The fish were fed twice daily (09:00 h and 17:00 h) using a commercial tilapia feed (Star feed T- 1L, 32% crude protein, Star Feedmill Sdn Bhd, Malaysia) throughout the acclimatisation period.

2.1 Experimental diets, design and feeding

Four experimental diets were formulated (Table 1) using a commercial feed (Star Feedmill Sdn Bhd, Malaysia). Briefly, the feed was grounded and divided into four parts. A portion was used as the control diet (basal diet only). For the *C. vulgaris* supplemented diets, the dry *C. vulgaris* powder (Daesang Corporation, South Korea) was added into the remaining three portions at the following supplementation levels; 1%, 3% and 5% as test diets I, II and III, respectively. The diets were formulated by mechanically stirring the ingredients into a homogenous mixture using a stand mixer (Faber, Malaysia). Cool distilled water was added to achieve a consistency suitable for cold pelletising, using a manual pelletiser (Ajanta, India). Diets were dried in an oven at

37°C for 24 h, as described by Gong et al. (2017). Fish were randomly distributed into twelve tanks (3 tanks for each treatment group) after the completion of acclimatisation, with 20 fish in each tank. The fish were hand fed at 3% of their body weight twice daily at 0930 h and 1700 h. An equal amount of feed was provided to the fish in the morning and afternoon (3% shared into two equal portions for morning and evening, respectively).

Table 1
Composition of the diets and the proximate nutrient/energy content of the control and tests as dry matter basis (mean ± SE).

Ingredients	Control Diet[b]	Test diet I[3]	Test diet II[4]	Test diet III[5]
Basal diet[a]	1000 g	1000 g	1000 g	1000 g
C. vulgaris	-	10 g	30 g	50 g
Proximate analysis				
Dry matter (%)	90.74 ± 0.20	92.56 ± 0.90	92.73 ± 0.73	92.08 ± 0.56
Ash (%)	11.06 ± 1.09	11.88 ± 1.52	11.00 ± 0.34	10.52 ± 1.56
Crude protein (%)	40.15 ± 1.02	40.30 ± 1.05	41.11 ± 2.00	40.8 ± 1.80
Ether extract (%)	10.90 ± 1.50	11.07 ± 1.30	10.95 ± 1.35	11.91 ± 0.71
Crude fibre (%)	7.00 ± 0.46	7.12 ± 1.81	8..02 ± 0.06	8.33 ± 2.04
Gross energy (KJ g^{-1})	17.95 ± 1.40	18.00 ± 1.23	17.02 ± 2.16	18.05 ± 1.68
[a]Basal diet; is a commercial feed (Star Feedmills (M) Sdn. Bhd, Selangor, Malaysia), which was formulated to contain; fish meal, soybean meal, rice bran, broken rice, fish oil, di-calcium phosphate, vitamin premix, and mineral premix. [2]Control diet = Basal diet + 0% *Chlorella vulgaris*,				
[b]Test diet I = Basal diet + 1% *Chlorella vulgaris*, [3]Test diet II = Basal diet + 3% *Chlorella vulgaris*, [5]Test diet III = Basal diet + 5% *Chlorella vulgaris*				

[Insert Table 1]

2.3 Collection of blood

The fish were kept off feed for 24 h before sampling. The fish were anaesthetised using MS-222 (tricaine methane sulphonate at 150 mg l^{-1}). Using a 1 ml plastic syringe, blood was collected from the caudal vein of five randomly selected fish from each tank at 30-day intervals (days 30, 60 and 90). The blood samples were transferred into plain sample vials. The samples were allowed to clot, centrifuged at 5000 g and the sera were collected and kept refrigerated at -80 °C until used. The samples collected were used to estimate growth hormone, hepatic function, some immunological and serum biochemical parameters.

2.4 Fish growth performance, survival and feed utilisation

The growth performance and survival of the fish fed the experimental diets were assayed. The growth performance parameters assayed were: weight gain (WG), absolute growth rate (AGR), specific growth rate (SGR), condition factor (CF), hepatosomatic (HSI) and viscerosomatic (VSI) indices. Whereas feed utilisation parameters taken were: feed conversion ratio (FCR) and mean daily feed intake (MDFI). The calculation was carried out using the following formulas.

$WG\ (g) = W_2 - W_1$

$AGR\ (g/day) = (W_2 - W_1)/t$

$SGR\ (\%\ /day) = [\ln (W_2) - \ln (W_1)/t] \times 100$

$CF\ (g\ cm^{-3}) = (W/L^3) \times 100$

HIS (%) = [liver weight/W] x 100

VSI (%) = [visceral weight/W] x 100

MDFI (g/fish) = (dry feed intake/Number of fish)

FCR = FI (g)/WG (g)

Survival (%) = [Number of survived fish/initial number of fish] x 100

Where W_1 is initial weight (g), W_2 is final weight (g), t is the feeding trial period (days), W is body weight (g), and L is total length (cm).

2.5 Growth hormone assay

The competitive inhibition enzyme immunosorbent assay technique was employed using the fish growth hormone (GH) Elisa kit (Cat. No. CSB-E1212Fh Cusabio Biotech Co., Ltd). Serum samples harvested at days 30, 60 and 90 were used for the assay. Serum GH levels were measured according to the manufacturer's instructions. All reactions were set for triplicates (Lu et al. 2016). Using the computer software elisaanalysis.com, a standard curve was generated using a four-parameter logistic (4-PL) curve fit and the growth hormone concentration computed.

2.6 Determination of serum proteins

Serum samples collected were analysed for total serum protein using the method described by Lowry et al. (1951), albumin content was analysed by methods described by Doumas et al. (1997), while the globulin content was determined by subtracting the serum albumin from the total serum protein.

2.7 Glucose and liver enzymes

The glucose concentration was assayed using a test-kit (Cat. No. 1245016, 1245023) as described elsewhere by Bartoňková et al. (2016). While the serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were carried out according to IFCC (International Federation of Clinical Chemistry and Laboratory Medicine) standard E.C 2.6.1.1, using a test-kit (Cat. No. 1251016, 1251023) as described by Jyotirmayee and Das (2015).

2.8 Respiratory burst activity

The respiratory burst assay was carried out following Anderson and Siwicki (1995) description. Briefly, 100 µl of blood was placed into a microtiter plate wells, and an equal volume of 0.2% NBT solution (life technologies, Ref. N6495, USA) was added to each well. The mixture was incubated for 30 minutes at room temperature. One hundred microliter of NBT-blood mixture was transferred into a glass tube containing 2.0 ml N, N-dimethyl formamide (DMF). The suspension was centrifuged for 5 minutes at 720 g. The supernatant was collected, and the optical density was read in a spectrophotometer (Shimadzu, Uv-1601, Cat. No. 206-67001-93) at 620 nm using a glass cuvette.

2.9 Lysozyme activity

The turbidimetric assay was carried out as described by Anderson and Siwicki (1995). Briefly, a buffer was prepared by dissolving 8.95 g of Na_2HPO_4 (0.05 M) in 500 ml of distilled water. The pH of the buffer was adjusted to 6.2 using concentrated HCl. A bacterial suspension of 0.01% *Micrococcus lysodeikticus* (ATCC No. 4698) was prepared using the buffer. The bacterial suspension (950 µl) was added into a plastic cuvette containing 50 µl of the serum. After mixing, the reduction in turbidity was measured at 0, 1, 2, 3, 4, 5 minutes at 530 nm at 22°C. One unit of the enzyme activity was defined as a decrease in absorbance of 0.001 unit/min.

2.10 Statistical analysis

Statistical analysis was performed using the general linear model (GLM) performed using the IBM SPSS Statistical package version 22.0 (USA). Data were analysed with supplementation level, duration and their two-way interactions as the main effects. Where significant effects were found, a comparison among means was made by Duncan's multiple range test. All statistical procedures were conducted at 95% confidence level.

3. Results

3.1 Weight gain (WG), average growth rate (AGR), specific growth rate (SGR) and condition factor (CF)

Significant interaction ($p < 0.05$) between the supplementation levels and duration of feeding was observed in WG and AGR (Table 2). The supplementation significantly affected the SGR ($p < 0.05$). Similarly, the duration of feeding had a significant effect on the SGR and CF ($p < 0.05$). The SGR values were consistently higher at 5% supplementation level regardless of the feeding duration (Table 2). The SGR outputs were significantly higher ($p < 0.05$) than the control diet (0%) at both the short and long-term duration of feeding. Significantly higher ($p < 0.05$) SGR values were consistently observed at medium and long-term feeding than at short-term feeding (Table 2). In addition, higher CF values were observed at the long-term duration of feeding in all the treatment groups.

Table 2

Differences in weight gain, average growth rate, specific growth rate, condition factor and organo-somatic indices values in red hybrid tilapia (*Oreochromis* spp.) fed diets supplemented with *C. vulgaris* during short (30 days), medium (60 days) and long term (90 days) feeding durations (mean ± SE)

Parameters	Duration (Days)	*C. vulgaris* (%) supplementation levels				Level of significance		
		0	1	3	5	Supplementation	Duration	Supplementation x Duration
Weight gain (g)	Short (30)	24.90 ± 8.05^{bz}	23.36 ± 6.34^{bz}	21.54 ± 4.10^{cz}	28.98 ± 4.78^{az}	< .001	< .001	0.006
	Medium (60)	60.80 ± 8.57^{cy}	61.13 ± 6.02^{cy}	68.73 ± 12.82^{by}	96.34 ± 12.23^{ay}	< .001	< .001	0.006
	Long (90)	100.77 ± 19.28^{dx}	119.51 ± 19.61^{cx}	120.27 ± 22.81^{bx}	140.82 ± 29.29^{ax}	< .001	< .001	0.006
Average growth rate (g)	Short (30)	0.829 ± 0.268^{bz}	0.779 ± 0.211^{cz}	$0.718 \pm 0.13dz$	0.966 ± 0.159^{ay}	< .001	< .001	0.012
	Medium (60)	1.013 ± 0.143^{cy}	1.019 ± 0.100^{cy}	1.146 ± 0.214^{by}	1.606 ± 0.204^{az}	< .001	< .001	0.012
	Long (90)	1.119 ± 0.214^{cx}	1.327 ± 0.218^{bx}	1.336 ± 0.254^{bx}	1.565 ± 0.325^{az}	<.001	< .001	0.012
Specific growth rate (g)	Short (30)	3.29 ± 0.71^{cx}	3.55 ± 0.61^{bx}	3.47 ± 0.38^{bx}	4.20 ± 0.37^{ax}	< .001	< .001	0.179
	Medium (60)	2.7 ± 50.19^{dy}	2.95 ± 0.14^{cy}	3.13 ± 0.26^{by}	3.67 ± 0.17^{ay}	< .001	< .001	0.179
	Long (90)	2.30 ± 0.18^{cz}	2.58 ± 0.17^{bz}	2.60 ± 0.18^{bz}	2.79 ± 0.22^{az}	< .001	< .001	0.179
Condition factor (cm^3)	Short (30)	1.62 ± 0.47^{az}	1.73 ± 0.49^{ay}	1.66 ± 0.45^{ay}	1.71 ± 0.40^{a}	0.942	0.009	0.590
	Medium (60)	1.73 ± 0.31^{acy}	1.76 ± 0.34^{ay}	1.87 ± 0.41^{ax}	1.96 ± 0.47^{ax}	0.942	0.009	0.590
	Long (90)	2.19 ± 0.72^{ax}	1.92 ± 0.17^{ax}	1.91 ± 0.18^{ax}	1.92 ± 0.18^{ax}	0.942	0.009	0.590
Hepatosomatic indices (%)	Short (30)	1.56 ± 0.31^{ax}	1.86 ± 0.52^{ax}	1.39 ± 0.36^{az}	1.47 ± 0.43^{ay}	0.203	< 0.001	0.116
	Medium (60)	1.15 ± 0.59^{ay}	1.39 ± 0.4^{ay}	1.76 ± 0.97^{ax}	1.31 ± 0.23^{ay}	0.203	< 0.001	0.116
	Long (90)	1.62 ± 0.66^{ax}	1.99 ± 0.37^{ax}	1.88 ± 0.73^{ax}	2.22 ± 0.51^{ax}	0.203	< 0.001	0.116
Viscerosomatic index (%)	Short (30)	6.78 ± 1.89^{bxy}	8.35 ± 2.08^{abx}	9.31 ± 1.85^{ax}	8.79 ± 0.99^{abx}	0.044	< 0.001	0.254
	Medium (60)	5.51 ± 1.51^{ay}	7.19 ± 1.99^{ay}	6.92 ± 3.14^{ay}	5.70 ± 1.74^{ay}	0.044	< 0.001	0.254
	Long (90)	7.60 ± 2.15^{ax}	7.57 ± 1.13^{ay}	7.59 ± 1.00^{ay}	7.11 ± 0.74^{ax}	0.044	< 0.001	0.254

a - d superscript, means in the same row with different letters are significantly different at $p < 0.05$.

x - z superscript, means in the same column with different letters are significantly different at $p < 0.05$.

3.2 Hepatosomatic and viscerosomatic indices

No significant interaction was observed (p 0.05) between the supplementation levels and duration of feeding in all the treatment groups. However, the supplementation level significantly affected the viscerosomatic index ($p < 0.05$). While the duration of feeding had a significant effect ($p < 0.05$) on both the hepatosomatic and viscerosomatic indices. Viscerosomatic index was significantly higher ($p < 0.05$) at 3% *C. vulgaris* inclusion level during the short-term feeding than the control group. The effects of the duration of feeding on both hepatosomatic and viscerosomatic indices were observed during the feeding periods, with long-term feeding presenting consistently higher hepatosomatic indices values (Table 2).

[Insert Table 2]

3.3 Feed conversion ratio (FCR) and mean daily feed intake (MDFI)

A significant interaction was observed in MDFI between the supplementation levels and the duration of feeding ($p < 0.05$), where both factors affected the MDFI value. The supplementation and duration of feeding had a significant effect ($p < 0.05$) on the FCR. Significantly higher FCR were observed at 0, 1 and 3% supplementation levels than at the 5% during the medium-term feeding. Irrespective of the supplementation levels, the FCR was significantly higher during the early days of the feeding trial (short-term). In addition, as the duration of feeding expands, the medium-term feeding presented a significantly higher FCR than the long-term feeding (Table 3)

Table 3
Differences in feed conversion ratio, mean daily feed intake, survival rate and growth hormone (GH) of red hybrid tilapia (*Oreochromis* spp.) fed diets supplemented with *C. vulgaris* during short (30 days), medium (60 days) and long term (90 days) feeding durations (mean ± SE)

Parameters	Duration (Days)	*C. vulgaris* (%) supplementation levels				Level of significance		
		0	1	3	5	Supplementation	Duration	Supplementation x Duration
Feed conversion ratio	Short (30)	1.15 ± 0.42^{ax}	1.28 ± 0.48^{ax}	1.36 ± 0.25^{ax}	1.00 ± 0.17^{ax}	0.004	< 0.001	0.486
	Medium (60)	0.72 ± 0.10^{ay}	0.82 ± 0.09^{ay}	0.81 ± 0.15^{ay}	0.59 ± 0.06^{by}	0.004	< 0.001	0.486
	Long (90)	0.68 ± 0.12^{ay}	0.68 ± 0.12^{az}	0.70 ± 0.11^{az}	0.65 ± 0.14^{az}	0.004	< 0.001	0.486
Mean daily feed intake	Short (30)	0.39 ± 0.02^{bz}	0.45 ± 0.01^{az}	0.48 ± 0.01^{az}	0.47 ± 0.01^{az}	< 0.001	< 0.001	< 0.001
	Medium (60)	0.60 ± 0.02^{dy}	0.75 ± 0.04^{cy}	0.88 ± 0.03^{by}	0.97 ± 0.03^{ay}	< 0.001	< 0.001	< 0.001
	Long (90)	0.94 ± 0.05^{cx}	1.20 ± 0.04^{abx}	1.13 ± 0.08^{bx}	1.28 ± 0.09^{ax}	< 0.001	< 0.001	< 0.001
Survival rate (%)	Short (30)	93.33 ± 4.36^{bx}	98.10 ± 1.65^{ax}	96.19 ± 1.65^{ax}	98.10 ± 1.65^{ax}	0.005	< 0.001	0.999
	Medium (60)	85.71 ± 2.86^{by}	92.38 ± 1.65^{ay}	89.52 ± 5.95^{ay}	92.38 ± 4.36^{ay}	0.005	< 0.001	0.999
	Long (90)	85.71 ± 2.86^{by}	92.38 ± 1.65^{ay}	89.52 ± 5.95^{ay}	92.38 ± 4.36^{ay}	0.005	< 0.001	0.999
Growth hormone (pg)	Short (30)	1381.59 ± 277.48^{cx}	1532.75 ± 199.20^{bx}	1746.74 ± 117.92^{ax}	1763.10 ± 227.80^{ax}	0.001	< 0.001	0.999
	Medium (60)	1121.23 ± 10.93^{cy}	1253.50 ± 95.00^{by}	1442.29 ± 339.47^{ay}	1480.99 ± 251.17^{ay}	0.001	< 0.001	0.999
	Long (90)	969.51 ± 89.99^{dz}	1100.76 ± 199.89^{cz}	1245.48 ± 80.97^{bz}	1379.44 ± 156.99^{az}	0.001	< 0.001	0.999
a - d superscript, means in the same row with different letters are significantly different at $p < 0.05$.								
x - z superscript, means in the same column with different letters are significantly different at $p < 0.05$								

3.4 Survival rate (SR) and growth hormone (GH)

The supplementation and duration of feeding had a significant effect ($p < 0.05$) on the survival rate and GH levels ($p < 0.05$). The survival rate at all the supplementation levels during short, medium and long-term feeding was significantly higher ($p < 0.05$) than the control. On the contrary, the short-term feeding had a higher survival rate ($p < 0.05$) in both the control and microalgae supplemented groups. Significantly higher ($p < 0.05$) GH levels were observed at 3 and 5% supplementation levels than at the 1% and control (0%) during short, medium and long-term feeding. In addition, at 1% the GH levels were significantly higher than the control. The highest levels of GH were observed in the short-term feeding, followed by the medium term. Long-term feeding presented the lowest growth hormone levels (Table 3).

[Insert Table 3]

3.5 Serum proteins

There was a significant interaction ($p < 0.05$) between the supplementation levels and duration of feeding on total serum protein, globulin and AG ratio outputs ($p < 0.05$). Total serum protein values were consistently higher ($p < 0.05$) than the control at 5% supplementation levels throughout the feeding. Significantly higher ($p < 0.05$) total serum protein levels were observed at 3% supplementation level during the short-term feeding. The values were higher ($p < 0.05$) than the other supplementation levels and the control. The total serum protein levels were significantly higher ($p < 0.05$) at 1 and 3% supplementation levels during the medium-term feeding than in the control. Similarly, the total serum protein values during the long-term feeding were significantly higher ($p < 0.05$) at 1 and 3% supplementation levels than the values observed in the control group. Significantly higher albumin values were observed at 5% supplementation level when compared with control during the short, medium and long-term feeding. At 3% supplementation, the albumin

values were consistently higher ($p < 0.05$) than in the control group throughout the trial. The globulin values were significantly higher ($p < 0.05$) than the control during the medium and long-term feeding at all the supplementation levels. In addition, the total serum protein, albumin and globulin values significantly increased as the period of the trial extended. During the short and medium-term feeding, the albumin/globulin ratio was not significantly different, except for 5% supplementation level, which was lower during the medium-term than the short-term feeding. The albumin/globulin ratio values stabilised during the long-term feeding, irrespective of the diet fed (Table 4).

Table 4
Differences in serum biochemistry in red hybrid tilapia (*Oreochromis* spp.) fed diets supplemented with *C. vulgaris* during short (30 days), medium (60 days) and long term (90 days) feeding durations (mean ± SE)

Parameters	Duration (Days)	*C. vulgaris* (%) supplementation levels				Level of significance		
		0	1	3	5	Supplementation	Duration	Supplementation x Duration
Total plasma protein (g/L)	Short (30)	31.24 ± 4.16^{cy}	31.36 ± 3.07^{cz}	34.48 ± 2.36^{az}	33.22 ± 2.24^{bz}	< 0.001	< 0.001	0.009
	Medium (60)	31.88 ± 2.23^{cy}	37.60 ± 4.03^{by}	38.16 ± 1.16^{by}	45.94 ± 5.57^{ax}	< 0.001	< 0.001	0.009
	Long (90)	36.28 ± 2.06^{cx}	42.68 ± 3.37^{ax}	41.64 ± 4.41^{bx}	42.80 ± 4.61^{ax}	< 0.001	< 0.001	0.009
Albumin (g/L)	Short (30)	9.88 ± 1.55^{cy}	10.26 ± 0.90^{cz}	12.18 ± 2.03^{ax}	11.32 ± 0.67^{by}	< 0.001	< 0.001	0.433
	Medium (60)	10.06 ± 0.98^{cy}	12.30 ± 0.77^{aby}	12.98 ± 0.15^{ax}	11.92 ± 0.44^{by}	< 0.001	< 0.001	0.433
	Long (90)	13.22 ± 0.37^{bx}	15.42 ± 1.45^{ax}	15.38 ± 1.48^{ax}	15.64 ± 1.92^{ax}	< 0.001	< 0.001	0.433
Globulin (g/L)	Short (30)	21.36 ± 2.98^{ax}	21.10 ± 2.41^{az}	22.30 ± 3.69^{ax}	21.90 ± 1.79^{az}	< 0.001	< 0.001	0.002
	Medium (60)	21.82 ± 2.00^{cy}	25.30 ± 3.35^{by}	25.18 ± 1.05^{bx}	34.02 ± 5.96^{ax}	< 0.001	< 0.001	0.002
	Long (90)	23.06 ± 1.69^{cx}	27.26 ± 2.10^{ax}	26.26 ± 3.10^{ax}	27.16 ± 2.89^{ax}	< 0.001	< 0.001	0.002
A:G (unit)	Short (30)	0.47 ± 0.06^{dy}	0.49 ± 0.04^{cy}	0.57 ± 0.15^{ax}	0.52 ± 0.04^{by}	0.020	< 0.001	0.039
	Medium (60)	0.46 ± 0.06^{cy}	0.49 ± 0.04^{by}	0.52 ± 0.02^{az}	0.36 ± 0.08^{dz}	0.020	< 0.001	0.039
	Long (90)	0.58 ± 0.03^{ax}	0.57 ± 0.03^{ax}	0.59 ± 0.04^{ax}	0.58 ± 0.04^{ax}	0.020	< 0.001	0.039
Glucose (mmol/L)	Short (30)	4.28 ± 1.26^{ax}	3.24 ± 0.63^{ax}	3.28 ± 0.35^{ax}	3.44 ± 0.35^{ax}	0.001	< 0.001	0.642
	Medium (60)	1.88 ± 0.24^{ay}	1.28 ± 0.73^{az}	1.82 ± 0.45^{az}	1.36 ± 0.23^{az}	0.001	< 0.001	0.642
	Long (90)	4.14 ± 1.49^{ax}	2.66 ± 0.23^{by}	2.90 ± 0.51^{by}	2.96 ± 0.76^{by}	0.001	< 0.001	0.642
Aspartate aminotransferase (u/L)	Short (30)	89.20 ± 31.73^{ax}	93.00 ± 9.46^{ax}	95.20 ± 14.08^{ax}	9620 ± 22.53^{ax}	0.216	< 0.001	0.443
	Medium (60)	86.80 ± 22.19^{ax}	73.20 ± 9.09^{ax}	84.20 ± 24.81^{ax}	89.60 ± 7.16^{ax}	0.216	< 0.001	0.443
	Long (90)	64.67 ± 21.18^{ax}	57.33 ± 16.00^{az}	65.00 ± 24.71^{az}	62.33 ± 16.28^{az}	0.216	< 0.001	0.443
Alanine aminotransferase (u/L)	Short (30)	4.20 ± 1.79^{az}	5.80 ± 1.92^{az}	4.40 ± 1.14^{az}	3.40 ± 1.67^{az}	0.635	< 0.001	0.273
	Medium (60)	12.80 ± 3.12^{ax}	12.40 ± 3.98^{ax}	12.20 ± 2.77^{ax}	11.20 ± 3.56^{ax}	0.635	< 0.001	0.273
	Long (90)	22.50 ± 4.51^{ax}	17.50 ± 4.68^{ax}	20.67 ± 4.08^{ax}	21.00 ± 1.41^{ax}	0.635	< 0.001	0.273

a - d superscript, means in the same row with different letters are significantly different at $p < 0.05$.

x - z superscript, means in the same column with different letters are significantly different at $p < 0.05$

The supplementation level had a significant effect ($p < 0.05$) on the glucose level. Similarly, the duration of feeding had also shown a significant effect ($p < 0.05$) on the glucose, AST and ALT levels. The control group presented a significantly higher glucose level than all the supplementation levels during the

long-term feeding. Overall, the short duration of feeding showed significantly higher ($p < 0.05$) glucose levels than the medium and long durations. While the long-term feeding presented significantly higher ($p < 0.05$) glucose level values than the medium term feeding at all the supplementation levels. The AST values were significantly higher ($p < 0.05$) across all groups during the early days of feeding (short-term), but the values significantly decreased as the feeding duration widened. The ALT values were significantly lower across all groups during the short-term feeding, but the values significantly increased as the feeding duration expanded (Table 4).

[Insert Table 4]

3.6 Respiratory burst and lysozyme activities

While there was no significant interaction ($p < 0.05$) between the supplementation levels and duration of feeding on respiratory burst and lysozyme activities. However, on individual basis the supplementation level and feeding duration had a significant effect (p 0.05) on the respiratory burst and lysozyme activities. Significantly higher ($p < 0.05$) respiratory burst activity was observed at 5% supplementation level across all the different feeding durations. At 1% and 3% supplementation levels, the respiratory burst activity values were significantly higher ($p < 0.05$) than the control during the short and long-term feeding. In addition, the respiratory burst activity at 3% supplementation level was significantly higher ($p < 0.05$) during the medium-term feeding than... Similarly, significantly higher ($p < 0.05$) lysozyme activity was observed at 5% supplementation level, which was greater than those in control and 1% supplementation level during the short and medium-term feeding. The same trend was observed during long-term feeding, being significantly higher than 3% supplementation level. Irrespective of the type of diet fed to fish the values of respiratory burst and lysozyme activities were significantly higher ($p < 0.05$) during the long-term feeding than during the short and medium-term feeding (Table 5).

Table 5
Differences in lysozyme and respiratory burst activity in red hybrid tilapia (*Oreochromis* spp.) fed diets supplemented with *C. vulgaris* during short (30 days), medium (60 days) and long term (90 days) feeding durations (mean ± SE)

Parameters	Duration (Days)	*C. vulgaris* (%) supplementation levels				Level of significance		
		0	1	3	5	Supplementation	Duration	Supplementation x Duration
Respiratory burst activity (O.D/mg)	Short (30)	0.3709 ± 0.03^{cy}	0.3867 ± 0.04^{bz}	0.4095 ± 0.05^{abz}	0.4433 ± 0.03^{az}	< 0.001	< 0.001	0.112
	Medium (60)	0.5018 ± 0.00^{cx}	0.5360 ± 0.01^{by}	0.5673 ± 0.01^{ay}	0.5887 ± 0.05^{ay}	< 0.001	< 0.001	0.112
	Long (90)	0.5066 ± 0.01^{bx}	0.5873 ± 0.01^{bx}	0.6146 ± 0.03^{abx}	0.6205 ± 0.03^{ax}	< 0.001	< 0.001	0.112
Lysozyme activity (U/ml)	Short (30)	159.05 ± 55.75^{by}	148.20 ± 45.13^{by}	205.51 ± 84.43^{aby}	249.69 ± 66.36^{ay}	< 0.001	< 0.001	0.972
	Medium (60)	169.97 ± 50.87^{by}	171.87 ± 43.45^{by}	205.28 ± 84.93^{aby}	256.58 ± 66.64^{ay}	< 0.001	< 0.001	0.972
	Long (90)	237.27 ± 32.24^{bx}	269.37 ± 44.95^{bx}	280.05 ± 25.18^{bx}	348.57 ± 84.18^{ax}	< 0.001	< 0.001	0.972
a - d superscript, means in the same row with different letters are significantly different at $p < 0.05$.								
x - z superscript, means in the same column with different letters are significantly different at $p < 0$.								

[Insert Table 5]

4. Discussion

The present study demonstrated the effects of dietary *C. vulgaris* supplementation and duration of feeding on growth performance, growth hormone levels, feed utilisation, and some biochemical parameters of red hybrid tilapia. Certain levels of interactions were observed between the supplementation levels and the duration of feeding. From the results (Table 2) there was significant interaction ($p < 0.05$) between supplementation levels and duration of feeding on weight gain (WG) and average growth rate (AGR) of the fish. The values were positively affected (increased weight gain) by increasing the supplementation levels during the medium and long durations of feeding. Similarly, the supplementation levels showed a significant effect ($p < 0.05$) on the fish's specific growth rate (SGR) and survival rate (SR). The highest effect was seen at 5% supplementation levels ($p < 0.05$) for the specific growth rate. Although the values for the specific growth rate decreased with extension of the feeding duration, the findings were in concordance with Enyidi's (2017) report. According to Enyidi, African catfish (*Clarias gariepinus*) subjected to medium-term feeding with a *C. vulgaris* supplemented diet (5% kg^{-1} of feed), showed a significantly higher weight gain, and specific growth rate. The findings were further supported by a study on olive flounder (*Paralichthys olivaceus*), which revealed the favourable effect of medium-term feeding with the *Chlorella* diet on weight gain and specific growth rate (Rahimnejad and Lee 2016). Similarly, Sergejevová and Masojídek (2012), observed that 2.5% *Chlorella* biomass dietary supplementation results in a significantly higher specific growth rate in starlet (*Acipenser ruthenus*) following a medium-term experimental feeding. In contrast, the short-term feeding of Nile tilapia (*Oreochromis niloticus*) with ≥ 30% *C. vulgaris* supplemented diet showed an unfavourable effect on daily weight gain and specific growth rate (Lupatsch and Blake 2013). The effect reported is not in line with the effects observed in the present study, as short-term feeding of 5% *C. vulgaris* presented an

appreciable effect on weight gain and specific growth rate. The conflicting effect may be due to the dietary supplementation levels of *C. vulgaris*, as higher inclusion levels of alternative proteins could result in decreased growth performance (Lupatsch and Blake 2013; Esmaeili 2021), perhaps due to low digestibility of the alternative protein sources.

Organo-somatic indices are part of the indicators of fish well-being (Kiron et al. 2016). In spite of the significant effect of duration of feeding on condition factor (CF) and hepatosomatic index (HSI), there was no significant effect (p 0.05) of supplementation levels on CF and HSI of the fish. The finding did not correspond with that of a study by Enyidi (2017), who observed a decrease in HSI of African catfish fed diet supplemented with 5% *C. vulgaris.* However, the finding corroborates with Kim et al. (2013), who reported that medium-term feeding with diets whose fishmeal was partially replaced with 15% *Spirulina pacifica* did not significantly affesct the HSI values of parrot fish (*Oplegnathus fasciatus*). However, the supplementation levels showed a significant effect on the viscerosomatic index (VSI) during the short-term feeding, but the effect diminishes and blends with that of the control group at the end of medium and long-term feedings, respectively. The finding is in line with that of Kiron et al. (2016), who observed VSI values that were not significantly different from the control after feeding Atlantic salmon (*Salmo salar)* with 10% *Desmodesmus* sp. supplemented diet for medium-term feeding. Therefore, the experimental fish in the current study efficiently utilise the *Chlorella* supplemented diet with no accompanied untoward effect on organo-somatic indices.

The supplementation level had significantly improved the feed utilisation ($p \leq 0.05$) of the red hybrid tilapia. The feed conversion ratio (FCR) of the Chlorella supplemented fish was not significantly different from the control during the short-term and long-term feeding trials, although, a lower FCR was seen at 5% supplementation level during the medium-term feeding. Nevertheless, the findings are in agreement with those of Enyidi (2017), and Lupatsch and Blake (2013), who reported significantly higher ($p < 0.05$) FCR in African catfish and Nile tilapia fed diets supplemented with *C. vulgaris* at 5 and ≥ 30% inclusion levels, respectively. Similarly, supplementation levels have showed a favourable effect on mean daily feed intake (MDFI). The MDFI increases with increased supplementation levels, with the highest value recorded at 5% supplementation level. The present finding seems to be consistent with the one reported by Lupatsch and Blake (2013), who reported that the daily feed intake of tilapia increases with the increased dietary inclusion of *C. vulgaris*. A study by Gouveia et al. (1996) reported that, a medium-term (67 days) dietary supplementation of *C. vulgaris* produces feed intake and weight gain values comparable to those produced by other feeds rich in pigments and vitamins. Therefore, the inclusion of *C. vulgaris* in fish diet could increase its acceptability in the diet.

Growth hormone in fish is associated with diverse roles in different tissues and cell types. The hormone mediates homeostasis, immunological pathways, reproduction, behavioural activities and neuroendocrine functions (Jönsson and Bjornsson, 2002). The current work presented a significant effect of dietary supplementation of *C. vulgaris* on the growth hormone (GH) concentration of red hybrid tilapia. The levels of the GH increase with increased supplementation levels. Similarly, a study by Khan et al. (2015) revealed an elevated serum GH concentration in juvenile mahseer (*Tor putitora*) supplemented with a graded level of L-Ascorbyl-2-Polyphosphate over a longer duration of feeding. Miao et al. (2012) reported the positive impact of exogenous growth hormone on body length and weight gain of Nile tilapia (*Oreochromis niloticus*). Similarly, the duration of feeding showed a significant effect on GH levels. The serum GH concentration drops as the feeding period extends, with the long-term feeding presenting the lowest GH concentrations across both the control and the treatment groups. The trend is directly proportional to FCR and SGR reported earlier. The present finding is in line with the report by Won & Borski, (2013), who reported that there is an increased secretion of GH during hyper-anabolism, which stimulates protein assimilation and raises the FCR and SGR values as a result of an elevated feeding. The secreted GH binds its receptor (growth hormone receptor GHR) and triggers the production of hepatic insulin-like growth factor I (IGF I). This increases somatic growth and ultimately sends negative feedback on growth hormone secretion and hence a drop in the GH concentration (Won and Borski 2013).

Certain plant products have a history of enhancing total serum protein and globulin levels in fish, which signals immune system activation (Das et al. 2009). There was a significant interaction effect between the supplementation levels and duration of feeding on total serum protein (TP) and albumin globulin ratio (A:G) values. These effects were more glaring at 5% supplementation levels during the medium and long-term feeding. Similarly, Xu et al. (2014) observed an elevated level of total protein in gibel carp (*Carassius auratus gibelio*) fed diets supplement with lower inclusion levels of *Chlorella*, following a medium-term feeding (60 days). Likewise, Das et al. (2009) observed an increase in total protein levels in rohu (*Labeo rohita)* fed diets supplemented with *Euglena viridis* at ≤ 1g/ kg after a long-term feeding. There was significant interaction ($p < 0.05$) effect between the supplementation levels and feeding duration on serum globulin concentration. The trend of globulin values was the same at 1 and 3% supplementation levels at the medium and long-term feeding. The highest globulin value was observed at 5% supplementation level during the medium-term feeding. An elevated globulin concentration could depict the capability of the *Chlorella s*upplemented diets to enhance the mononuclear phagocyte system, hence an improved immune response (Kumar et al. 2012). The result of the present study concerning higher globulin values in the *Chlorella* supplemented diet is similar to the findings of Xu et al. (2014); that medium-term feeding of gibel carp with Chlorella supplemented diets elevates the levels of globulin in the fish serum. The result also confirmed the observation of Das et al. (2009), who observed increased serum globulin levels following feeding rohu (*Labeo rohita*) diets supplemented with *Euglena viridis* over short, medium and long-terms feeding.

Nevertheless, the serum albumin concentration of the red tilapia supplemented with *Chlorella* differed from total protein and globulin concentrations by not presenting any interaction effect between the supplementation levels and feeding duration. The significant effect of supplementation on the albumin concentration increased with the increased supplementation level. The result was more prominent during the medium and long-term feeding. In addition, there was a significant effect of duration of feeding on albumin levels; the effect was conspicuous at long-term feeding. Similarly, short and medium-term supplementation of *Chlorella* spp. could not cause a marked change in albumin levels of gibel carp (Xu et al. 2014). In addition, Saberi et al. (2017) suggested that only long-term feeding with low-level *C. vulgaris* supplementation could cause a marked change in albumin concentration in tilapia. Interestingly, the result conforms with Haghighi et al. (2017) findings in rainbow trout (*Oncorhynchus mykiss*) fed diets supplemented with *Aloe vera* at

lower inclusion levels over a long duration of feeding. Therefore, long-term feeding with *C. vulgaris* supplemented diet could result in higher total serum protein concentration in tilapia.

The welfare and productivity of fish are dependent on the level of stressors in fish, and a rise in the level of blood glucose is one of the bio-indicators of heightened stress in fish (Jiang et al. 2017). The present study indicated the significant effect of supplementation levels of *C. vulgaris* on the serum glucose level of the experimental fish. After a long feeding duration, the dietary *C. vulgaris* supplementation significantly lowered the serum glucose level, relative to the control group's values. The feeding duration has also shown an effect on glucose concentration, with the lowest glucose concentration values observed at the medium-term feeding irrespective of the supplementation levels. The result is in concordance with that of Rahimnejad and Lee (2016), who reported that medium-term feeding on diets supplemented with *C. vulgaris* at 5–10% does not significantly affect the plasma glucose concentration of olive flounder (*Paralichthys olivaceus*). In addition, the findings of the current study are in line with that of Koo et al. (2001). They observed a significantly lower serum glucose level in Juvenile Olive Flounder (*Paralichthys olivaceus*) fed diets supplemented with *C. vulgaris* at 2–4% for long-term feeding. The result also agrees with the findings of Henrique et al. (1998). The authors noticed that long-term feeding of seabream (*Sparus aurata*) with vitamin C supplemented diet significantly lowers the plasma glucose level. Therefore, long-term feeding of fish with diets supplemented with *C. vulgaris* could reduce the stress in fish.

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are the main liver enzymes used as bio-indicators of the functionality of a liver. An increase in the serum concentration of these enzymes is signalling damage to the liver, muscles, kidney and/or gills (Jyotirmayee and Das 2015). The supplementation levels did not affect the serum AST and ALT levels in the present study. The finding is similar to that of Rahimnejad and Lee (2016), who observed that medium-term feeding of Olive Flounder (*Paralichthys olivaceus*) with *C. vulgaris* diet at 5–15% supplementation levels showed no significant effect on AST and ALT serum levels. Although, there was a significant effect of duration of feeding on liver enzymes levels, with the AST serum levels appearing significantly higher during the short-term feeding, as the period of feeding extended, the concentration decreased. On the other hand, the ALT serum concentration was lower during the short-term feeding, and the value increased as the duration of feeding extend. The trend is similar to the pattern observed in the control group of rohu (*Labeo rohita*) fed *C. vulgaris* diet for 90 days (long-term feeding) (Jyotirmayee and Das 2015). AST is found in many organs such as; the liver, pancreas, kidneys, cardiac muscle, brain, lungs, white blood cells and red blood cells, while, ALT is present in abundance in the liver (Küpeli et al. 2006). Therefore, the dietary supplementation of *C. vulgaris* in the current study appeared safer by not causing an observable damaging effect on the vital organs of red hybrid tilapia.

Respiratory burst activity is mainly mediated by neutrophils and macrophages (Uribe et al. 2011). Neutrophils are the first cells deployed when there is an inflammatory reaction and are integral components of innate immunity in teleost. In addition, they also limit the abundance of several pathogens (Sowmya and Sachindra 2013). These cells eliminate bacteria during the respiratory burst via the production of reactive oxygen species that are toxic to the pathogenic agents (Uribe et al. 2011). The result of the present study confirmed the significant effect of the supplementation levels of *C. vulgaris* on the respiratory burst activity of tilapia. The activity steadily increased with the extension of the feeding period; the long-term feeding presented the highest respiratory burst activity values. Medium-term feeding with *C. vulgaris* supplemented diets enhances the free radicals scavenging activity of Olive Flounder (*Paralichthys olivaceus*) (Rahimnejad and Lee, 2016). Similarly, medium-term feeding of a diet supplemented with garlic enhances the respiratory burst activity of Nile tilapia (*Oreochromis niloticus*) (Aly et al. 2008).

Lysozyme is a bactericidal enzyme identified in various fishes. The enzyme is present in mucus secretion/membranes, lymphoid tissue, and serum of most aquatic vertebrates. Lysozymes are mainly found in monocytes/macrophages and neutrophils. The enzyme act by hydrolysing the peptidoglycans of bacterial cell walls, eventually resulting in cell lysis. In addition, it also acts via the opsonisation pathway (Uribe et al. 2011; Hayball and Puccetti 2012). Moreover, its activity is not related to other immune substances (Hayball and Puccetti 2012). The current study revealed the significant effect of supplementation levels on serum lysozyme activity, with a 5% supplementation level being consistently higher during short, medium and long-term feeding. Similarly, the duration of feeding has indicated an effect on lysozyme activity. Although, the lysozyme activities were not significantly different in the short and medium-term feeding, but significantly higher in long-term feeding. A similar result of elevated lysozyme activity was seen in rohu (*Labeo rohita*) fed diets supplemented with a microalgae (*E. viridis*), the effect persisted throughout the short, medium and long durations of feeding (Das et al. 2009). correspondingly, long-term feeding with diets supplemented with *Dunaliella salina*, significantly increased the lysozyme concentration of *Oncorhynchus mykiss* (Amar et al. 2004). Long-term feeding of diets supplemented with carotenoid elevates the serum lysozyme activity of common carp, *Cyprinus carpio* (Sowmya and Sachindra 2013). The same trend was observed following medium-term feeding of koi carp (*Cyprinus carpio*) with diets supplemented with *C. vulgaris* at 2–10% (Khani et al. 2017).

Conclusions

The current study showed the significant effect of *C. vulgaris* supplementation on growth performance and feed utilisation in red hybrid tilapia. The effect increases with a corresponding increase in the feeding duration. Besides, the diets did not negatively affect the fish's organo-somatic indices and liver enzymes. The study also revealed the positive effect of diets on growth hormone concentration. The growth hormone levels dropped with the extension of the feeding period, with the least value recorded during long-term feeding. Moreover, the diets showed an improved effect on TP and globulin levels of the fish. The effects were more pronounced at 5% supplementation levels for the medium and long-term feeding periods. The diets also improved the respiratory burst activity, with the effect increasing with the feeding period extension.

Declarations

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Data availability

All datasets analysed in this study are available from the corresponding author on reasonable request.

Ethical approval

The Institutional Animal Care and Use Committee (IACUC) of the Universiti Putra Malaysia (UPM/IACUC/AUP - R052/2016) reviewed and approved the experimental protocol.

Declaration of interest

The authors have no competing interests to declare that are relevant to the content of this article.

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