

Growth, intestinal health, nutritional profile and molecular mechanism of the hybrid grouper, *E. lanceolatus* × *E. fuscoguttatus* juveniles reared with microalgae *Chlorella pyrenoidosa*

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Abstract

The effects of microalgae *Chlorella pyrenoidosa* on the hybrid groupers' (*Epinephelus lanceolatus* × *E. fuscoguttatus*) growth, gut microbiome and transcriptome were examined in this study. Feeding trials with duration of 15-days (15d) and 60-days (60d) were conducted on three experimental groups (n=3) as follow; (T1) grouper fed with basal diet and reared in filtered seawater (control treatment), (T2) grouper fed with basal diet and reared in *C. pyrenoidosa* monoculture water and (T3) grouper fed with basal diet partially replaced with 15% of dried *C. pyrenoidosa* (CRM) and reared in filtered seawater. The findings revealed that groupers reared 15d in T2 treatment (T2-15d) had a higher fat content, with apparent shift of microbial composition and functional pathways in the gut. Groupers reared 60d in T2 treatment (T2-60d) displayed an increased NADH dehydrogenases and cytochrome c oxidases gene expression, indicating more robust oxidative phosphorylation activity and ATP production crucial for the metabolic homeostasis. The reduction in the total amino acid content was also detected in groupers raised in T2-60d. Gastritis, enteritis and lipid malabsorption syndrome were observed in groupers raised 60d in T3 treatment (T3-60d), with the condition likely due to a higher intake of omega-6 to omega-3 fatty acids ratio in grouper raised 15 days in T3 (T3-15d) and T3-60d. This result indicated that grouper intestinal inflammation could arise from the inclusion of dried *C. pyrenoidosa*. Overall, the study outcomes demonstrated that introducing live *C. pyrenoidosa* to the culture water is advantageous in the digestion and enhance the energy metabolism of juvenile groupers.

Introduction

Grouper (Family Serranidae, Subfamily Epinephelinae) is a popular and valuable food fish for human consumption. The farming of grouper started more than three decades ago and had since expanded to meet the increasing market demand and reduce over-catching particularly in the Southeast Asia and East Asia regions (Sadovy 2000). Between 2010 to 2019, the production of groupers increased from 77,199 tonnes to 229,379 tonnes, with annual trade value of more than USD 760 million, where 92% of the production were contributed by China, Taiwan and Indonesia (Rimmer and Glamuzina 2019; FAO 2021). Currently, at least 47 grouper species have been cultured (Rimmer and Glamuzina 2019; Megarajan et al. 2022) and more than 66% of all culture are wild-caught (Liao et al. 2001; Tupper and Sheriff 2008). Hybrid groupers have become more popular in Asian aquaculture since 2006 due to their successful production and better organoleptic qualities, resulting in a high commercial value (Ching et al. 2018). The successful breeding of hybrid groupers (Rimmer and Glamuzina 2019) has enabled hatcheries to produce fingerlings and market-sized groupers for every stage of development (Rimmer and Glamuzina 2019). Since then, research has focused on improving rearing conditions (Sutthinon et al. 2015; Ching et al. 2018) and feed composition (Ye et al. 2019a; Chen et al. 2020; Azfar-Ismail et al. 2021; Nankervis et al. 2022) to further enhance the production of hybrid groupers.

It has been shown in the recent decades that green water system provides many benefits during the culture of marine fish species, including grouper (Chen et al. 2012). Green water consists mainly of microalgae, and they are unicellular micro-organisms capable of performing photosynthesis which are

collectively known as phytoplankton. In aquaculture, microalgae production is performed under intensive or extensive monoculture (Duerr et al. 1998). Various microalgae species are used as starter or grow-out feeds for shrimp, fish and bivalves (Alagawany et al. 2021; Ansari et al. 2021; Chen et al. 2021) and they can also be used in live food enrichment (Banerjee et al. 2010). In addition to stabilizing water quality and acting as a light shield during fish larviculture (Chen et al. 2012; Anita and Dewi 2020), microalgae are used in fish hatcheries to prevent disease occurrence. Disease and infection were identified as one of the factors that caused mass mortality in grouper culture (Liao et al., 2001).

As one of the most studied types of microalgae in aquaculture, *Chlorella*'s biomass provides vital nutrients like protein, fat, and carbohydrates that could be used as sustainable ingredients in aquaculture feed.. Up to 50% replacement of fish meal by *Chlorella* has been shown to successfully boost the protein content of fish (Deng et al. 2021). They also produce a specific phytonutrient known as *Chlorella* Growth Factor, which benefits many economically important fish by improving their growth rate, nutritional value, and meat quality (Ahmad et al. 2020). On other aspect, the introduction of live *Chlorella* culture in the rearing water serves as water cleaner by assimilating feed residue and fish waste, subsequently maintain the water quality and ecological balance (Deng et al. 2021). Adding microalgae *Chlorella* to the rearing water or utilizing them in formulated diets as a replacement meal has also been shown to increase feed digestibility and stress tolerance during aquaculture (Chen et al. 2012; Ahmad et al. 2022).

The present study was conducted to investigate the effects of *Chlorella pyrenoidosa* on the growth performance, gut health and gene regulation of the hybrid grouper (*E. lanceolatu* × *E. fuscoguttatus*). Three experimental groups, T1 control group was fed with basal diet only; T2 group was consistently reared in *C. pyrenoidosa* monoculture water containing 10^7 cells mL⁻¹; T3 group was fed with basal diet containing 15% of dried *C. pyrenoidosa* powder (CRM). The growth performance, gastrointestinal health and transcriptomic changes of these groups were examined on day 15 (15d) and 60 (60d) (Fig. 1). Henceforth, the experimental groups will be addressed as T1-15d, T2-15d and T3-15d in 15days trial and T1-60d, T2-60d and T3-60d in 60-days trial. The outcomes will justify the potential of *C. pyrenoidosa*, in form of processed powder or green water, in the hybrid grouper culture.

Results

Growth performance and chemical composition of whole fish body

The WGR, SGR and HIS indexes of T2 (T2-15d and T2-60d) and T3 (T3-15d and T3-60d) groups had no significant difference ($p > 0.05$) when compared to T1 control group (Table 1; Fig. 1b and 1c). The chemical composition analysis (Table 2) demonstrated that the moisture content was higher in T2-15d and T3-15d groups ($p < 0.05$). Crude ash was higher in T2-15d group ($p < 0.05$), while crude protein and fat were lower in T2-15d and T3-15d groups ($p < 0.05$), as compared to the T1-15d. The moisture content was lower in T2-60d and T3-60d groups ($p < 0.05$), whereas the highest crude ash content occurred in T2-

60d group. The crude lipid in T2-60d and T3-60d groups was significantly higher ($p < 0.05$) than T1-60d. The difference in the protein content among three groups were insignificant ($p > 0.05$).

Table 1

The growth performance of T1, T2 and T3 groups in 15-days and 60-days trials. Data represented in mean $\pm$ SE (n = 3).

	T1-15d	T2-15d	T3-15d	T1-60d	T2-60d	T3-60d
W ₀ (g)	10.01 $\pm$ 0.43 ^a	10.00 $\pm$ 0.30 ^a	9.45 $\pm$ 0.32 ^a	10.01 $\pm$ 0.43 ^a	10.00 $\pm$ 0.30 ^a	9.45 $\pm$ 0.32 ^a
WGR (%)	66.99 $\pm$ 15.13 ^a	59.15 $\pm$ 17.05 ^a	53.25 $\pm$ 26.64 ^a	321.36 $\pm$ 70.58 ^a	321.04 $\pm$ 69.95 ^a	287.67 $\pm$ 32.80 ^a
SGR (% d ⁻¹)	3.40 $\pm$ 0.58 ^a	3.06 $\pm$ 0.72 ^a	2.47 $\pm$ 1.20 ^a	9.51 $\pm$ 1.10 ^a	9.50 $\pm$ 1.16 ^a	9.01 $\pm$ 0.56 ^a
HSI (%)	3.42 $\pm$ 0.43 ^a	3.39 $\pm$ 0.94 ^a	3.52 $\pm$ 0.99 ^a	3.45 $\pm$ 1.23 ^a	3.61 $\pm$ 1.72 ^a	4.09 $\pm$ 1.34 ^a
W0: initial body weight; WGR: weight gain rate; SGR: specific growth rate; HIS: hepatosomatic index						
a,b Different letters indicate significant difference ($p < 0.05$) among means within the same row.						
T1 group, fed with basal diet; T2 group, reared in <i>C. pyrenoidosa</i> monoculture water; T3 group, fed with basal diet containing 15% of dried <i>C. pyrenoidosa</i> powder (CRM).						

Table 2

The body composition of T1, T2 and T3 groups on the 15th and 60th day. Data represented in mean $\pm$ SE (n = 3)

	T1-15d	T2-15d	T3-15d	T1-60d	T2-60d	T3-60d
Moisture content (%)	72.83 $\pm$ 0.49 ^b	74.63 $\pm$ 0.45 ^a	75.10 $\pm$ 0.75 ^a	74.87 $\pm$ 0.25 ^a	73.90 $\pm$ 0.61 ^b	74.20 $\pm$ 0.17 ^{ab}
Crude ash (%)	4.80 $\pm$ 0.10 ^b	6.23 $\pm$ 0.06 ^a	4.87 $\pm$ 0.06 ^b	5.00 $\pm$ 0.10 ^b	5.93 $\pm$ 0.12 ^a	4.93 $\pm$ 0.12 ^b
Crude protein(%)	16.13 $\pm$ 0.64 ^a	14.83 $\pm$ 0.32 ^b	15.57 $\pm$ 0.50 ^{ab}	15.63 $\pm$ 0.32 ^a	15.30 $\pm$ 0.46 ^a	15.90 $\pm$ 0.26 ^a
Crude fat (%)	4.63 $\pm$ 0.15 ^a	3.27 $\pm$ 0.06 ^c	4.10 $\pm$ 0.20 ^b	3.57 $\pm$ 0.06 ^b	4.23 $\pm$ 0.21 ^a	4.43 $\pm$ 0.15 ^a
a,b,c Different letters indicate significant difference ($p < 0.05$) among means within the same row.						
T1 group, fed with basal diet; T2 group, reared in <i>C. pyrenoidosa</i> monoculture water; T3 group, fed with basal diet containing 15% of dried <i>C. pyrenoidosa</i> powder (CRM).						

The amino acid profiles among the three feeding groups were different (Table 3). The level of total amino acid (TAA), essential amino acid (EAA) and non-essential amino acid (NEAA) in 15d and 60d trials was in the order of T1-15d > T2-15d > T3-15d and T3-60d > T1-60d > T2-60d. Notably T3-60d also recorded with substantially higher amount of lysine and glutamic acid than the other two groups.

Table 3

The amino acid composition of the T1, T2 and T3 groups on the 15th and 60th day. Data represented in mean $\pm$ SE (n = 3).

<i>Amino acid</i>	15d (g kg ⁻¹)			60d (g kg ⁻¹)		
	T1	T2	T3	T1	T2	T3
Σ EAA	77.97 $\pm$ 0.05 ^a	75.31 $\pm$ 0.07 ^b	69.97 $\pm$ 0.39 ^c	63.03 $\pm$ 0.12 ^b	55.45 $\pm$ 0.11 ^c	65.03 $\pm$ 0.39 ^a
Met	5.74 $\pm$ 0.03 ^a	5.78 $\pm$ 0.01 ^a	5.44 $\pm$ 0.05 ^b	5.01 $\pm$ 0.02 ^a	4.48 $\pm$ 0.04 ^b	4.35 $\pm$ 0.03 ^c
Val	8.68 $\pm$ 0.01 ^a	8.40 $\pm$ 0.02 ^b	8.00 $\pm$ 0.01 ^c	7.48 $\pm$ 0.07 ^a	6.71 $\pm$ 0.03 ^c	7.44 $\pm$ 0.05 ^b
Ile	9.59 $\pm$ 0.02 ^a	9.26 $\pm$ 0.02 ^b	8.70 $\pm$ 0.03 ^c	8.02 $\pm$ 0.04 ^a	7.21 $\pm$ 0.04 ^c	7.87 $\pm$ 0.05 ^b
Phe	8.57 $\pm$ 0.03 ^a	8.27 $\pm$ 0.01 ^b	7.75 $\pm$ 0.03 ^c	7.47 $\pm$ 0.03 ^a	6.81 $\pm$ 0.04 ^c	7.18 $\pm$ 0.05 ^b
Leu	16.81 $\pm$ 0.03 ^a	16.25 $\pm$ 0.04 ^b	15.27 $\pm$ 0.06 ^c	14.00 $\pm$ 0.04 ^a	12.68 $\pm$ 0.09 ^b	13.93 $\pm$ 0.05 ^a
Thr	9.40 $\pm$ 0.01 ^a	9.28 $\pm$ 0.06 ^b	8.72 $\pm$ 0.07 ^c	8.87 $\pm$ 0.01 ^a	8.12 $\pm$ 0.01 ^b	7.88 $\pm$ 0.03 ^c
Lys	19.20 $\pm$ 0.02 ^a	18.09 $\pm$ 0.07 ^b	16.06 $\pm$ 0.15 ^c	12.18 $\pm$ 0.10 ^b	9.44 $\pm$ 0.15 ^c	16.37 $\pm$ 0.17 ^a
Σ NEAA	101.19 $\pm$ 0.18 ^a	97.41 $\pm$ 0.11 ^b	85.96 $\pm$ 0.31 ^c	79.71 $\pm$ 0.10 ^b	66.60 $\pm$ 0.03 ^c	92.21 $\pm$ 0.46 ^a
Asp	18.45 $\pm$ 0.03 ^a	16.72 $\pm$ 0.05 ^b	13.97 $\pm$ 0.08 ^c	11.98 $\pm$ 0.02 ^b	9.07 $\pm$ 0.06 ^c	16.42 $\pm$ 0.11 ^a
Glu	28.62 $\pm$ 0.05 ^a	27.69 $\pm$ 0.03 ^b	23.36 $\pm$ 0.09 ^c	19.82 $\pm$ 0.02 ^b	15.91 $\pm$ 0.03 ^c	24.52 $\pm$ 0.14 ^a
Ser	7.71 $\pm$ 0.02 ^a	7.53 $\pm$ 0.02 ^b	6.69 $\pm$ 0.03 ^c	5.61 $\pm$ 0.01 ^b	4.71 $\pm$ 0.01 ^c	6.79 $\pm$ 0.02 ^a
Gly	8.21 $\pm$ 0.02 ^b	8.70 $\pm$ 0.01 ^a	7.80 $\pm$ 0.02 ^c	9.54 $\pm$ 0.04 ^b	8.56 $\pm$ 0.01 ^c	9.78 $\pm$ 0.02 ^a

EAA: Essential amino acid; NEAA: Non-essential amino acid; TAA: Total amino acid

a,b,c Different letters indicate significant difference ($p < 0.05$) among means within the same row.

T1 group, fed with basal diet; T2 group, reared in *C. pyrenoidosa* monoculture water; T3 group, fed with basal diet containing 15% of dried *C. pyrenoidosa* powder (CRM).

<i>Amino acid</i>	15d (g kg ⁻¹)			60d (g kg ⁻¹)		
	T1	T2	T3	T1	T2	T3
His	4.06 ± 0.02 ^a	3.92 ± 0.01 ^b	3.73 ± 0.02 ^c	3.35 ± 0.01 ^b	2.84 ± 0.01 ^c	3.52 ± 0.02 ^a
Arg	11.65 ± 0.01 ^a	11.23 ± 0.01 ^b	10.26 ± 0.06 ^c	8.85 ± 0.03 ^b	7.21 ± 0.02 ^c	10.90 ± 0.08 ^a
Ala	10.22 ± 0.03 ^a	9.76 ± 0.01 ^b	8.95 ± 0.04 ^c	8.21 ± 0.02 ^b	7.22 ± 0.02 ^c	8.87 ± 0.04 ^a
Pro	5.65 ± 0.02 ^a	5.46 ± 0.01 ^b	5.28 ± 0.03 ^c	6.68 ± 0.03 ^a	6.09 ± 0.04 ^c	6.27 ± 0.01 ^b
Tyr	6.62 ± 0.03 ^a	6.40 ± 0.05 ^b	5.91 ± 0.04 ^c	5.67 ± 0.03 ^a	4.99 ± 0.03 ^c	5.13 ± 0.04 ^b
TAA	179.16 ± 0.23 ^a	172.72 ± 0.06 ^b	155.89 ± 0.70 ^c	142.75 ± 0.06 ^b	122.05 ± 0.09 ^c	157.24 ± 0.85 ^a
EAA/TAA (%)	43.52 ± 0.03 ^b	43.60 ± 0.06 ^b	44.86 ± 0.05 ^a	44.16 ± 0.05 ^b	45.43 ± 0.72 ^a	41.36 ± 0.74 ^c
NEAA/TAA (%)	56.48 ± 0.03 ^b	56.40 ± 0.06 ^b	55.14 ± 0.05 ^a	55.84 ± 0.05 ^b	54.57 ± 0.72 ^a	58.64 ± 0.74 ^c
EAA/NEAA	0.77	0.77	0.81	0.79	0.83	0.71
EAA: Essential amino acid; NEAA: Non-essential amino acid; TAA: Total amino acid						
a,b,c Different letters indicate significant difference ($p < 0.05$) among means within the same row.						
T1 group, fed with basal diet; T2 group, reared in <i>C. pyrenoidosa</i> monoculture water; T3 group, fed with basal diet containing 15% of dried <i>C. pyrenoidosa</i> powder (CRM).						

The highest levels of saturated (SFA) and polyunsaturated (PUFA) fatty acids were found in the T3-15d group, whereas the highest levels of monounsaturated fatty acids (MUFA) occurred in the T1-15d group (Table 4). All fatty acid classes were present at higher levels in the T2-60d and T3-60d groups than in the T1-60d groups ($p < 0.05$). The largest ratio of omega-6 (ω -6) to omega-3 fatty acids (ω -3) was found in the T3-15d and T3-60d samples.

Table 4

The fatty acid profiles of the T1, T2 and T3 groups on the 15th and 60th day. Data represented in mean $\pm$ SE (n = 3).

Fatty acid (mg kg⁻¹)	T1-15d	T2-15d	T3-15d	T1-60d	T2-60d	T3-60d
Σ SFA	137.93 $\pm$ 4.44 ^a	88.63 $\pm$ 2.06 ^b	144.97 $\pm$ 6.16 ^a	79.47 $\pm$ 4.80 ^b	87.63 $\pm$ 4.90 ^b	118.67 $\pm$ 6.33 ^a
C12:0	0.57 $\pm$ 0.06 ^a	0.50 $\pm$ 0.10 ^a	0.50 $\pm$ 0.00 ^a	0.50 $\pm$ 0.01 ^b	0.50 $\pm$ 0.01 ^b	0.60 $\pm$ 0.01 ^a
C14:0	15.07 $\pm$ 0.46 ^a	6.10 $\pm$ 0.10 ^c	12.00 $\pm$ 0.50 ^b	6.97 $\pm$ 0.35 ^c	8.83 $\pm$ 0.59 ^b	15.07 $\pm$ 0.80 ^a
C15:0	1.93 $\pm$ 0.06 ^a	1.20 $\pm$ 0.00 ^c	1.73 $\pm$ 0.06 ^b	1.27 $\pm$ 0.06 ^b	1.37 $\pm$ 0.12 ^b	1.87 $\pm$ 0.12 ^a
C16:0	81.03 $\pm$ 2.91 ^a	51.00 $\pm$ 1.31 ^b	86.23 $\pm$ 3.81 ^a	46.80 $\pm$ 3.16 ^b	52.60 $\pm$ 2.72 ^b	72.10 $\pm$ 4.19 ^a
C17:0	1.77 $\pm$ 0.12 ^a	1.20 $\pm$ 0.00 ^b	1.73 $\pm$ 0.12 ^a	1.03 $\pm$ 0.06 ^b	1.03 $\pm$ 0.06 ^b	1.37 $\pm$ 0.06 ^a
C18:0	35.10 $\pm$ 1.14 ^b	26.70 $\pm$ 0.75 ^c	40.37 $\pm$ 1.70 ^a	21.10 $\pm$ 1.11 ^b	21.33 $\pm$ 1.50 ^b	25.10 $\pm$ 1.15 ^a
C20:0	1.60 $\pm$ 0.17 ^a	1.10 $\pm$ 0.00 ^b	1.50 $\pm$ 0.00 ^a	1.07 $\pm$ 0.06 ^b	1.17 $\pm$ 0.12 ^b	1.53 $\pm$ 0.06 ^a
C22:0	0.87 $\pm$ 0.06 ^a	0.83 $\pm$ 0.06 ^a	0.90 $\pm$ 0.00 ^a	0.73 $\pm$ 0.06 ^b	0.80 $\pm$ 0.00 ^b	1.03 $\pm$ 0.06 ^a
Σ MUFA	90.00 $\pm$ 1.78 ^a	49.57 $\pm$ 0.81 ^c	85.33 $\pm$ 3.46 ^b	46.03 $\pm$ 1.88 ^c	52.57 $\pm$ 3.14 ^b	71.00 $\pm$ 3.42 ^a
C16:1	21.40 $\pm$ 0.61 ^a	9.50 $\pm$ 0.40 ^c	17.50 $\pm$ 0.85 ^b	8.97 $\pm$ 0.47 ^c	10.97 $\pm$ 0.35 ^b	17.73 $\pm$ 0.90 ^a
C17:1	2.33 $\pm$ 0.15 ^a	1.10 $\pm$ 0.00 ^c	2.00 $\pm$ 0.10 ^b	1.20 $\pm$ 0.10 ^c	1.43 $\pm$ 0.06 ^b	2.30 $\pm$ 0.10 ^a

SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid

a,b,c Different letters indicate significant difference ($p < 0.05$) among means within the same row.

T1 group, fed with basal diet; T2 group, reared in *C. pyrenoidosa* monoculture water; T3 group, fed with basal diet containing 15% of dried *C. pyrenoidosa* powder (CRM).

Fatty acid (mg kg⁻¹)	T1-15d	T2-15d	T3-15d	T1-60d	T2-60d	T3-60d
C18:1n9c	50.00 ± 0.66 ^a	29.73 ± 1.00 ^b	50.47 ± 2.00 ^a	26.60 ± 1.31 ^b	29.83 ± 2.12 ^b	37.17 ± 1.80 ^a
C20:1	12.40 ± 0.56 ^a	5.73 ± 0.15 ^c	10.73 ± 0.31 ^b	6.03 ± 0.31 ^c	7.07 ± 0.47 ^b	9.60 ± 0.61 ^a
C24:1	3.87 ± 0.12 ^b	3.50 ± 0.35 ^b	4.63 ± 0.23 ^a	3.23 ± 0.06 ^b	3.27 ± 0.25 ^b	4.20 ± 0.10 ^a
Σ PUFA	171.80 ± 4.98 ^b	122.33 ± 3.07 ^c	187.73 ± 7.75 ^a	97.93 ± 5.42 ^b	98.13 ± 6.85 ^b	125.13 ± 6.06 ^a
C18:2n6c	14.73 ± 0.25 ^b	9.80 ± 0.26 ^c	18.43 ± 0.85 ^a	9.33 ± 0.50 ^b	10.17 ± 0.74 ^b	16.80 ± 1.05 ^a
C18:3n6	0.80 ± 0.10 ^a	0.43 ± 0.06 ^b	0.73 ± 0.06 ^a	0.57 ± 0.06 ^b	0.60 ± 0.00 ^b	0.77 ± 0.06 ^a
C18:3n3	7.17 ± 0.55 ^a	2.87 ± 0.06 ^b	6.50 ± 0.26 ^a	3.03 ± 0.21 ^b	3.50 ± 0.26 ^b	7.07 ± 0.35 ^a
C20:2	1.47 ± 0.12 ^b	1.10 ± 0.10 ^c	2.00 ± 0.17 ^a	1.00 ± 0.10 ^b	1.03 ± 0.06 ^b	1.27 ± 0.12 ^a
C20:3n6	1.23 ± 0.12 ^b	1.10 ± 0.00 ^b	1.47 ± 0.06 ^a	0.97 ± 0.06 ^a	0.93 ± 0.06 ^a	1.00 ± 0.00 ^a
C20:3n3	9.93 ± 0.51 ^a	3.40 ± 0.10 ^c	7.07 ± 0.25 ^b	3.93 ± 0.21 ^b	4.60 ± 0.26 ^b	8.33 ± 0.51 ^a
C20:4n6	15.00 ± 1.31 ^a	10.63 ± 0.31 ^b	16.40 ± 0.70 ^a	8.93 ± 0.45 ^b	9.23 ± 0.71 ^b	11.97 ± 0.45 ^a
C20:5n3	36.73 ± 1.18 ^a	19.93 ± 0.47 ^b	37.03 ± 1.45 ^a	16.10 ± 0.85 ^b	17.27 ± 1.19 ^b	24.80 ± 1.21 ^a
C22:6n3	84.73 ± 2.44 ^b	73.07 ± 1.83 ^c	98.10 ± 4.05 ^a	54.07 ± 3.00 ^a	50.80 ± 3.58 ^a	53.13 ± 2.36 ^a

SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid

a,b,c Different letters indicate significant difference ($p < 0.05$) among means within the same row.

T1 group, fed with basal diet; T2 group, reared in *C. pyrenoidosa* monoculture water; T3 group, fed with basal diet containing 15% of dried *C. pyrenoidosa* powder (CRM).

Fatty acid (mg kg ⁻¹)	T1-15d	T2-15d	T3-15d	T1-60d	T2-60d	T3-60d
ω-6/ω-3	0.23 ± 0.01b	0.22 ± 0.00b	0.25 ± 0.00a	0.26 ± 0.00c	0.27 ± 0.00b	0.33 ± 0.00a
SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid						
a,b,c Different letters indicate significant difference ($p < 0.05$) among means within the same row.						
T1 group, fed with basal diet; T2 group, reared in <i>C. pyrenoidosa</i> monoculture water; T3 group, fed with basal diet containing 15% of dried <i>C. pyrenoidosa</i> powder (CRM).						

Histological analysis of gut, muscle and intestine

The gut structural changes of three experimental groups were shown in Fig. 2. Remarkable shrinkage of gastric glands was observed in T2-15d, T3-15d, T2-60d and T3-60d, as compared to the T1-15d and T1-60d fishes. The reduced mucous membrane fold, excessive digesta and lipid accumulation were detected in T3-60d.

The muscle histology of all specimens was demonstrated in Fig. 3. There was no significant difference among three experimental groups during the 15d trial. Smaller muscle fibre bundles and larger intra muscular spaces were observed in fishes of the T3-60d treatment. The muscular fibre appeared to be more compact than T1-60d and T2-60d, respectively.

Enormous intestinal structure changes occurred in fishes of the T1, T2 and T3 experimental groups (Fig. 4). The goblet cells of the fishes in T2-15d and T2-60d were enlarged, with damaged epithelial tissue coupled with haemorrhage observed in T3-15d and T3-60d treatments. The accumulation of lipid droplets within the epithelial tissues in T3-60d treatment was apparent as compared to T1-60d and T2-60d (Fig. 4bi).

The intestinal 16S rDNA deep sequencing analysis

The intestinal microbiome analysis was carried out for all experimental groups (n = 3). The presence of *C. pyrenoidosa* did not induce any change in alpha diversity, as all the Simpson, Shannon, Chao1 and ACE indices revealed insignificant difference among T1, T2 and T3 groups.

The effects of *C. pyrenoidosa* on the intestinal microbial community in all the experimental groups were analysed at phylum level (Fig. 5a). Proteobacteria remains the most abundant phylum in T1-15d and T1-60d groups. The Fusobacteria dominance in T2-15d was reduced and replaced by Proteobacteria in T2-60d as the most abundant phylum in the community. Firmicutes consistently survived as the most

dominant phylum in T3-15d and T3-60d, along with the suppression of Fusobacteria and Proteobacteria communities.

The level 3 pathways of gut functional community in all experimental groups were compared with Welsh's t-test significant difference flagged at p value > 0.05 . (Fig. 5b-d). Three processes were enhanced among intestinal community in the T2-15d group, including galactose metabolism, dioxin and xylene degradation, whereas brassinosteroid biosynthesis and mRNA surveillance pathways were slightly deteriorated as compared to the control group (Fig. 5b). Parkinson disease-associated function was reduced in T2-60d (Fig. 5c).

Many functional pathways declined in the intestinal community in T3-60d group (Fig. 5d), including xenobiotics biodegradation and metabolism (toluene and ethylbenzene degradation), lipid metabolism (primary and secondary bile acid biosynthesis), metabolism of cofactors and vitamins such as biotin, riboflavin, porphyrin and chlorophyll, nicotinate and nicotinamide, the biosynthesis of flavonoid, polyketide sugar, lipopolysaccharide, lysosome, as well as diseases-related processes (African trypanosomiasis, Parkinson and biofilm formation of *Vibrio cholerae*).

Transcriptome analysis

A total of 405 and 2273 genes were upregulated, while 107 and 2273 genes were downregulated in T2-15d and T3-15d, respectively. When compared to the control, T2-60d and T3-60d demonstrated increased expression of the 64 and 130 genes and decreased expression of the 144 and 70 genes.

Figure 6 demonstrated the enriched GO terms and its number of annotated DEGs in respective experimental group. Based on the top 20 (ranked by lowest q value) out of 182 enriched GO terms, GO functions related to the reproduction, immunity and cell death were upregulated in T2-15d fishes, of which the GO term with the highest number of upregulated DEGs was response to organic substance (Fig. 6a). This positive effect in T2-15 did not last long to 60 days, with only eight GO terms linked to extracellular matrix were differentially regulated in T2-60d (Fig. 6b). In contrast, most of the 67 enriched GO functions in T3-15d were all declined, especially the GO terms that associated with metabolic processes and nuclear (Fig. 6c). The downregulation was recovered in T3-60d fishes, where 16 enriched GO terms were linked to regulation of hormone secretion and response to chemical and organic substance.

Three pathways were upregulated in T2-15d group, namely ferroptosis, IL-17 signalling pathway and legionellosis (Fig. 7a), while six enriched pathways were identified in T2-60d, including the three human neurodegenerative diseases, amoebiasis, oxidative phosphorylation and retrograde endocannabinoid signalling (Fig. 7b). All 16 enriched pathways were downregulated in T3-15d, especially cellular signalling pathways and human diseases (Fig. 7c), while only one pathway, Alzheimer disease was enriched in T3-60d.

Discussion

In order to reduce the operational expense of mariculture accounted for expensive fish meal, substitutes from microbes (Cui et al. 2013; Sun et al. 2019), plants (Shi et al. 2017; Chen et al. 2020; Hansen et al. 2020) and animals (Millamena 2002) were formulated in the past to facilitate continuous production. Unfortunately, these trials of replacement diet failed due to antinutritional components and imbalance nutritional ingredients. Replacement meal with high percentages of non-fish materials had been reported to impair fish health and reduced survival rate (Chen et al. 2020; Yin et al. 2020; Yong et al. 2020). Previous studies showed that as high as 45–75% plant protein replacement meal (Chen et al. 2020; Yin et al. 2020) triggered inflammation, decreased growth and survival rate, and 80% animal protein blend replacement meal (Ye et al. 2019b) induced pathogenic hepatic steatosis in hybrid groupers. In the present study, although unlethal gastrointestinal irritations were observed, the lower percentage of fish meal replacement with *C. pyrenoidosa* did not adversely affect the growth of hybrid groupers. This indicates that 15% of CRM is a better aquafeed alternative for marine fish, as compared to other plant-sourced proteins. Positive effects of 30% microalgae replacement meal had also been showed in gilthead seabream (Karapanagiotidis et al. 2022).

C. pyrenoidosa green water improved gut health

The function of intestinal goblet cells is to secrete anti-inflammatory mucin and maintain the mucous barrier in between the intestinal microbiome and underlying epithelial tissue (Alemão et al. 2021). The enlarged intestinal goblet cells in T2 fishes implied that the *C. pyrenoidosa* monoculture water had activated the protective mechanism by producing more intestinal mucus and strengthen the barrier. Besides that, live *C. pyrenoidosa* acts as nutrient scavenger and water cleaner in the rearing water (Deng et al. 2021), may have supported the dominance of intestinal Fusobacteria and Proteobacteria abundance in both T2-15d and T3-60d. Both phylum were reported to assist the fish host in digestion by synthesizing useful compounds such as enzymes and vitamins (Romero et al. 2014). The elevated dominance of Proteobacteria in T3-60d allegedly increased oxygen consumption to support the beneficial survival of anaerobes for healthy gut function, as found in young mammals (Shin et al. 2015). Indeed, the activities of energy metabolism, toxin removal (dioxin and xylene degradation) and protective mechanism (mRNA surveillance pathway and brassinosteroid pathway were improved in T2-15d gut functional community, though these activities were back to normal after 60 days.

RNA sequencing also conformed the positive effect of *C. pyrenoidosa* monoculture water, when all processes including immunity and reproduction were greatly improved in T2-15d. Higher expression of multiple NADH dehydrogenases and cytochrome c oxidases genes detected in T2-60d cued higher ATP production. Both enzymes catalyze oxidative phosphorylation pathway in ATP production, which determine the electron transfer from the intramitochondrial NADH to molecular oxygen (Wilson 2017). This process is important for the life support and metabolic homeostasis in higher animals (Wilson 2017).

However, long duration of culture with *C. pyrenoidosa* resulted in decreased level of TAA, including the EAA and NEAA. Protein is one of the four macromolecules that serve as crucial cell components. Though

the reason beyond the reduced level of AA was unknown, the protein deficiency had likely led to the downregulation of extracellular matrix structural protein genes such as collagen (*col1a1* and *col1a2*) and fibronectin (*fn1*).

The gastrointestinal changes in groupers in response to partial *C. pyrenoidosa* replacement meal (CRM)

The fish gastrointestinal morphology was known to change in response to stressors, which was often associated with the higher vertebrates' "General Adaptation Syndrome" (Peters 1982). Fish that fed with diet composed of high plant ingredients such as soy (Chen et al. 2020) and cottonseed proteins (Li et al. 2022) were frequently found to exhibit a certain degree of gastrointestinal inflammation. In fact, high percentage of non-fish supplement was not recommended to reduce the possibility of gastrointestinal health impairment (Ye et al. 2019a; Chen et al. 2020; Yong et al. 2020; Deng et al. 2021). For instance, 50% fish meal replacement by soy and cottonseed protein concentrates induced low growth rate, intestinal inflammation and reduced gut microbial diversity in juvenile hybrid groupers (*Epinephelus fuscoguttatus* × *E. lanceolatus*) (Mohd Faudzi et al. 2018; Yin et al. 2020). Another study showed that fish replacement meal containing unoptimized amount of soy and microalgae-derived ingredients could reduce growth and induce enteritis in giant groupers (Kissinger 2014; García-Ortega et al. 2016; Deng et al. 2021). In this study, the gastrointestinal histology revealed irritation with symptoms such as shrinkage of gastric glands and reduced mucous membrane fold (Fig. 2), damaged and haemorrhaging intestine epithelial layer (Fig. 4) due to CRM. The gastrointestinal structural changes described above were similar to the atrophic gastritis depicted in the stress-induced European eel (Peters 1982) and fish that fed with high plant ingredients as aforementioned. Furthermore, the ratio of Bacteroidetes to Firmicutes (B/F) in gut microbiota community were lower in T3 fishes (Fig. 5a), indicated the deterioration of gastrointestinal health (Li et al. 2023). Many digestive activities supported by intestinal functional community (Fig. 5d), such as the metabolism of fatty acids (bile acid biosynthesis) and cofactor (biotin and riboflavin), toxin removal (toluence and enthybenzene degradation) were greatly affected due to the intestinal microbial composition change induced by CRM. The macromolecules and primary metabolic pathways were also downregulated in T3-15d fishes (Fig. 6c), indicated that CRM suppressed the hybrid grouper gastrointestinal health.

The reason beyond the suppression may be the higher ratio of ω -6/ ω -3 triggered by CRM (Table 4). Balanced proportion of pro-inflammatory ω -6 and anti-inflammatory ω -3 was important as in maintaining gastrointestinal health (Løvmo et al. 2021) and reduced the risk of cytokine storm in human (DiNicolantonio and O'Keefe 2020). In this study, it was postulated that significant higher dietary ω -6/ ω -3 ratio (Table 4) had triggered stress responses, including shutdown of major signalling pathways in T3-15d (Fig. 6c), activated multiple cytokine-associated pathways to regulate the intestinal tight junction barrier and permeability (Li et al. 2023) (Fig. 6d) and the secretion and transport of corticosteroids (also called cortisol, a type of steroid hormone) in T3-60d (Fig. 6d) to cope with the inflammation induced by CRM. The anti-inflammatory role of cortisol was well documented in teleost fish (Mommensen et al. 1999; Tokarz et al. 2015). The mediation of cortisol synthesis and secretion-related genes (Table 5) in T3-60d may have attempted to curtail the event of tissue damage. Besides, the upregulation of cAMP-dependent

protein kinase catalytic subunit alpha gene (*prkaca*) in retrograde endocannabinoid signalling also depicted the attempt to cope with the pain and inflammation, which is similar to the human responses in rheumatic disease (Barrie and Manolios 2017).

Table 5

The genes associated with cortisol synthesis and secretion that were differentially expressed in T3-60d group (fed with *C. pyrenoidosa* powder).

<i>Unigene ID</i>	<i>Gene name</i>	<i>Log2FD</i>	<i>Function</i>
Unigene0051501	PREDICTED: glucocorticoid receptor isoform X1	2.36	regulation of glucocorticoid metabolic process; corticosteroid receptor signaling pathway
Unigene0013664	high affinity cAMP-specific and IBMX-insensitive 3',5'-cyclic phosphodiesterase 8A isoform X1	1.83	Cortisol synthesis and secretion
Unigene0049162	tyrosine-protein phosphatase non-receptor type 11	1.54	regulation of cortisol secretion
Unigene004720	cAMP-dependent protein kinase catalytic subunit alpha	1.41	Cortisol synthesis and secretion
Unigene0040586	Nuclear receptor subfamily 4 group A member 1	-1.14	Cortisol synthesis and secretion
Unigene0021393	POMC	-8.71	Cortisol synthesis and secretion
Note: Log2FD: Log 2-fold change			

Poor digestibility and excessive lipid supplied by CRM (7% higher than T1 diet) had presumably led to the appearance of pale and foamy lipid accumulation in the gut and intestine of T3-60d groupers (Fig. 8). This histopathology was highly similar to the lipid malabsorption syndrome found in Atlantic salmon that was overfed with plant ingredients (Hansen et al. 2020). When fed with CRM, the dominance of Firmicutes in gut microbial community was supported (Fig. 5a). The finding also found that the abundance of Firmicutes was positively correlated with the lipid content, including the crude fat content (Table 2), the level of ω -6 and the ratio of dietary ω -6/ ω -3 ratio (Table 4). The vital role of gut Firmicutes in regulating the host lipid metabolism had been reported in various animal models, such as human (Mohammadzadeh et al. 2021), mice (Chen et al. 2019), zebrafish (Jiang et al. 2020) and swine (Cui et al. 2013). It was speculated that the higher lipid content supplied by CRM shifted the gut microbial community proportion to speed up the excessive lipid storage. Fortunately, previous research found that choline supplement may help in mitigating the lipid malabsorption syndrome in fish fed with plant ingredient (Hansen et al. 2020). On an applied note, the presence of firm microalgal cell walls may be partly responsible for the poor digestibility of dried *C. pyrenoidosa* on diet palatability. An earlier study demonstrated that enzymatic and mechanical processing steps during the manufacturing process of

feed can significantly increase the digestibility of dried microalgae biomass by weakening the cell wall (Batista et al. 2020).

Conclusion

In conclusion, the growth of hybrid groupers was unaffected by the presence of *C. pyrenoidosa*. The hybrid groupers' crude fat content significantly rose after *C. pyrenoidosa* was introduced, along with their intramuscular spaces and muscle fiber bundle sizes. The muscular alterations suggested softer flesh texture, which is ideal for human consumption. Because it increased the diversity of the beneficial gut microbial community, enhanced intestinal protection, and generally improved the digestive system over the course of at least 15 days, rearing juvenile groupers in *C. pyrenoidosa* monoculture water was beneficial to their overall gut health. On the other hand, CRM caused enteritis and gastritis, decreased mucous membrane fold, intestinal tissue damage, increased fat accumulation, and altered gut microbial composition, all of which had a negative impact on digestion. Farmers might use alternating feeding between CRM and basal diet, explore the use of choline supplements or prior microalgae cell wall processing to prevent the negative effects of CRM on hybrid groupers.

Materials and methods

The preparation of experimental diets

The commercial pellets (EP feed YouShi #2, Santong Bio-engineering (Weifang) Co., Ltd.) was used as basal diet. Both live *C. pyrenoidosa* cells and *C. pyrenoidosa* powder were supplied by Qingdao Langyatai Group Co., Ltd. China to ensure the identical species used in both treatments. To prepare CRM, the commercial pellets were crushed into fine powder and mixed with 15% of *C. pyrenoidosa* powder. The mixture was then compressed into pellets and kept under 4°C until further use. The composition of basal diet and *Chlorella* meal was shown in Table 6. Live *C. pyrenoidosa* was consistently restocked and kept under 4°C until further use. All the diets were freshly prepared biweekly.

Table 6
Biochemical composition of T1, T2 and T3 diets.

Nutrient level	T1	T2	T3
Crude protein	32.01	32.01	30.42
Crude fat	12.00	12.00	12.88
Crude Ash	13.05	13.05	10.77
Moisture content	6.76	6.76	11.44
T1 group, fed with basal diet; T2 group, reared in <i>C. pyrenoidosa</i> monoculture water; T3 group, fed with basal diet containing 15% of dried <i>C. pyrenoidosa</i> powder (CRM).			

Fish rearing and experimental design

The juvenile hybrid groupers (*E. lanceolatu* × *E. fuscoguttatus* , average body weight: 9.91 ± 0.33 g; average body length: 8.86 ± 0.57 cm) used in this study were supplied by Tianjin Lida Sea Water Resources Development Co., Ltd. China. Two weeks prior to the experiment, the fish were fed twice daily (8:00 and 16:00 hours) with the basal diet (3% of the total body weight) and acclimatized in one culture tank under temperature of $20 \pm 3^\circ\text{C}$ and dissolved oxygen kept above 5 mg L^{-1} .

After obtained the initial measurement of body weight (IBW) and length (IBL), the fish were randomly allocated and cultured in the tanks (80 cm diameter × 80 cm height) that filled with 200 L of filtered seawater and fed twice a day (3% of the fish's body weight) (Fig. 1a). The T1 control group was fed with basal diet only, while the T2 group was consistently reared in the filtered seawater containing 10^7 cells mL^{-1} of fresh *C. pyrenoidosa*. Meanwhile, the T3 group was fed with *Chlorella* pellets only. All experiments were performed in triplicate in two feeding trials, 15 days and 60-days in duration. The tank condition was maintained at $25 \pm 1^\circ\text{C}$ with dissolved oxygen (DO) $> 5 \text{ mg/L}$, pH 7.0 ± 0.1 and 26 ± 2 PSU salinity. Half volume of the culture water was replaced with fresh filtered seawater twice daily in all groups and the *C. pyrenoidosa* cell density in T2 group was measured and adjusted after each water change.

Assessment parameters and approach

Growth performance

On the 15th day and 60th day, the fish was captured and anaesthetized as described elsewhere (Ng et al. 2021), followed by the final body weight and length measurement (Fig. 1). The growth parameters including weight gain rate (WGR), specific growth rate (SGR) and hepatosomatic index (HIS) were calculated as described elsewhere (Ren et al. 2020).

Proximate composition analysis and biochemical indices

The whole fish sample was homogenized and used to carry out the proximate composition analysis, including the content of moisture, ash and crude lipid, were determined according to Wang et al. (2022). The fish was dried in the oven at 105°C until constant weight and the moisture content was the determined by gravimetric assay. The ash content was weighed from the ash produced from complete combustion of samples until constant weight at 550°C in a muffle furnace. Crude protein content was quantified by the Kjeldahl method (Barbano et al. 1990) utilizing the Kjeldahl nitrogen analyzer (Kjeltec 2200, FOSS, Denmark). The crude lipid was extracted by using chloroform-methanol approach (Folch et al. 1957) and the fatty acid profiling (in form of fatty acid methyl ester) was conducted by using Agilent 7890A gas chromatograph (Agilents Technologies, USA) with a CNW CD-2560 fused silica capillary column (Hewlett Packard, USA) and a flame ionization detector. Meanwhile, the amino acid profile was determined by Agilent 1260 liquid chromatograph (Agilents Technologies, USA).

Muscle, gut and intestinal histology

Prior to the dissection, the fish were rinsed in sterile dH₂O and 75% ethanol, followed by three repeated rinse by sterile dH₂O. To prepare the tissues for histological analysis (Bancroft and Gamble 2008), the cross-section of fish posterior muscles (between dorsal and causal fins), gut and anterior intestines were fixed in 10% formalin, dehydrated in a series of alcohol solutions (70–100%) and embedded in paraffin wax. Final fixing block of 3 µm thickness was cut and stained by haematoxylin and eosin. The morphological features of the specimens were then captured by Motic BA 600 Mot automatic microscope equipped with the DSAssistant Lite software.

Intestinal microbiota analysis

High-throughput 16S rDNA sequencing

One-gram of pooled intestinal specimen was collected for each biological replicate and kept under – 80 °C. The total genomic DNA of intestinal specimen was extracted by HiPure Stool DNA extraction kit (Magen, China) in accordance of manufacturer's protocol. Then, the extracted DNA was used as DNA template for the PCR amplification of the V3-V4 hypervariable regions of prokaryotic 16S rDNA gene in the following PCR cycles: 94°C for 2 min, followed by 30 cycles at 98°C for 10 s, 62–66°C for 30 s, and 68°C for 30 s and a final extension at 68°C for 5 min. The primer pairs used were 341 F and 806R with sequences 5' CCTACGGGNGGCWGCAG 3' and 5' GGACTACHVGGGTATCTAAT 3'. The 50 µL PCR reaction mixture (TOYOBO, Japan) was composed of 1× KOD Buffer, 10 mM dNTPs, 75 mM MgSO₄, 15 µM of each forward and reverse primer, 1 unit of KOD polymerase and 100 ng DNA template.

Then, the PCR amplicons were purified using the AxyPrep DNA Gel extraction kit (Axygen Biosciences, USA) and quantified by using ABI StepOnePlus Real-Time PCR System (Life Technologies, USA). Purified amplicons were then pooled in equimolar and paired-end sequenced (250 bp) on Illumina Novaseq 6000 platform. The generated raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP*****).

Data analysis

Raw reads generated from sequencing were filtered by fastp (Chen et al. 2018) to remove ambiguous nucleotides and low quality bases. The paired-end clean reads were merged as raw tags with FLASH (Magoč and Salzberg 2011) with a minimum overlap of 10bp and mismatch error rates of 2% and were filtered by QIIME pipeline (Caporaso et al. 2010). The high quality tags were queried against the reference database to perform chimera checking via UCHIME algorithm (Edgar et al. 2011) to remove any chimeric tag. The remaining effective tags were clustered into operational taxonomic units (OTU) at 97% similarity via UPARSE (Edgar 2013). The highest abundance tag sequence was selected as representative sequence for each cluster. Unique and common OTUs were identified with UpSetR package (Conway et al. 2017).

The Chao1, Simpson, Shannon and goods indices were determined by QIIME (Caporaso et al. 2010). Taxonomic richness was performed by QIIME and the microbiota functional profiles were generated by using PICRUST (Langille et al. 2013).

Transcriptomic analysis

A total of 4 mL of whole blood was collected from all experimental groups by puncturing through the caudal vessels with citrate sodium used as the blood anticoagulant. The blood specimens were flash frozen in liquid nitrogen and kept in -80 °C until further use.

RNA extraction, library construction and sequencing

Total RNA of whole blood specimens was extracted using TRIzol™ reagent (Invitrogen, USA) according to the manufacturer's protocol. The RNA quality was assessed by Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and RNase free agarose gel electrophoresis. The eukaryotic mRNA was enriched by Oligo(dT) beads and fragmented into short fragments using fragmentation buffer. The short RNA fragments were then reverse transcribed to synthesize cDNA libraries with random primers. The second-strand of cDNA was synthesized and purified with QiaQuick PCR extraction kit (Qiagen, The Netherlands). The cDNA was then ligated to Illumina sequencing adapters and the ligation products were sequenced on Illumina NovaSeq 6000 system (Gene Denovo Biotechnology Co., China).

RNA-Seq data processing and unigenes annotation

The raw reads generated from paired-end sequencing were trimmed and quality-checked with fastp (Chen et al. 2018) to get rid of adaptor sequences, ambiguous nucleotides and low quality reads. Clean reads were pooled for *de novo* assembly by Trinity software (Grabherr et al. 2011). The assembled unigenes were subjected to BUSCO v5 (Simão et al. 2015) for completeness assessment against the actinopterygii lineage.

The unigenes were annotated in five public databases, including non-redundant protein (Nr) database (<http://www.ncbi.nlm.nih.gov>), the Swiss-Prot protein database (<http://www.expasy.ch/sprot>), the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.genome.jp/kegg>), COG/KOG database (<http://www.ncbi.nlm.nih.gov/COG>) and Gene Ontology (GO) Resource (<http://geneontology.org/>). The highest similarity hit was selected for each unigene.

Gene expression quantification and downstream analysis

The clean reads were mapped to the unigenes by using Bowtie2 (Langmead and Salzberg 2012) with default settings. The transcript abundance was quantified by RSEM (Li and Dewey 2011).

Comparative analysis between control (T1) and two treatment groups (T2 and T3) was performed by using DESeq2 (Love et al. 2014). The cutoff values for differentially expressed gene (DEG) were false discovery rate (*q*value) < 0.05 and log 2-fold change (log2FC) ≥ 1 and ≤ -1 . The DEG list was subjected to GO and KEGG pathway enrichment analysis through Omicsmart web-based tools (<https://www.omicsmart.com/>) by using unigenes as the background gene set. The cutoff values for enriched GO terms and KEGG pathway were *q*value < 0.05 and gene count ≥ 2 .

Statistical analysis

The differences among the treatment and control groups were evaluated by One-way Analysis of Variance (ANOVA) using SPSS 16.0 software (SPSS Inc.) and Duncan's multiple range test was employed for comparison among the groups. All the data are presented as mean $\pm$ standard error (SE) in three replicates and the p -value < 0.05 was regarded as statistically significant.

Declarations

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Data availability statement

Raw data of transcriptomic and 16S amplicon sequencing generated from this study is deposited into NCBI SRA under BioProject accession: PRJNA917794.

Authors' Contributions

MWJ and YYS designed the experiments, supervised the projects and revised the manuscripts. ZWH performed experiments and wrote the manuscript. LCC analysed data and wrote the manuscript. ZWL, GJW and JZF acquired financial support and supervised the project. Final manuscript was approved by all the authors.

Ethics declarations

The handling of animals complied with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) and was approved by the Institutional Animal Care and Use Committee (IACUC) of Universiti Malaysia Terengganu.

Disclosure statement

No potential conflict of interest was reported by the author(s).

References

1. Trupp, M., et al., *Functional receptor for GDNF encoded by the c-ret proto-oncogene*. Nature, 1996. **381**(6585): p. 785-789.
2. Ibanez, C.F., *Structure and physiology of the RET receptor tyrosine kinase*. Cold Spring Harb Perspect Biol, 2013. **5**(2).
3. Trupp, M., et al., *Complementary and Overlapping Expression of Glial Cell Line-Derived Neurotrophic Factor (GDNF), c-ret Proto-Oncogene, and GDNF Receptor- α Indicates Multiple Mechanisms of Trophic Actions in the Adult Rat CNS*. The Journal of Neuroscience, 1997. **17**: p. 3554-3567.

4. Wang, X., *Structural studies of GDNF family ligands with their receptors-Insights into ligand recognition and activation of receptor tyrosine kinase RET*. Biochim Biophys Acta, 2013. **1834**(10): p. 2205-12.
5. Aron, L. and R. Klein, *Repairing the parkinsonian brain with neurotrophic factors*. Trends Neurosci, 2011. **34**(2): p. 88-100.
6. Murakumo, Y., et al., *RET and neuroendocrine tumors*. Pituitary, 2006. **9**(3): p. 179-92.
7. Anders, J., S. Kjar, and C.F. Ibanez, *Molecular modeling of the extracellular domain of the RET receptor tyrosine kinase reveals multiple cadherin-like domains and a calcium-binding site*. J Biol Chem, 2001. **276**(38): p. 35808-17.
8. Knowles, P.P., et al., *Structure and chemical inhibition of the RET tyrosine kinase domain*. J Biol Chem, 2006. **281**(44): p. 33577-87.
9. Treanor, J.J.S., et al., *Characterization of a multicomponent receptor for GDNF*. Nature, 1996. **382**(6586): p. 80-83.
10. Jing, S., et al., *GDNF-Induced Activation of the Ret Protein Tyrosine Kinase Is Mediated by GDNFR- α , a Novel Receptor for GDNF*. Cell, 1996. **85**(7): p. 1113-1124.
11. Li, J., et al., *Cryo-EM analyses reveal the common mechanism and diversification in the activation of RET by different ligands*. Elife, 2019. **8**.
12. Hsu, J.Y., et al., *Non-homeostatic body weight regulation through a brainstem-restricted receptor for GDF15*. Nature, 2017. **550**(7675): p. 255-259.
13. Yang, L., et al., *GFRAL is the receptor for GDF15 and is required for the anti-obesity effects of the ligand*. Nat Med, 2017. **23**(10): p. 1158-1166.
14. Airaksinen, M.S. and M. Saarma, *The GDNF family: signalling, biological functions and therapeutic value*. Nat Rev Neurosci, 2002. **3**(5): p. 383-94.
15. Encinas, M., et al., *c-Src Is Required for Glial Cell Line-Derived Neurotrophic Factor (GDNF) Family Ligand-Mediated Neuronal Survival via a Phosphatidylinositol-3 Kinase (PI-3K)-Dependent Pathway*. The Journal of Neuroscience, 2001. **21**(5): p. 1464-1472.
16. Encinas, M., et al., *Tyrosine 981, a novel ret autophosphorylation site, binds c-Src to mediate neuronal survival*. J Biol Chem, 2004. **279**(18): p. 18262-9.
17. Zhang, Y., et al., *Interaction of SH2-B β with RET is involved in signaling of GDNF-induced neurite outgrowth*. J Cell Sci, 2006. **119**(Pt 8): p. 1666-76.
18. Crowder, R.J., et al., *Dok-6, a Novel p62 Dok family member, promotes Ret-mediated neurite outgrowth*. J Biol Chem, 2004. **279**(40): p. 42072-81.
19. Cheng, Y., C. Duan, and C. Zhang, *New perspective on SH2B1: An accelerator of cancer progression*. Biomed Pharmacother, 2020. **121**: p. 109651.
20. Uchida, M., et al., *Dok-4 regulates GDNF-dependent neurite outgrowth through downstream activation of Rap1 and mitogen-activated protein kinase*. J Cell Sci, 2006. **119**(Pt 15): p. 3067-77.

21. Kurokawa, K., et al., *Identification of SNT/FRS2 docking site on RET receptor tyrosine kinase and its role for signal transduction*. *Oncogene*, 2001. **20**(16): p. 1929-1938.
22. Culpier, M., J. Anders, and C.F. Ibanez, *Coordinated activation of autophosphorylation sites in the RET receptor tyrosine kinase: importance of tyrosine 1062 for GDNF mediated neuronal differentiation and survival*. *J Biol Chem*, 2002. **277**(3): p. 1991-9.
23. Drinkut, A., et al., *Ret is essential to mediate GDNF's neuroprotective and neuroregenerative effect in a Parkinson disease mouse model*. *Cell Death Dis*, 2016. **7**(9): p. e2359.
24. Chang, K.Y., et al., *Light-inducible receptor tyrosine kinases that regulate neurotrophin signalling*. *Nat Commun*, 2014. **5**: p. 4057.
25. Kim, N., et al., *Spatiotemporal control of fibroblast growth factor receptor signals by blue light*. *Chem Biol*, 2014. **21**(7): p. 903-12.
26. Grusch, M., et al., *Spatio-temporally precise activation of engineered receptor tyrosine kinases by light*. *EMBO J*, 2014. **33**(15): p. 1713-26.
27. Woo, D., et al., *Locally Activating TrkB Receptor Generates Actin Waves and Specifies Axonal Fate*. *Cell Chem Biol*, 2019. **26**(12): p. 1652-1663 e4.
28. Ingles-Prieto, A., et al., *Optogenetic delivery of trophic signals in a genetic model of Parkinson's disease*. *PLoS Genet*, 2021. **17**(4): p. e1009479.
29. Park, H., et al., *Optogenetic protein clustering through fluorescent protein tagging and extension of CRY2*. *Nat Commun*, 2017. **8**(1): p. 30.
30. Kim, S., et al., *Non-invasive optical control of endogenous Ca(2+) channels in awake mice*. *Nat Commun*, 2020. **11**(1): p. 210.
31. Hong, J. and W.D. Heo, *Optogenetic Modulation of TrkB Signaling in the Mouse Brain*. *J Mol Biol*, 2020. **432**(4): p. 815-827.
32. Liu, H., et al., *Photoexcited CRY2 Interacts with CIB1 to Regulate Transcription and Floral Initiation in *Arabidopsis**. *Science*, 2008. **322**(5907): p. 1535-1539.
33. Park, W.S., et al., *Comprehensive identification of PIP3-regulated PH domains from *C. elegans* to *H. sapiens* by model prediction and live imaging*. *Mol Cell*, 2008. **30**(3): p. 381-92.
34. Riedl, J., et al., *Lifeact: a versatile marker to visualize F-actin*. *Nat Methods*, 2008. **5**(7): p. 605-7.
35. Regot, S., et al., *High-sensitivity measurements of multiple kinase activities in live single cells*. *Cell*, 2014. **157**(7): p. 1724-34.
36. Hyeon, B., M.K. Nguyen, and W. Do Heo, *Optogenetic Control of Membrane Trafficking Using Light-Activated Reversible Inhibition by Assembly Trap of Intracellular Membranes (IM-LARIAT)*, in *Membrane Trafficking*. 2022. p. 309-331.
37. Burotto, M., et al., *The MAPK pathway across different malignancies: a new perspective*. *Cancer*, 2014. **120**(22): p. 3446-56.
38. Hayashi, Y., et al., *Activation of BMK1 via Tyrosine 1062 in RET by GDNF and MEN2A Mutation*. *Biochemical and Biophysical Research Communications*, 2001. **281**(3): p. 682-689.

39. Hayashi, H., et al., *Characterization of intracellular signals via tyrosine 1062 in RET activated by glial cell line-derived neurotrophic factor*. *Oncogene*, 2000. **19**(39): p. 4469-4475.
40. Yang, H.W., et al., *Cooperative activation of PI3K by Ras and Rho family small GTPases*. *Mol Cell*, 2012. **47**(2): p. 281-90.
41. Golden, J.P., et al., *Expression of neurturin, GDNF, and their receptors in the adult mouse CNS*. *Journal of Comparative Neurology*, 1998. **398**(1): p. 139-150.
42. Bonafina, A., et al., *GDNF and GFRalpha1 Are Required for Proper Integration of Adult-Born Hippocampal Neurons*. *Cell Rep*, 2019. **29**(13): p. 4308-4319 e4.
43. Wilson, M.Z., et al., *Tracing Information Flow from Erk to Target Gene Induction Reveals Mechanisms of Dynamic and Combinatorial Control*. *Mol Cell*, 2017. **67**(5): p. 757-769 e5.
44. Gross, S.M. and P. Rotwein, *Mapping growth-factor-modulated Akt signaling dynamics*. *J Cell Sci*, 2016. **129**(10): p. 2052-63.
45. Bugaj, L.J., et al., *Optogenetic protein clustering and signaling activation in mammalian cells*. *Nat Methods*, 2013. **10**(3): p. 249-52.
46. Richardson, D.S., A.Z. Lai, and L.M. Mulligan, *RET ligand-induced internalization and its consequences for downstream signaling*. *Oncogene*, 2006. **25**(22): p. 3206-11.
47. Tsui, C.C. and B.A. Pierchala, *The differential axonal degradation of Ret accounts for cell-type-specific function of glial cell line-derived neurotrophic factor as a retrograde survival factor*. *J Neurosci*, 2010. **30**(15): p. 5149-58.
48. Sariola, H. and M. Saarma, *Novel functions and signalling pathways for GDNF*. *J Cell Sci*, 2003. **116**(Pt 19): p. 3855-62.
49. Takaku, S., et al., *GDNF promotes neurite outgrowth and upregulates galectin-1 through the RET/PI3K signaling in cultured adult rat dorsal root ganglion neurons*. *Neurochem Int*, 2013. **62**(3): p. 330-9.
50. Bogucka-Janczi, K., et al., *ERK3/MAPK6 dictates Cdc42/Rac1 activity and ARP2/3-dependent actin polymerization*. *bioRxiv*, 2022.
51. Lin, L.-F.H., et al., *GDNF: a Glial Cell Line-Derived Neurotrophic Factor for Midbrain Dopaminergic Neurons*. *Science*, 1993. **260**(5111): p. 1130-1132.
52. Kowsky, S., et al., *RET signaling does not modulate MPTP toxicity but is required for regeneration of dopaminergic axon terminals*. *Proceedings of the National Academy of Sciences*, 2007. **104**(50): p. 20049-20054.
53. Jung, H., et al., *Noninvasive optical activation of Flp recombinase for genetic manipulation in deep mouse brain regions*. *Nat Commun*, 2019. **10**(1): p. 314.
54. Mishra, A.K. and A. Dixit, *Dopaminergic Axons: Key Recitalists in Parkinson's Disease*. *Neurochem Res*, 2022. **47**(2): p. 234-248.
55. Kjaer, S., et al., *Self-association of the transmembrane domain of RET underlies oncogenic activation by MEN2A mutations*. *Oncogene*, 2006. **25**(53): p. 7086-95.

56. Ladbury, J.E., C.C. Lin, and K.M. Suen, *Phase separation enhances probability of receptor signalling and drug targeting*. Trends Biochem Sci, 2023.
57. Murphy, J.E., et al., *Endosomes: A legitimate platform for the signaling train*. Proceedings of the National Academy of Sciences, 2009. **106**(42): p. 17615-17622.
58. Cui, B., et al., *One at a time, live tracking of NGF axonal transport using quantum dots*. Proceedings of the National Academy of Sciences, 2007. **104**(34): p. 13666-13671.
59. Fukuda, T., K. Kiuchi, and M. Takahashi, *Novel mechanism of regulation of Rac activity and lamellipodia formation by RET tyrosine kinase*. J Biol Chem, 2002. **277**(21): p. 19114-21.
60. Mesa-Infante, V., et al., *Long-term exposure to GDNF induces dephosphorylation of Ret, AKT, and ERK1/2, and is ineffective at protecting midbrain dopaminergic neurons in cellular models of Parkinson's disease*. Mol Cell Neurosci, 2022. **118**: p. 103684.
61. Barker, R.A., et al., *GDNF and Parkinson's Disease: Where Next? A Summary from a Recent Workshop*. J Parkinsons Dis, 2020. **10**(3): p. 875-891.
62. Georgievska, B., D. Kirik, and A. Bjorklund, *Aberrant sprouting and downregulation of tyrosine hydroxylase in lesioned nigrostriatal dopamine neurons induced by long-lasting overexpression of glial cell line derived neurotrophic factor in the striatum by lentiviral gene transfer*. Exp Neurol, 2002. **177**(2): p. 461-74.
63. Tereshchenko, J., et al., *Pharmacologically controlled, discontinuous GDNF gene therapy restores motor function in a rat model of Parkinson's disease*. Neurobiol Dis, 2014. **65**: p. 35-42.
64. Chtarto, A., et al., *A regulatable AAV vector mediating GDNF biological effects at clinically-approved sub-antimicrobial doxycycline doses*. Mol Ther Methods Clin Dev, 2016. **5**: p. 16027.

Figures



Figure 1

The experiments were conducted to study the effects of partial *C. pyrenoidosa* replacement meal (CRM) and *C. pyrenoidosa* monoculture water on hybrid groupers. (a) The hybrid groupers were cultured in tanks with dimensions of 80 cm in diameter × 80 cm in height. The weight and length of the hybrid groupers were measured at the end of (b) 15-days and (c) 60-days trials. From the left: T1, T2 and T3 fishes. Note: T1 group, fed with basal diet; T2 group, reared in *C. pyrenoidosa* monoculture water; T3 group, fed with basal diet containing 15% of dried *C. pyrenoidosa* powder (CRM).

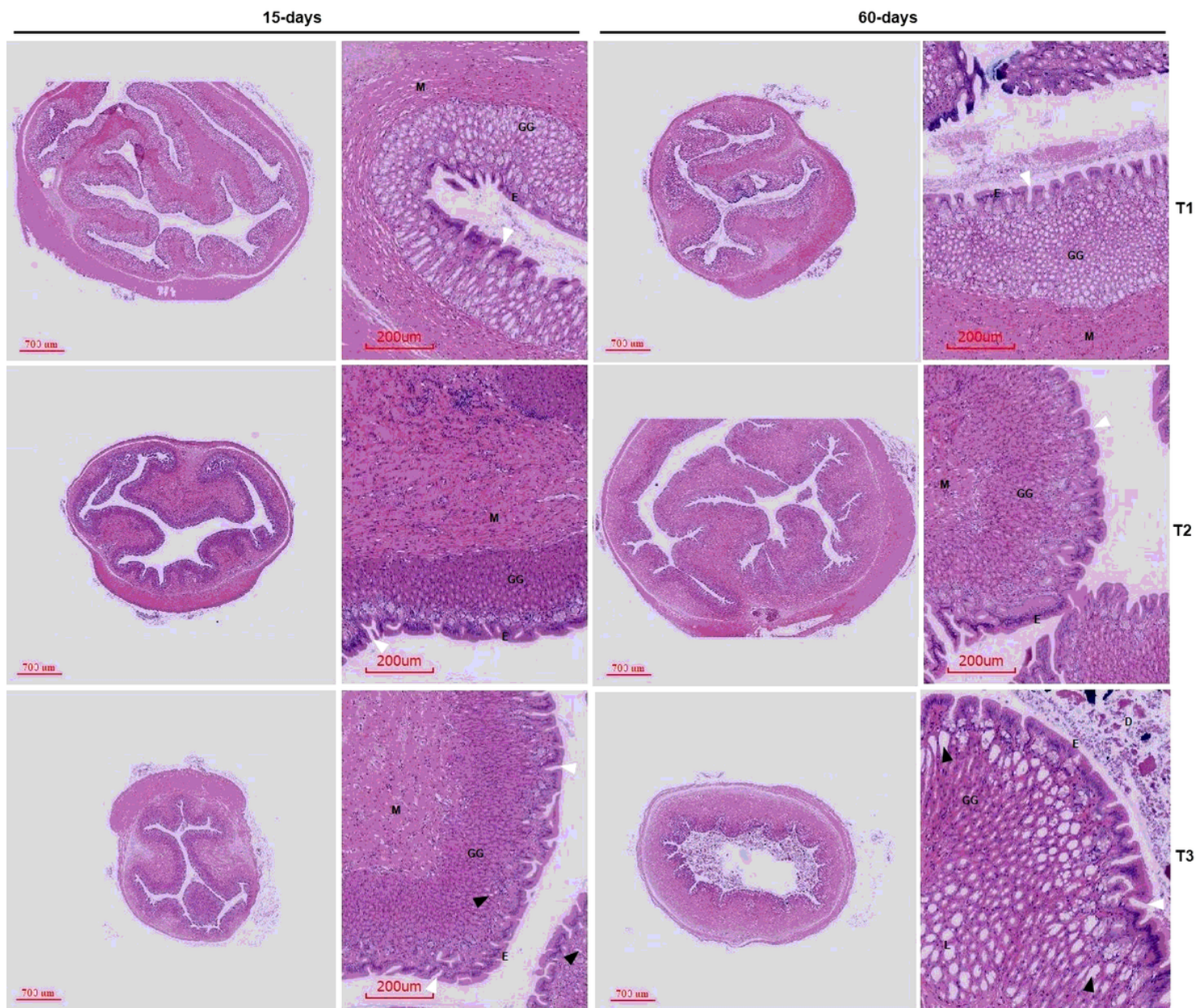


Figure 2

The stomach histological analysis in T1, T2 and T3 fishes examined in 15-days (15d) and 60 days (60d) trials. Fold significantly reduced in T3-60d stomach. Both T2 and T3 fishes demonstrated significant shrinkage of gastric glands (GG) in 15-days and 60-days trials, as compared to the T1 fishes. Lipid accumulation (L) was observed within the gastric gland layer of T3-60d. Vacuolar degeneration (black arrowhead) was observed in both T3-15d and T3-60d fishes. D: digesta; M: muscularis; white arrowhead: gastric pit; E: Epithelial layer. HE staining. Note: T1 group, fed with basal diet; T2 group, reared in *C. pyrenoidosa* monoculture water; T3 group, fed with partial *C. pyrenoidosa* replacement meal (CRM) basal diet containing 15% of dried *C. pyrenoidosa* powder (CRM).

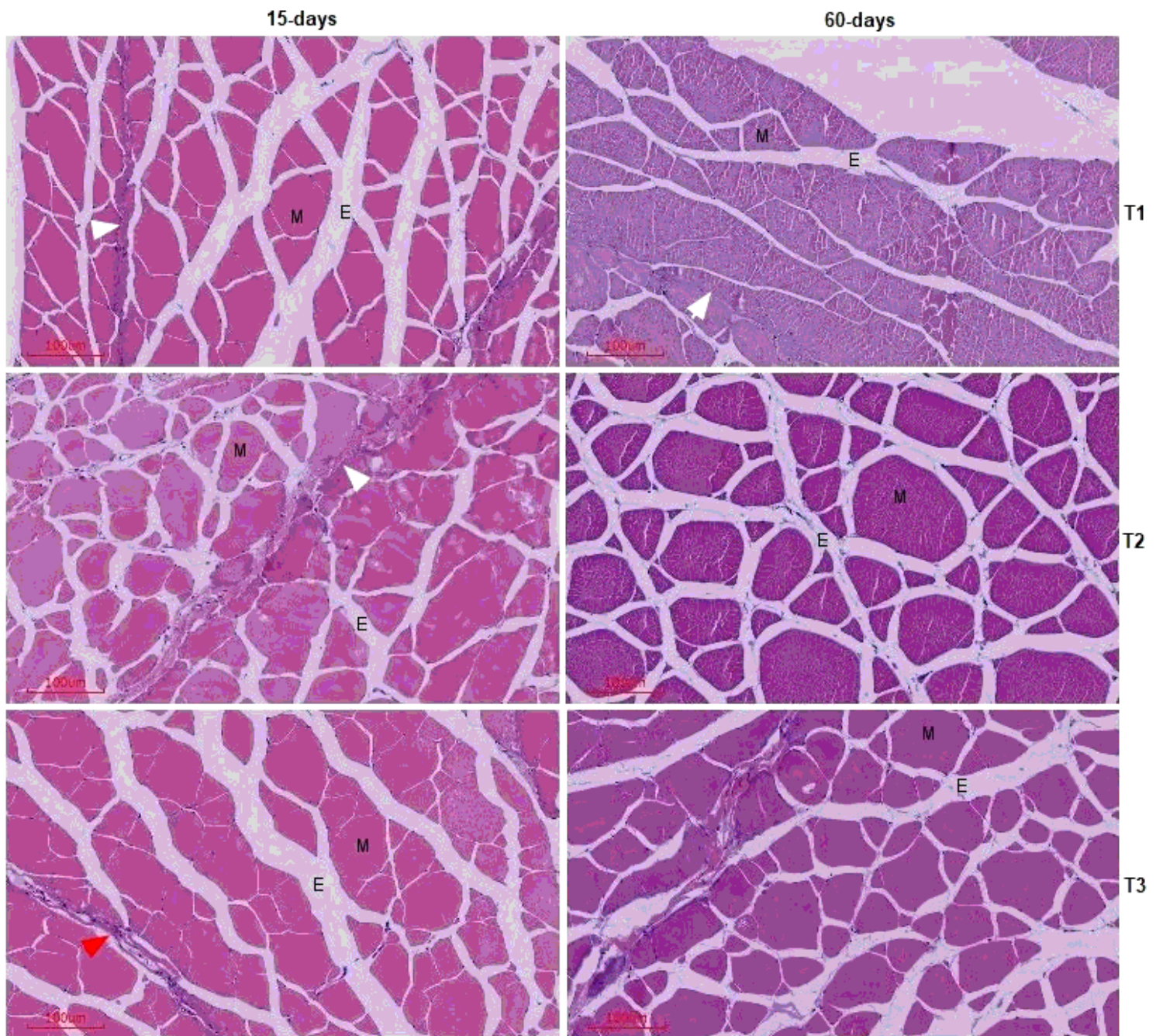


Figure 3

The muscle histological analysis in T1, T2 and T3 fishes examined in 15-days (15d) and 60 days (60d) trials. The muscular bundles (M) in T3-60d were smaller in shape. The larger inter muscular spaces, or called endomysium (E) were observed in T2-60d and T3-60d. White arrowhead: connective septum; red arrowhead: blood vessel. HE staining. Note: T1 group, fed with basal diet; T2 group, reared in *C. pyrenoidosa* monoculture water; T3 group, fed with basal diet containing 15% of dried *C. pyrenoidosa* powder (CRM).

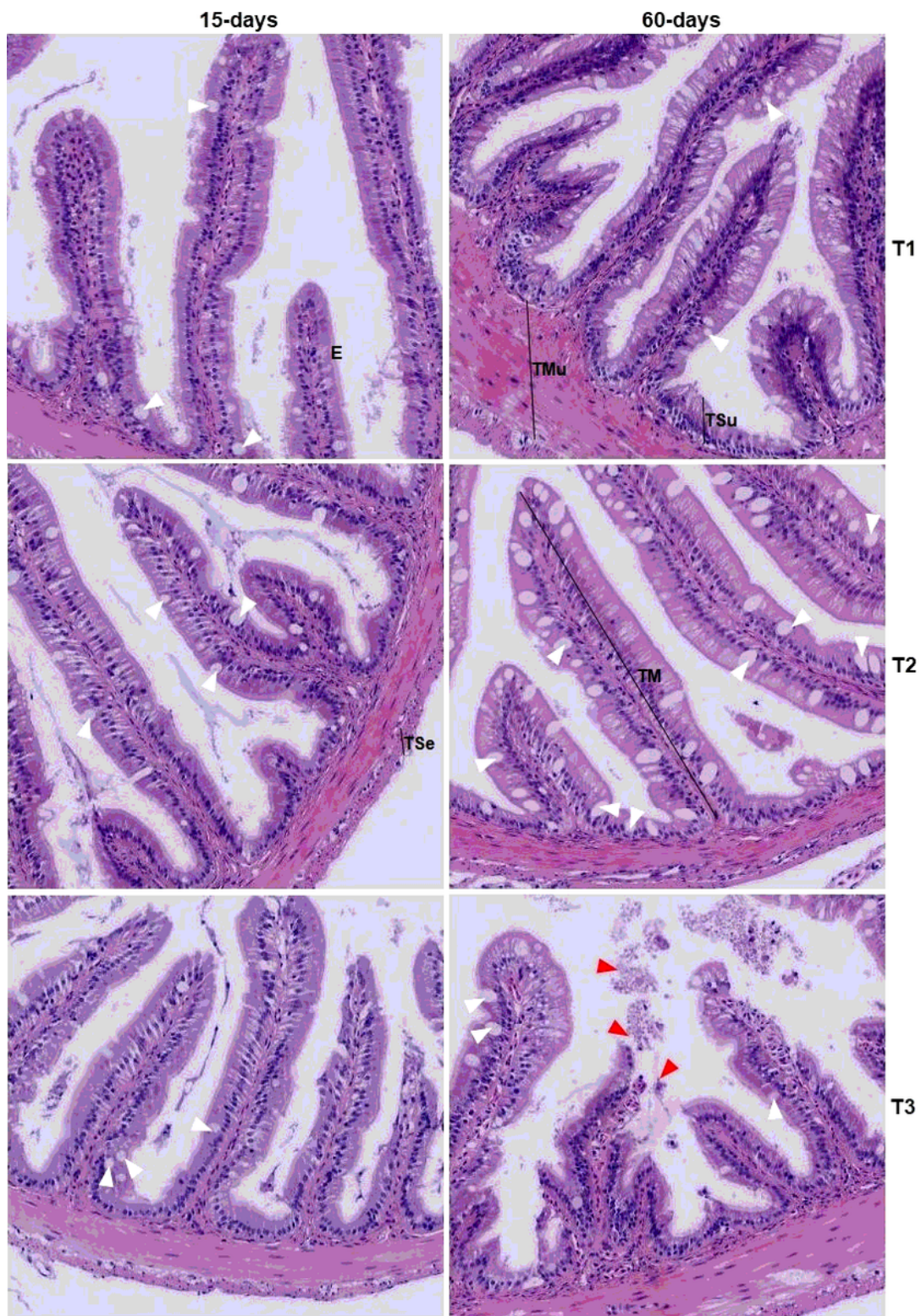


Figure 4

The interior intestine histological analysis in T1, T2 and T3 fishes examined in 15-days (15d) and 60 days (60d) trials. Enlarged goblet cells (white arrowhead) were observed in T2-15d and T2-60d. Damaged epithelial layer with haemorrhages (red arrowhead) was found in T3-15d and T3-60d. TM: tunica mucosa; TMu: tunica muscularis; TSu: tunica submucosa; TSe: tunica serosa; E: simple columnar epithelial layer.

HE staining. Note: T1 group, fed with basal diet; T2 group, reared in *C. pyrenoidosa* monoculture water; T3 group, fed with basal diet containing 15% of dried *C. pyrenoidosa* powder (CRM).

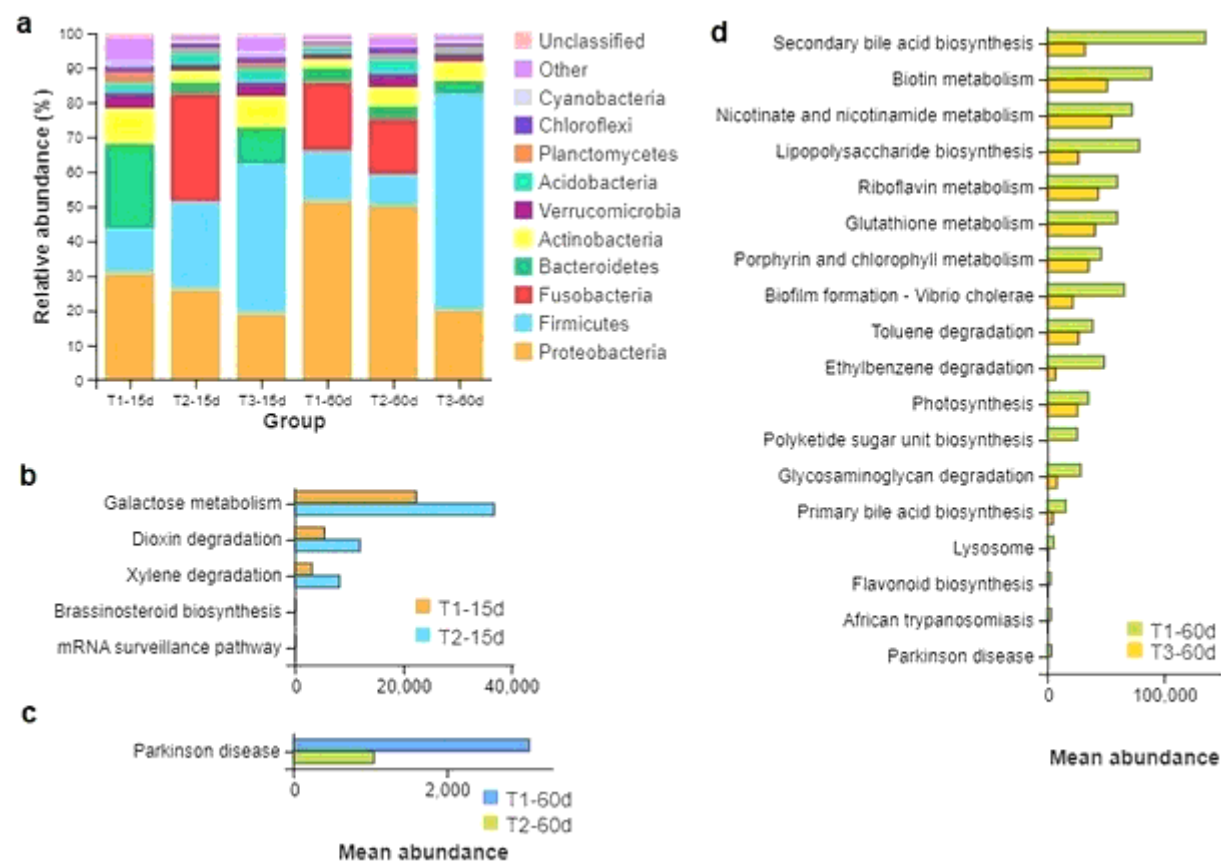


Figure 5

The gut microbial community analysis in T1, T2 and T3 fishes examined in 15-days (15d) and 60 days (60d) trials. (a) the abundance of gut microbial community at phylum level. Fusobacteria was dominant phylum in T2-15d, which replaced by Proteobacteria in T2-60d. Firmucutes had the highest abundance in both T3-15d and T3-60d. (b, c, d) the gut functional community in T2-15d, T2-60d and T3-60d, as compared to the T1 control group. Note: T1 group, fed with basal diet; T2 group, reared in *C. pyrenoidosa* monoculture water; T3 group, fed with basal diet containing 15% of dried *C. pyrenoidosa* powder (CRM).

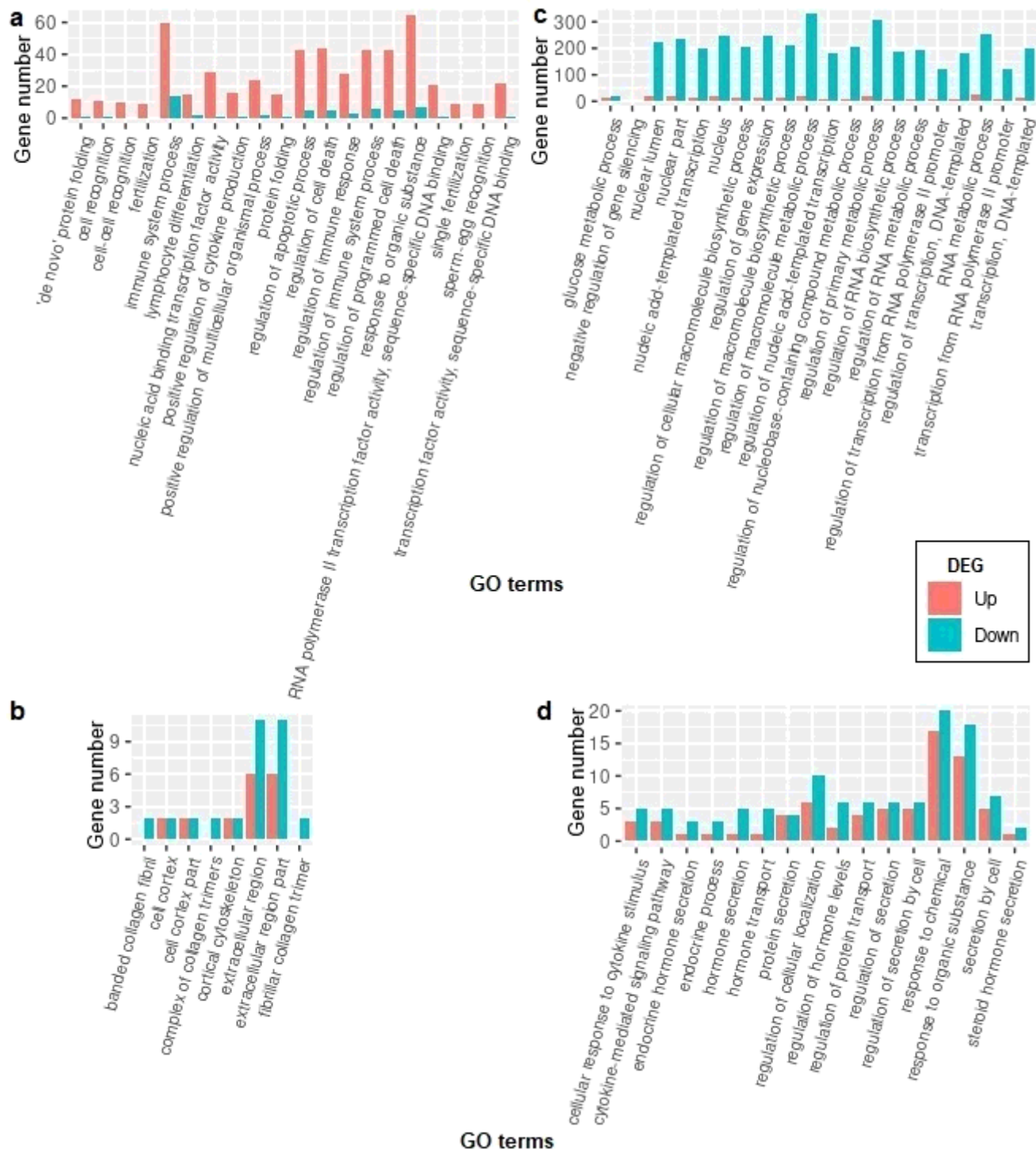


Figure 6

The enriched GO terms explored in the differentially expressed genes (DEG) obtained from comparative transcriptomic analysis in T1, T2 and T3 fishes examined in 15-days (15d) and 60 days (60d) trials. (a,c) Top 20 enriched GO terms (ranked from the lowest qvalue) in T2-15d and T3-15d. (b, d) Enriched GO terms in T2-60d and T3-60d. Note: T1 group, fed with basal diet; T2 group, reared in *C. pyrenoidosa* monoculture water; T3 group, fed with basal diet containing 15% of dried *C. pyrenoidosa* powder (CRM).

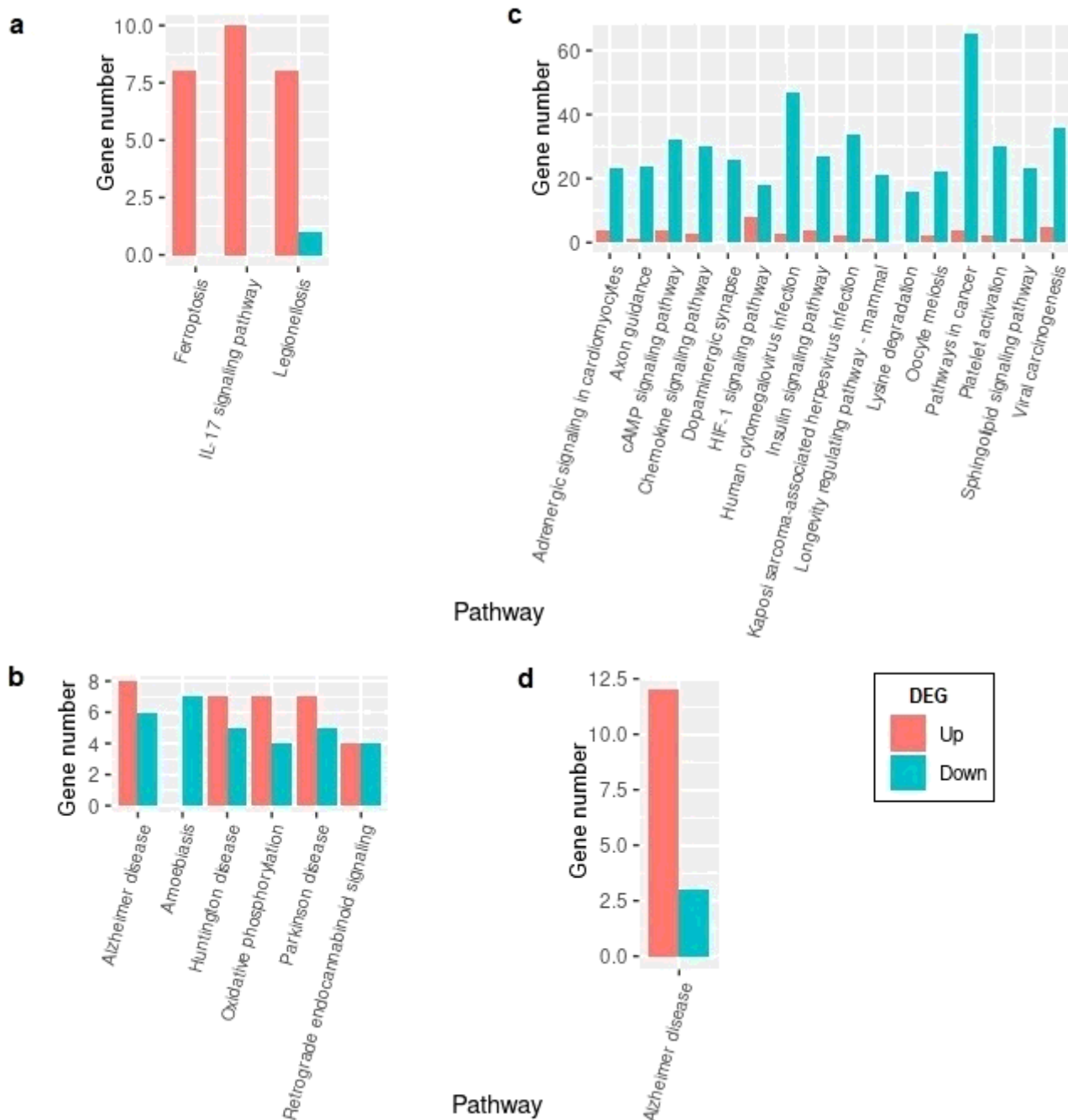


Figure 7

The enriched KEGG pathways explored in the differential expressed genes (DEG) obtained from comparative transcriptomic analysis in 15-days (15d) and 60 days (60d) trials of (a) T2-15d, (b) T2-60d, (c) T3-15d and (d) T3-60d. Note: T1 group, fed with basal diet; T2 group, reared in *C. pyrenoidosa* monoculture water; T3 group, fed with basal diet containing 15% of dried *C. pyrenoidosa* powder (CRM).

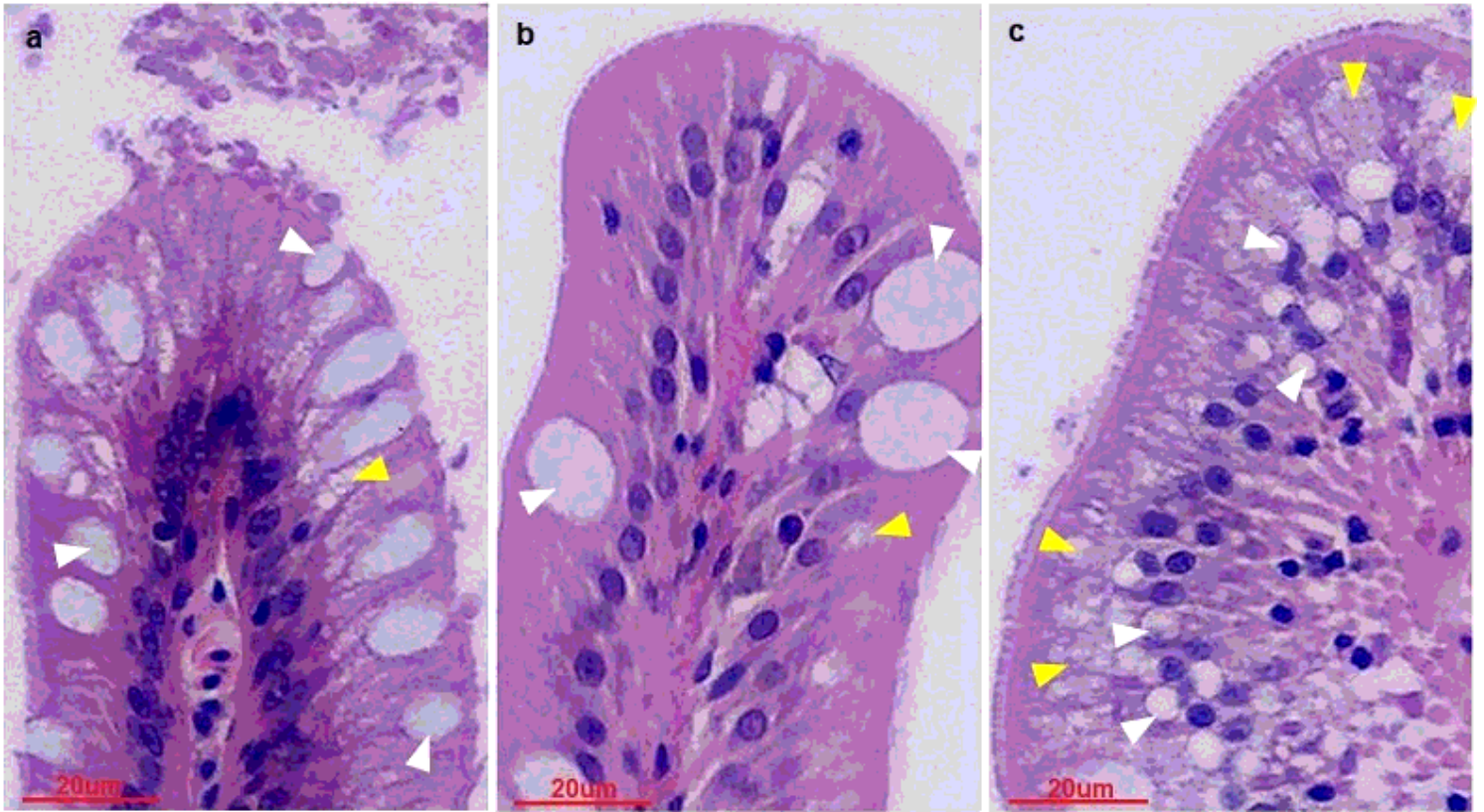


Figure 8

The histology analysis of villus tip in T1, T2 and T3 fishes examined in 60 days (60d) trials. (a) T1-60d showed normal distribution of goblet cells (white arrowhead). (b) Enlarged goblet cells in the T2-60d. (c) Pale and abundant bubbly lipid accumulation (yellow arrowhead) in T3-60d. HE staining. Note: T1 group, fed with basal diet; T2 group, reared in *C. pyrenoidosa* monoculture water; T3 group, fed with basal diet containing 15% of dried *C. pyrenoidosa* powder (CRM).