



Dietary Open Water *Chlorella vulgaris* Meal Modulates Growth, Antioxidant Defenses and Metabolic Responses of *Clarias magur*

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Abstract

The present study evaluated the open raceway-cultured *Chlorella vulgaris* meal (CVM) as an ingredient in the diet of *Clarias magur* fingerlings. Five iso-nitrogenous (35% crude protein), isolipidic (6%), and isoenergetic (350 kcal digestible energy/100 g) experimental diets by replacing fish meal (FM) gradually with *C. vulgaris* meal (CVM) on protein equivalent basis viz., C (Control with 20% FM, 0% CVM), T₂₅ (15% FM, 7.30% CVM), T₅₀ (10% FM, 14.60% CVM), T₇₅ (5% FM, 21.80% CVM), and T₁₀₀ (0% FM, 29.13% CVM) were formulated. A total of 150 acclimatised fingerlings were randomly distributed among five treatments (C, T₂₅, T₅₀, T₇₅, and T₁₀₀) in triplicate using 15 experimental tanks at a stocking density of 10 fish/200 L tank and fed for 60 days. The 50% fishmeal replacement group showed the highest growth, with 11–12% greater ($p < 0.05$) final body weight and weight gain than the control, while the 75% replacement group maintained comparable growth. Activities of amylase, ALT, LDH, and SOD were unaffected, whereas those of chymotrypsin, ALP, ACP, MDH, and catalase increased in CVM-fed groups (50%, 75% & 100%). Overall, inclusion of 21.8% open raceway-cultured *C. vulgaris* effectively replaced 75% of fishmeal without compromising growth or physiological health, supporting its use as a sustainable aquafeed ingredient.

Keywords: Open water algal culture, sustainable aquaculture, digestive enzymes, alternative protein source, fishmeal replacement

Introduction

Global population is projected to reach 9.7 billion by 2050, requiring food production to rise by 25–60% (Chouhan et al., 2021). Fisheries must help meet this demand by using water resources efficiently. While capture fisheries have plateaued, aquaculture is the fastest-growing food sector, with global fish production reaching 184.6 million tonnes in 2022, a 17% increase over the past decade (Statista Research Department, 2023). It is estimated that global fish production will reach 209 million tonnes by 2030 (FAO, 2018). Feed-based intensified aquaculture could be one of the essential options to achieve this (Tacon, Metian, & McNevin, 2022). However, feed cost usually contributes 60–70% of the operational expenses in feed-based aquaculture; thus, this practice should be carried out judiciously to make this venture sustainable (Kari et al., 2022). Fishmeal (FM) remains the preferred aquafeed ingredient due to its high-quality protein, balanced essential amino acids, digestibility, and growth-promoting factors (Maisashvili et al., 2015). However, increasing aquaculture demand, reliance on wild fisheries, stagnant capture production, competition from livestock feeds, rising costs, and declining quality have created major economic and sustainability challenges for aquafeed production (Tacon & Metian, 2008). Consequently, considerable research has focused on identifying sustainable, reasonably priced, and nutritionally adequate fishmeal substitutes with

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high protein content and balanced fatty acid profiles, particularly for carnivorous fish.

Plant-protein sources have been preferred as FM alternatives due to their low cost, high protein content, and regular availability. In this regard, soybean meal has been widely accepted as an alternative to FM for aquafeed. However, an imbalanced essential amino acid profile, high fibre content, and the presence of anti-nutritional factors have limited its use in aquafeed (Owen, Jayasuriya, & Preston, 1993). Thus, researchers are evaluating other plant protein sources as alternatives to FM. Due to their favourable nutritional composition, researchers have emphasized evaluating microalgae as an alternative to FM in aquafeeds. Apart from being a rich source of protein and essential amino acids, microalgae also contain carotenoids and omega-3 fatty acids to positively influence growth, lipid metabolism, immunity and disease resistance, skin colouration, and carcass quality in fish (Raja et al., 2018).

In this regard, *C. vulgaris* (CV) has gained attention as a fishmeal alternative due to its high protein content, balanced amino acid profile, essential fatty acids, vitamins, and minerals (Alagawany, Taha, Noreldin, El-Tarabily, & El-Hack, 2021; Parashar, Shah, Rane, & Shastri, 2023). Dietary inclusion of CV improved growth and pigmentation in largemouth bass and enhanced growth in olive flounder (Rahimnejad, Lee, Park, & Choi, 2017; Xi et al., 2022). However, higher inclusion levels reduced growth in tilapia and showed limited benefits in sterlet sturgeon (Sergejevoá & Masojídek, 2012; Teuling, Schrama, Gruppen, & Wierenga, 2017). In addition, the main limitation of using algal meal, including CVM, is its availability on a large scale. Large-scale cultivation in open water systems could be a feasible strategy to improve its availability. But environmental variation and nutrient composition of the culture medium influence the nutrient composition of CV (Wild et al., 2019). Open water culture would be feasible for establishing mass production of CV. As the nutritional composition and bioactive compound content of CV vary with the culture environment, it is essential to evaluate its effect in aquafeeds.

The endangered freshwater air-breathing omnivorous fish with a preference towards animal matter, *C. magur*, is highly valued in the Indian subcontinent for its high protein and PUFA content, and

therapeutic qualities (Borah, 2020; Bhandarkar & Paliwal, 2022). Its capacity to produce long-chain PUFA from C18 fatty acids makes it an appropriate model for assessing algal meal as a fishmeal substitute as its culture grows to meet demand. Hence, the present study aimed to evaluate open-system cultivated *C. vulgaris* meal as a fishmeal alternative in the diet of *C. magur* fingerlings with respect to growth, nutrient utilisation, body composition, and the activities of digestive, metabolic, and antioxidant enzymes.

Materials and Methods

C. vulgaris obtained from the DBT–Pan IIT Centre for Bioenergy, IIT Bombay, India, was progressively scaled up from 100 mL laboratory cultures to a 4000 L pilot-scale open raceway pond using BG-11 medium (Stanier, Kunisawa, Mandel, & Cohen-Bazire, 1971). A 10% inoculum from each batch was used for subsequent cultures. The pond was covered with a net and mixed continuously using a motor-driven paddle to prevent settling. Water losses were compensated twice weekly. Cultivation was carried out from February to May 2024 under natural sunlight (24–37 °C). Biomass concentration was monitored weekly following the protocol of Parashar et al. (2023).

Once the cultivation cycle was completed after 28 days, the biomass was allowed to settle under gravity for 24 hours. Excess water was removed from the pond using a pump. The biomass-rich slurry left behind was collected, washed with water twice, and kept for natural drying in a shaded area for 2 days. The dried biomass thus obtained was then ground to a fine powder using a kitchen grinder and subjected to nutritional profiling and feed preparation. In the present study, the culture conditions were standardised in the open water system, and each batch produced contained 40–42% crude protein and 4–6% crude lipid.

Five isonitrogenous (35% crude protein), isolipidic (6%) and isoenergetic (350 kcal digestible energy/100 g) experimental diets, viz., C (Control with 20% FM, 0% CVM), T₂₅ (15% FM, 7.30% CVM), T₅₀ (10% FM, 14.60% CVM), T₇₅ (5% FM, 21.80% CVM), and T₁₀₀ (0% FM, 29.13% CVM), were formulated (Table 1) and prepared. The methodology used for feed preparation was similar to that performed by Chuphal et al. (2021). Finely ground ingredients were mixed, steam-cooked (121 °C, 20 min),

supplemented with oil and additives, pelleted (1 mm die), dried to ~10% moisture, packed in airtight bags, and stored at 4 °C until use in feeding trials.

Feeding trials were conducted in a static aquaculture system using *C. magur* fingerlings (0.6–0.9 g) acclimatised for 15 days and fed a 35% protein control diet. A total of 150 fish were randomly distributed into five triplicate treatments (10 fish/tank; 200 L) and fed experimental diets to satiation twice daily for 60 days. Water quality was maintained within optimal ranges through aeration and regular water exchange. Fish were weighed fortnightly to adjust feeding rates. All procedures were conducted in accordance with ICAR-CIFE, Mumbai animal ethics guidelines.

After the 60-day feeding trial, initial and final body weights were recorded following one day of starvation. Five fish per tank were sampled for whole-body proximate analysis, while three fish were anaesthetised and dissected for collection of intestine, liver, muscle, and stomach tissues. Tissue homogenates (5%) were prepared in chilled sucrose solution, centrifuged (Heraeus Megafuge 8R Centrifuge, Thermo Fisher Scientific) at 2800 g for 10 min at 4 °C, and the supernatants were stored at -20 °C for subsequent biochemical analyses.

At the Fish Nutrition Laboratory, ICAR-CIFE, Mumbai, India, the proximate composition of feed ingredients, experimental diets, and fish samples was analysed on a dry matter basis following AOAC methods (Cunniff, 1997).

$$NFE (\%) = 100 - \{CP (\%) + EE (\%) + CF (\%) + TA (\%)\} \quad (1)$$

$$TC (\%) = 100 - \{CP (\%) + EE (\%) + TA (\%)\} \quad (2)$$

$$DE (Kcal \text{ per } 100g) = \{CP (\%) \times 4 + EE (\%) \times 9 + NFE (\%) \times 4\} \quad (3)$$

In Equations (1), (2), and (3), NFE is the nitrogen-free extract, CP is crude protein, EE is ether extract, CF is crude fibre, TA is total ash, TC is total carbohydrate, and DE is digestible energy.

The samples were prepared according to the methodology explained in the literature. (Cuchiaro & Laurens, 2019). The amino acid profile of CVM and FM was analysed using an amino acid analyser (Biochrome 30+, Cambridge, United Kingdom) in the Fish Nutrition laboratory, ICAR-CIFE, Mumbai, following the standard procedure (Garg et al., 2022).

Mineral analysis of oven-dried feed was performed using ICP-AES at SAIF, IIT Bombay, following the reported procedure (Kraugerud, Jørgensen, & Svihus, 2011).

Growth performance parameters such as weight gain (WG), weight gain percentage (WG%), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), and body indices such as hepatosomatic and intestinal somatic index, were calculated using the following standard formulae.

$$WG (g) = W_f (g) - W_i (g) \quad (4)$$

$$WG\% = \frac{W_f (g) - W_i (g)}{W_i (g)} \times 100 \quad (5)$$

$$SGR\% = \frac{\ln \text{ of } W_f (g) - \ln \text{ of } W_i (g)}{W_i (g)} \times 100 \quad (6)$$

$$FCR = \frac{\text{Feed intake (dry weight in g)}}{\text{Body weight gain (wet weight in g)}} \quad (7)$$

$$PER = \frac{\text{Body weight gain (wet weight in g)}}{\text{Protein intake (dry weight in g)}} \quad (8)$$

$$HSI\% = \frac{\text{Liver wet weight (g)}}{\text{Wet weight of fish (g)}} \times 100 \quad (9)$$

$$GSI\% = \frac{\text{Wet weight of the int (g)}}{\text{Wet weight of fish (g)}} \times 100 \quad (10)$$

In Equations (4), (5), and (6), W_f is the final weight of the fish, W_i is the initial weight of the fish, and T is the total duration of the experiment (days). In Equation (10), Wet weight of the int denotes the wet weight of the intestine.

The quantification of protein in the different tissue homogenates was carried out using Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951). The standard curve was generated using bovine serum albumin as the standard, facilitating the determination of unknown protein concentration in the tissue samples.

Digestive enzyme activities were analysed from intestinal homogenates. Protease activity was determined using the casein digestion method of Drapeau (1976), chymotrypsin activity by monitoring *p*-nitroaniline production following Delmar, Largman, Brodrick, and Geokas (1979), and amylase activity using the reducing sugar method of Rick and Stegbauer (1974). Alkaline phosphatase (ALP)

and acid phosphatase (ACP) activities were estimated using *p*-nitrophenyl phosphate as substrate according to Garen and Levinthal (1960). Enzyme activities were expressed relative to tissue protein content.

Hepatic antioxidant enzyme activities were evaluated using liver homogenates. Superoxide dismutase (SOD) activity was determined based on inhibition of epinephrine auto-oxidation following Misra and Fridovich (1972), while catalase (CAT) activity was estimated by measuring the decomposition of hydrogen peroxide according to Takahara et al. (1960). Enzyme activities were expressed as unit/min/mg protein. Hepatic and muscular metabolic enzyme activities were assessed using tissue homogenates. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were estimated following King and Wootton (1964). Lactate dehydrogenase (LDH) activity was determined according to Wróblewski and Ladue (1955), while malate dehydrogenase (MDH) activity was measured using the methods of Ochoa, Mehler, and Kornberg (1948) and Ochoa (1955). Enzyme activities were expressed as unit/min/mg protein content under specified assay conditions.

The data were analysed using the statistical software SPSS version 20 and expressed as mean $\pm$ standard error (SE). After confirming the homogeneity of the data, they were subjected to one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) for post hoc comparison of means at a 5% level of significance ($p < 0.05$).

Results & Discussion

Fig. 1 shows that the biomass concentration increased rapidly from 0.3 g L⁻¹ at day 7 to 1.4 g L⁻¹ at day 21. The growth rate then slowed to reach 1.6 g L⁻¹ at the end of the 28-day cultivation period, yielding approximately 6.2 kg of dried biomass. The proximate analysis (Table 1) revealed that the CVM contained 41.20% crude protein (CP) and 4.50% ether extract (EE) or lipid. However, the fishmeal used in the experimental diet contained 59.98% CP and 6.5% EE. CVM and fishmeal (FM) had similar amino acid profiles (Table 1).

According to the proximate composition (Table 2), the experimental diets were isonitrogenous (35% CP), isolipidic (6%), and isocaloric (350 kcal DE/100 g). The mineral contents of CVM and the experimental diets are reported in Table 3. However, the

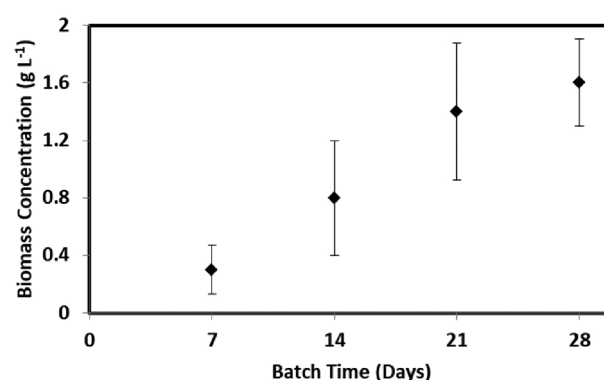


Fig. 1. Changes in biomass concentration during batch cultivation of *C. vulgaris*. Data are presented as mean $\pm$ standard deviation (SD). Error bars represent the standard deviation.

moisture, TA, CF, and NFE contents ranged from 7.22 to 8.10%, 9.79 to 11.57%, 8.13 to 8.77%, and 39.45 to 40.15%, respectively. The whole-body proximate composition of magur fingerlings fed the different experimental diets (Table 4) did not vary significantly ($p > 0.05$).

Feeding different levels of dietary CVM as an alternative to FM had a significant ($p < 0.05$) effect on FBW (g), WG (g), WG (%), SGR, and PER of magur fingerlings, but FCR, HSI, and GSI did not vary significantly among the experimental groups (Table 5). The fish in the T₅₀ group showed significantly higher ($p < 0.05$) FBW, WG, WG%, and SGR than the T₂₅ and T₁₀₀ groups but were similar ($p > 0.05$) to those of the C and T₇₅ groups. However, the PER values of fish in the C, T₂₅, T₅₀, and T₁₀₀ groups were similar ($p > 0.05$), as were those of the T₅₀, T₇₅, and T₁₀₀ groups. However, the T₇₅ group showed a significantly lower ($p < 0.05$) PER than the C and T₂₅ groups.

Amylase activity did not vary significantly ($p > 0.05$) among the treatments, whereas protease, chymotrypsin, ALP, and ACP activities were significantly affected ($p < 0.05$) by the replacement of dietary FM with CVM (Table 6). Although the protease activity of the T₂₅ group was significantly lower ($p < 0.05$) than that of the C group, there was no significant difference ($p > 0.05$) in protease enzyme activity among the C, T₅₀, T₇₅, and T₁₀₀ groups. On the other hand, the higher CVM-fed groups (T₅₀, T₇₅, and T₁₀₀) exhibited significantly higher ($p < 0.05$) activities of chymotrypsin, ALP, and ACP than the C group.

Although hepatic and muscle AST, muscle ALT, and hepatic MDH activities were significantly affected (p

Table 1. Proximate composition and amino acid profile (on dry weight basis) of *C. vulgaris* meal (CVM) and fishmeal (FM) used in the experimental diets

Proximate composition (%)	<i>C. vulgaris</i> meal	Fishmeal
Crude protein	41.20 ± 1.24	59.98 ± 2.24
Ether extract	4.50 ± 0.72	6.50 ± 0.45
Crude fibre	17.60 ± 1.65	0.98 ± 0.01
Total Ash	13.02 ± 1.02	18.16 ± 2.53
Nitrogen-free extract	23.90 ± 1.11	14.38 ± 1.24
Amino acids (g/100 g)		
Essential amino acids		
Arginine	4.68	4.84
Histidine	1.18	1.22
Leucine	4.06	4.23
Isoleucine	2.50	2.59
Lysine	4.95	5.11
Methionine	1.55	1.60
Phenylalanine	2.11	2.18
Threonine	2.40	2.48
Valine	2.83	2.92
Non-essential amino acids		
Alanine	2.20	2.28
Aspartic acid	3.55	3.67
Cystine	0.36	0.38
Glutamic acid	10.10	10.45
Glycine	4.43	4.58
Proline	3.03	3.13
Serine	2.30	2.38
Tyrosine	1.58	1.64

All proximate values are expressed as mean ± SE (n=3); Amino Acid values are average values (n=2)

< 0.05) by dietary CVM levels, hepatic ALT and LDH activities did not vary significantly among the treatments (Table 7). There was no significant difference ($p > 0.05$) in liver AST activity among the C, T₅₀ and T₁₀₀ groups; however, the activity of this enzyme in the T₇₅ group was significantly higher ($p < 0.05$) than that in the C group. In contrast, the muscle AST activity of the T₁₀₀ group was significantly lower ($p < 0.05$) than that of the control (C). However, the muscle AST activity of other groups (T₂₅, T₅₀ and T₇₅) was similar ($p > 0.05$) to that of the C group. The muscle ALT activity of the T₂₅ and

T₅₀ groups was similar ($p > 0.05$) to that of the control (C). The hepatic MDH activity of all CVM-fed groups (T₂₅, T₅₀, T₇₅, and T₁₀₀) was similar ($p > 0.05$) to the C group. However, the hepatic MDH activity of the T₅₀ and T₇₅ groups was significantly higher ($p < 0.05$) than that of the T₂₅ and T₁₀₀ groups.

No significant difference ($p > 0.05$) was observed in the hepatic SOD activity among the treatments, but the hepatic CAT activity of magur fingerlings was significantly affected ($p < 0.05$) by different levels of dietary CVM (Table 7). The hepatic CAT activity did

Table 2. Formulation and proximate composition of experimental diets fed to *C. magur*

Ingredient composition (%)	Diets ¹				
	C	T ₂₅	T ₅₀	T ₇₅	T ₁₀₀
Fishmeal	20.00	15.00	10.00	5.00	0.00
CVM ²	0.00	7.30	14.60	21.80	29.13
DSBM ³	29.00	29.00	29.00	29.00	29.00
GNOC ⁴	11.00	11.00	11.00	11.00	11.00
DORB ⁵	21.00	20.70	20.10	19.50	21.70
Wheat flour	6.50	5.50	4.50	3.50	1.50
Cellulose	5.00	4.00	3.30	2.70	0.27
Soya oil	3.25	3.25	3.25	3.25	3.25
CMC ⁶	2.00	2.00	2.00	2.00	2.00
Vitamin-Mineral mixture ⁷	2.00	2.00	2.00	2.00	2.00
BHT ⁸	0.02	0.02	0.02	0.02	0.02
Choline chloride	0.23	0.23	0.23	0.23	0.23
Total	100	100	100	100	100
Proximate Composition (on dry matter basis) (%)					
Moisture	8.10	7.42	7.62	7.75	7.22
Crude protein	35.39	35.22	35.00	34.74	34.89
Ether extract	6.20	6.14	6.09	6.02	5.96
Total ash	9.79	10.08	10.62	10.89	11.57
Crude fibre	8.47	8.69	8.77	8.76	8.13
Nitrogen-free extract	40.15	39.87	39.52	39.59	39.45
DE ⁹ (kcal/100 g)	357.96	355.62	352.89	351.50	351.00

Proximate compositions are expressed as mean values of triplicate

Diets¹, C {Control with 20% fish meal (FM)}, T₂₅ {7.30% *C. vulgaris* meal (CVM) with replacement of 25% FM protein equivalent}, T₅₀ (14.60% CVM with replacement of 50% FM protein equivalent), T₇₅ (21.80% CVM with replacement of 75% FM protein equivalent) and T₁₀₀ (29.13% CVM with replacement of 100% FM protein equivalent); CVM², *C. vulgaris* meal; DSBM³, De-fatted soybean meal; GNOC⁴, Groundnut oil cake; DORB⁵, De-oiled rice bran; CMC⁶, Carboxymethylcellulose; Vitamin-mineral mixture⁷ was from AGRIMIN FORTE; BHT⁸, Butylated hydroxytoluene; DE⁹, Digestible energy

not vary significantly ($p > 0.05$) among the CVM-fed groups. However, the activity in the T₇₅ and T₁₀₀ groups was significantly higher ($p < 0.05$) than that in the lower CVM-fed groups (T₂₅ and T₅₀) and was similar ($p > 0.05$) to that of the control (C) group.

The present study showed that the biomass concentration of *C. vulgaris* increased rapidly between days 7 and 21 and slowed down thereafter, likely due to the onset of the stationary growth phase (Fatemeh & Mohsen, 2016). Biomass yield exceeded that

reported for open raceway ponds (1–1.3 g L⁻¹), possibly because of the protective shed-net enclosure (Jeevanandam, Harun, Lau, Sewu, & Danquah, 2020). Despite challenges in economically viable mass cultivation, the standardised raceway system produced biomass with favourable nutritional profile. The resulting meal contained 41% crude protein and 4.5% lipid, with an amino acid profile comparable to fishmeal (Karapanagiotidis et al., 2022; Siddik et al., 2024).

Table 3. Mineral profile of various feeds used in the study

Mineral Profile (%)	CVM	C	T ₂₅	T ₅₀	T ₇₅	T ₁₀₀
Sodium (Na)	0.99	0.51	0.50	0.47	0.48	0.47
Potassium (K)	0.06	1.16	1.13	1.03	1.03	1.00
Zinc (Zn)	0.005	0.013	0.012	0.009	0.012	0.012
Iron (Fe)	1.34	0.08	0.14	0.20	0.28	0.35
Magnesium (Mg)	0.39	0.52	0.47	0.44	0.45	0.48
Cobalt (Co)	0.0003	0.0003	0.0003	0.0003	0.0004	0.0005
Phosphorous (P)	0.32	1.74	1.58	1.36	1.33	1.22
Sulphur (S)	0.50	0.38	0.36	0.34	0.36	0.35
Molybdenum (Mo)	ND	ND	ND	ND	ND	ND

Data are expressed as mean values of triplicate; ND, Not detected.

Table 4. Proximate composition (on wet weight basis) of the whole body of *C. magur* fingerlings fed with different experimental diets for 60 days

Treatments	Dry matter	Crude protein (%)	Lipid (%)	Total ash (%)	Total carbohydrate (%)
C	19.29±1.70	14.06±0.87	1.34± 0.13	2.87±0.02	1.02±0.11
T ₂₅	18.94±1.17	13.81±1.39	1.20±0.16	2.97±0.08	0.95±0.09
T ₅₀	19.94± 1.33	15.22±1.51	1.09±0.14	2.85±0.09	0.77±0.07
T ₇₅	19.43±1.27	14.32±1.07	1.48±0.12	2.83±0.15	0.79±0.06
T ₁₀₀	20.05±1.42	14.65±1.57	1.76±0.08	2.76±0.10	0.86±0.02
<i>p</i> value	0.084	0.095	0.553	0.188	0.861

Data expressed as mean ± SE (n=3)

In the present study, the whole-body proximate composition of magur fingerlings was not altered by replacing FM with CVM in the diet. Thus, this finding indicates that CVM could be a suitable alternative to FM in the catfish diet without compromising fish flesh quality. A similar trend has been reported for seabream and tilapia, (Mustafa, Wakamatsu, Takeda, Umino, & Nakagawa, 1995; Badwy, Ibrahim, & Zeinhom, 2008; Karapanagiotidis et al., 2022). Moreover, the dietary inclusion of 4% *Chlorella* meal did not alter the whole-body proximate composition of Crucian carp (Luo et al., 2018). Similarly, Rahimnejad et al. (2017) found no difference in the muscle and liver composition of olive flounder following dietary inclusion of algal meal. Another study using dietary *C. fusca* also showed no significant difference in the proximate composition of fish (García-Márquez et al., 2023).

The current study showed that CVM could replace 75% of FM protein on a protein-equivalent basis without compromising the growth performance (FBW, WG, WG%, and SGR) of *C. magur* fingerlings. The higher growth observed in the 50% replacement group compared with the control may be attributed to the high digestible protein content (> 80%), well balanced essential amino acid profile, and the presence of folate, vitamin D and B₁₂, minerals, peptides, sugars, nucleic acids, and amino acids in CVM (Sergejevová & Masojídek, 2012; Alagawany et al., 2021, Albaqami, 2025), whereas the fish in the 100% replacement group showed similar PER to the control. This indicates that 50% replacement of fishmeal with CVM can complement the bioavailable protein and micronutrients, such as zinc and iron, that contribute to growth. The FCR and body indices (HSI and GSI) were not significantly affected

Table 5. Growth performance and body indices of *C. magur* fingerlings fed with different experimental diets for the period of 60 days

Treatments	FBW ¹ (g)	WG ² (g)	WG ³ (%)	SGR ⁴	FCR ⁵	PER ⁶	HSI ⁷ (%)	GSI ⁸ (%)
C	5.22 ^{ab} ± 0.41	4.55 ^{ab} ± 0.35	665.67 ^{ab} ± 22.76	3.43 ^{ab} ± 0.09	1.72 ± 0.15	4.84 ^a ± 0.12	1.04 ± 0.12	3.92 ± 0.32
T ₂₅	4.74 ^{bc} ± 1.03	3.74 ^{bc} ± 1.06	575.01 ^{bc} ± 144.88	3.10 ^b ± 0.44	1.95 ± 0.23	4.72 ^a ± 0.21	1.12 ± 0.10	3.84 ± 0.33
T ₅₀	5.78 ^a ± 0.4	5.08 ^a ± 0.44	744.67 ^a ± 125.33	3.52 ^a ± 0.23	1.68 ± 0.04	4.69 ^{ab} ± 0.06	1.13 ± 0.009	3.63 ± 0.21
T ₇₅	5.18 ^{ab} ± 0.44	4.48 ^{ab} ± 0.43	651.33 ^{ab} ± 74.17	3.26 ^{ab} ± 0.16	1.64 ± 0.05	4.53 ^b ± 0.05	1.07 ± 0.11	4.08 ± 0.21
T ₁₀₀	3.86 ^c ± 0.22	3.16 ^c ± 0.19	503.33 ^c ± 40.48	2.83 ^b ± 0.11	1.93 ± 0.10	4.67 ^{ab} ± 0.16	0.98 ± 0.10	4.05 ± 0.11
<i>p</i> value	0.016	0.014	0.023	0.003	0.067	0.007	0.860	0.055

Data expressed as mean ± SE (n=3); Mean values in the same column with different superscripts differ significantly (*p* < 0.05).

FBW¹, Final body weight; WG², Weight gain; WG³, Weight gain percentage; SGR⁴, Specific growth rate; FCR⁵, Feed conversion ratio; PER⁶, Protein efficiency ratio; HSI⁷, Hepato-somatic index; GSI⁸, Gastrosomatic index.

Table 6. Digestive enzyme activities in the intestine of *C. magur* fingerlings fed with different experimental diets for the period of 60 days

Treatments	Amylase ¹	Protease ²	Chymotrypsin ³	Alkaline phosphatase (ALP) ⁴	Acid phosphatase (ACP) ⁵
C	0.31 ± 0.09	5.24 ^a ± 0.10	1.24 ^b ± 0.30	10.51 ^c ± 1.80	15.03 ^b ± 0.06
T ₂₅	0.34 ± 0.03	3.96 ^b ± 0.16	1.31 ^b ± 1.01	11.62 ^{bc} ± 2.05	15.92 ^b ± 0.09
T ₅₀	0.53 ± 0.07	4.47 ^{ab} ± 0.36	1.61 ^a ± 0.40	12.80 ^b ± 2.09	17.83 ^a ± 0.01
T ₇₅	0.34 ± 0.06	4.55 ^{ab} ± 0.31	1.62 ^a ± 0.36	14.93 ^a ± 1.04	18.54 ^a ± 0.18
T ₁₀₀	0.31 ± 0.09	4.81 ^{ab} ± 0.17	1.68 ^a ± 0.73	15.69 ^a ± 1.51	18.96 ^a ± 0.09
<i>p</i> value	0.564	0.031	< 0.001	0.030	0.024

Data expressed as mean ± SE (n=3); Mean values in the same column with different superscripts differ significantly (*p* < 0.05)

Amylase activity is expressed as micromole maltose released/min/mg protein¹; Protease activity is expressed as millimole of tyrosine released/min/mg protein²; Chymotrypsin activity is expressed as mg protein/min³; ALP activity is expressed as micromoles p-nitrophenol released/min/mg protein⁴; ACP activity is expressed as micromoles p-nitrophenol released/min/mg protein⁵

by dietary CVM inclusion. However, Shi et al. (2017) reported that complete (100%) replacement of dietary FM protein with CVM, resulting in better growth performance in *Carassius auratus*. At a 10% inclusion level, *Chlorella* meal supported higher growth in olive flounder than in the control group (Rahimnejad et al., 2017). However, CVM could replace 30% of FM protein on protein-equivalent basis in the diet of juvenile seabream

(Karapanagiotidis et al., 2022) and 50% in the tilapia diet (Badwy et al., 2008) without deterioration of growth performance. Moreover, dietary inclusion of 30% CVM resulted in similar growth in Nile tilapia and enhanced growth in African catfish (Teuling et al., 2017). Additionally, dietary *Chlorella* protein was reported to be digested and utilised well by Atlantic salmon (Tibbetts, Mann, & Dumas, 2017), resulting in improved PER values. The fish in the 100%

replacement group in the present study showed lower growth, which might be due to the lower dry matter digestibility by the fish. In agreement, the higher structural carbohydrate fraction of dietary algal meal resulted in low nutrient digestibility in *Paraliticus olivaceus* (Kim, Bai, Koo, Wang, & Kim, 2002) and *C. auratus* (Xu et al., 2014). Additionally, according to Kose, Ozen, Elibol, and Oncel (2017), *C. vulgaris* has a hard, multi-layered cell wall, composed mainly of cellulose and hemicellulose, which forms a physical barrier that prevents digestive enzymes from accessing the biomass, resulting in poor digestibility of the raw biomass (frequently as low as 35% to 50%). Microalgae such as *Chlorella* sp. and *Nannochloropsis* sp. have low digestibility because of their rigid cell walls and non-starch components (Ahmad, Shariff, Yusoff, Goh, & Banerjee, 2020). This might be one of the reasons for the slower growth observed in the 100% replacement group. Mueller et al. (2024) also reported that higher inclusion of *C. vulgaris* in the diet of Atlantic salmon adversely affected FCR and microbiota composition in the mucosa and gut digesta. Similar results were observed in juvenile Nile tilapia (Huang et al., 2023).

In the present study, the comparable activities of protease and chymotrypsin between the higher FM protein-equivalent replacement groups and the control indicated that higher dietary inclusion levels of CVM could be used as an FM alternative without affecting the activity of protein digestive enzymes required to support fish growth. However, in this study, growth was not compromised up to the 75% replacement group but was reduced in the 100%

replacement group compared with the control. It was reported that dietary microalgal carbohydrate fractions could act as prebiotics in the gastrointestinal tract and thus could stimulate the intestinal microflora to secrete supplementary digestive enzymes from endogenous sources, thereby supporting nutrient digestion and growth (Reitan, Rainuzzo, Øie, & Olsen, 1993). In line with our results, Xu et al. (2014) also observed increased protease activity in *C. auratus*. However, contrary to our observation, the same study showed increased amylase activity in fish. Koi carp fed with 5% *Chlorella* sp. meal exhibited enhanced digestive enzyme activity (Khani, Soltani, Mehrjan, Foroudi, & Ghaeni, 2017).

Alkaline and acid phosphatases are involved in mineral release, nutrient absorption, and amino acid transport, serving as important indicators of intestinal integrity and absorptive capacity in teleosts (Cahu & Infante, 2001; Vizcaino et al., 2014). Similar to the current study, feeding 15% *C. fusca* increased the ALP activity in thick-lipped grey mullets (García-Márquez et al., 2023). The increased ALP and ACP activities may indicate active phosphorous metabolism in the high *Chlorella* sp. fed groups. Moreover, CV contains bioactive compounds such as chlorophylls, carotenoids, polysaccharides, vitamins, nucleotides, and minerals that can enhance intestinal development and nutrient absorption. Increased ACP activity may indicate greater lysosomal activity, cellular turnover, and innate immune responses. Therefore, elevated phosphatase activities in high-*Chlorella* groups likely reflect improved intestinal health, immune function, and efficient utilisation of algal nutrients (Saleh et al., 2022) The

Table 7. The activities of metabolic and antioxidant enzymes of *C. magur* fingerlings fed with different experimental diets for the period of 60 days

Treatments	Aspartate aminotransferase (AST) ¹		Alanine aminotransferase (ALT) ²		Lactate dehydrogenase (LDH) ³	Malate dehydrogenase (MDH) ⁴	Superoxide dismutase (SOD) ⁵	Catalase (CAT) ⁶
	Liver	Muscle	Liver	Muscle	Liver	Liver	Liver	Liver
C								
T ₂₅	1.38 ^c ± 0.09	6.15 ^a ± 0.30	0.67 ± 0.03	9.79 ^a ± 1.08	4.06 ± 0.22	3.07 ^b ± 0.04	4.97 ± 0.25	13.07 ^{ab} ± 1.58
T ₅₀	2.15 ^{ab} ± 0.19	7.19 ^a ± 1.20	0.65 ± 0.09	9.55 ^a ± 1.14	5.43 ± 0.37	6.64 ^a ± 0.22	4.87 ± 0.43	12.44 ^{ab} ± 3.22
T ₇₅	2.32 ^a ± 0.21	6.04 ^{ab} ± 1.08	0.70 ± 0.10	8.12 ^b ± 1.02	5.08 ± 0.49	6.78 ^a ± 0.30	5.26 ± 0.45	17.41 ^a ± 3.27
T ₁₀₀	1.98 ^{ab} ± 0.13	5.32 ^b ± 1.50	0.66 ± 0.10	8.16 ^b ± 1.37	4.57 ± 0.23	3.28 ^b ± 0.03	4.42 ± 0.25	21.91 ^a ± 3.03
<i>p</i> value	0.029	0.036	0.197	0.042	0.474	0.001	0.474	0.001

Data expressed as mean ± SE (n=3); Mean values in the same column with different superscripts differ significantly (*p* < 0.05)

The activity is expressed as nanomoles of oxaloacetate released/min/mg protein¹; The activity is expressed as nanomoles of sodium pyruvate released/min/mg protein²; The activity is expressed as in unit/min/mg protein^{3,4,5,6}

current study showed a non-significant hepatic LDH activity but significant MDH activity, indicating active aerobic metabolism in fish.

The antioxidant defense system in fish neutralises ROS, especially with the help of antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase (Nazeemashahul, Sahu, Sardar, & Fawole, 2020; Luo et al., 2021). *Chlorella* sp. is rich in antioxidants, including flavonoids, carotenoids, polyphenols, and vitamin E, which help scavenge reactive oxygen species, inhibit lipid peroxidation, and protect cellular macromolecules from oxidative damage (Cha et al., 2010). Since aquaculture-related stressors can induce oxidative stress and impair fish health, dietary *Chlorella* sp. may enhance antioxidant defense and resilience under culture conditions (Sheikhzadeh, Soltani, Heidarieh, & Ghorbani, 2024). In the present study, superoxide dismutase activity was similar among the treatments, while an increase in the catalase activity was observed in the groups fed higher levels of *Chlorella* sp. This might be due to the action of several antioxidant substances present in *Chlorella* sp., as suggested by Vijayavel, Anbuselvam, and Balasubramanian (2007) and Safi et al. (2013). According to Zahran and Risha (2014), feeding *C. vulgaris* can increase the catalase activity in tilapia. Similarly, an elevated antioxidant defence was reported for rohu (Andrews, Sahu, Pal, & Kumar, 2009) and olive flounder (Rahimnejad et al., 2017) fed a *C. vulgaris* based diet.

By preserving cellular redox equilibrium and promoting regular physiological processes, antioxidant qualities of *C. vulgaris* can help enhance immunological responses, stress tolerance, and disease resistance. Therefore, *C. vulgaris* may be used as a functional feed element that improves fish resistance under intensive aquaculture conditions, in addition to its nutritional importance as a source of protein.

Cultivation of *C. vulgaris* in open raceway ponds proved viable, yielding nutrient-rich biomass suitable for aquafeeds. Feeding trials in *C. magur* showed that 21.8% CVM can replace 75% fishmeal at 20% dietary inclusion without affecting growth or health, highlighting its potential as a sustainable protein source for aquaculture. Nonetheless, comprehensive field trials at larger scales and over extended durations are imperative to inform definitive recommendations.

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