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Research Article

Chlorella vulgaris-supplemented Diet Enhances Growth, Immunity, and Survival in Red Hybrid Tilapia

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Abstract

Microalgae are increasingly explored as feed additives in aquaculture due to their potential benefits for fish health. This study evaluated the effects of a microalgae, *Chlorella vulgaris*-supplemented diet on the growth performance, immune-related gene expression, histopathology, and protective efficacy of red hybrid tilapia (*Oreochromis* sp.) following intraperitoneal injection with *Streptococcus agalactiae* and *Aeromonas hydrophila*. A total of 330 red hybrid tilapia, *Oreochromis* sp. were assigned to two groups: a control group fed a commercial diet (Group 1) and a treatment group receiving a diet supplemented with 2.5% C. vulgaris (Group 2) for seven weeks. Fish in the treatment group exhibited significantly higher growth rates than those in the control group. Moreover, the relative percentage survival (RPS) post-infection with *S. agalactiae* and *A. hydrophila* was 68.89 ± 1.52% and 88.89 ± 3.00%, respectively, significantly higher than in the control group (28.89 ± 0.57% and 40.00 ± 1.00%, respectively). Notably, fish fed the microalgae-supplemented diet displayed a higher expression of immune-related genes and exhibited reduced lesion severity across examined organs, indicating enhanced resistance to infection. These findings show that dietary *C. vulgaris* improves growth, boosts immune function, and enhances disease resistance in red hybrid tilapia, supporting its use as a sustainable functional feed ingredient. This highlights strong potential for commercial adoption, and future research should refine optimal inclusion levels and evaluate performance under real farm conditions.

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1. Introduction

Aquaculture is the most rapidly expanding segment of global food production, contributing 51% of the world's supply of food fish (FAO, 2024). Inland aquaculture contributes 62.6% of total farmed aquatic animal production, underscoring the need for sustainable and cost-effective protein sources to support its continued expansion (FAO, 2024). This growth is driven by increasing global demand for fish as a protein source, given its rich nutritional profile, including essential minerals and omega-3 fatty acids, which have been linked to reduced risks of cancer and cardiovascular disease and improved brain function (Gammone *et al*., 2019; Prem and Tewari, 2020).

Tilapia (*Oreochromis* spp.) plays a central role in meeting this demand and is one of the most widely cultivated freshwater fish, reaching an estimated 6.5 million tonnes of global production across more than 135 countries (Akter *et al*., 2025). Its widespread cultivation is attributed to its adaptability to diverse environments, resilience to disease, strong market demand, and ability to grow efficiently on both natural and formulated feeds (Huicab-Pech *et al*., 2016). In Malaysia, red hybrid tilapia accounts for more than 90% of the aquaculture industry, driven by strong consumer demand (Mohamad *et al*., 2021). However, the intensification required to sustain growing demand has also amplified disease pressures. Bacterial pathogens such as *Aeromonas hydrophila* and *Streptococcus agalactiae* continue to cause major outbreaks, resulting in substantial economic losses (Amal *et al*., 2018; Azzam-Sayuti *et al*., 2021a; Ali *et al*., 2023).

The extensive and frequent use of antibiotics to manage these pathogens has led to growing concerns regarding antibiotic resistance, environmental consequences, and food safety. As a result, there is an urgent need for sustainable and effective solutions to improve fish health and enhance disease resistance without dependence on antibiotics (Cai *et al.,* 2025). Within this context, microalgae, particularly *Chlorella vulgaris*, have gained attention as promising functional feed additives. Their rich nutritional profile, comprising essential amino acids, bioactive metabolites, and immunostimulatory compounds, supports improved immunity, enhanced disease resistance, and better flesh quality in fish (Ahmad *et al*., 2020; Nagappan *et al*., 2021). Additionally, previous studies have shown that microalgae supplementation in fish diets enhances weight gain and feed conversion efficiency compared to conventional diets (Nagappan *et al*., 2021). Several recent studies provide additional evidence of their functional benefits. For instance, Ferzola *et al.* (2025) demonstrated that *C. vulgaris*-supplemented diets significantly enhanced protein and energy digestibility, nutrient retention, and overall growth performance in Nile tilapia (*O. niloticus*). Similarly, Bayati *et al.* (2025) reported that replacing 10% of fishmeal with *C. vulgaris* improved growth, antioxidant capacity, and immune responses in Siberian sturgeon (*Acipenser baerii*).

However, limited research has investigated its role in boosting disease resistance and modulating immune responses in red hybrid tilapia. Therefore, this current study aims to evaluate the impact of a *Chlorella vulgaris*-supplemented diet on fish growth performance, feed utilization, histopathological changes, immune responses, and survival rates following bacterial infections. The findings will provide critical insights into sustainable, antibiotic-free strategies for enhancing fish health and productivity, thereby supporting the long-term sustainability of the aquaculture industry.

2. Materials and Methods

2.1 Materials

2.1.1 The equipment

The equipment used for feed preparation included a high-capacity mixer (Golden Bull B10-A Universal Mixer, Malaysia), a mini pelleting machine (Golden Avill, Guangdong, China), and a hot air oven (Memmert, Schwabach, Germany). Real-time PCR was performed using the Rotor-Gene Q Real-Time PCR system (Qiagen, Hilden, Germany), while histological analysis was conducted using a light microscope (Fein Optic RB30 Phase Contrast Microscope, USA).

2.1.2 The materials

Juvenile red hybrid tilapia (*Oreochromis* sp.), with an average initial weight of 6.67 ± 0.07 g, were obtained from a commercial supplier (Kam Sing Fish Farm, Selangor, Malaysia). The materials used for feed preparation included powdered commercial feed (30.85 ± 3.27% protein; Star Feed, Selangor, Malaysia) and *Chlorella vulgaris* culture, which was obtained from laboratory storage in freeze-dried form and maintained at 4 °C. For real-time PCR, the reagents used were TRIzol reagent (Ambion, USA), OneScript Hot cDNA Synthesis Kit (ABM, Richmond, Canada), and the SYBR Green-based QuantiNova RT-PCR Kit (Qiagen, Hilden, Germany). For histological analysis, the materials included 10% neutral-buffered formalin (Merck, Darmstadt, Germany) and bacterial cultures prepared using tryptic soy broth (TSB; Oxoid, Hampshire, UK) and tryptic soy agar (TSA; Oxoid, Hampshire, UK).

2.1.3 Ethical approval

This study requires ethical approval because it used experimental animals. All experimental procedures involving red hybrid tilapia (*Oreochromis* sp.) were conducted in compliance with the guidelines approved by the Animal Care and Use Committee of Universiti Putra Malaysia (UPM/IACUC/AUP-R024/2024). To minimise stress and discomfort, fish were anesthetised using MS-222 (Sigma-Aldrich, Missouri, USA) at a concentration of 100 mg/L before sample collection and bacterial challenge. Upon completion of the trial, all remaining fish were humanely euthanised by administering an overdose of MS-222 (400 mg/L) for a minimum duration of 10 minutes. Following euthanasia, fish carcasses were treated with 25% sodium hypochlorite for 30 minutes and subsequently disposed of as clinical waste, following institutional biosafety procedures.

2.2 Methods

2.2.1 Chlorella vulgaris culture preparation

Chlorella vulgaris culture was obtained from the Aquatic Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia, and was prepared following the method described by Pantami *et al.* (2020). Briefly, the microalgae were cultivated in Conway medium under controlled conditions at 27°C for three months. Following cultivation, the biomass was collected by centrifugation, freeze-dried, and kept at 4°C for later use.

2.2.2 Feed preparation

The freeze-dried *Chlorella vulgaris* was blended with powdered commercial feed (30.85 ± 3.27% protein; Star Feed, Selangor, Malaysia) at a 2.5% (w/w) inclusion rate. The ingredients were thoroughly mixed using a high-capacity mixer (Golden Bull B10-A Universal Mixer, Malaysia) and reformed into pellets (2 × 2 mm) using a mini pelleting machine (Golden Avill, Guangdong, China). The prepared feed was then dried at 40°C for 12 hours in a hot air oven (Memmert, Schwabach, Germany) and stored at 4°C until further use. A commercial diet without *Chlorella vulgaris* supplementation served as the control feed for the feeding trial.

2.2.3 Proximate analysis of commercial and microalgae-supplemented diet

Chlorella vulgaris powder, commercial, and microalgae-supplemented diets were analyzed for their nutrient-proximate composition, focusing on key nutritional parameters, including crude protein, crude lipid, ash, moisture, and carbohydrate content. Moisture, protein, ash, and lipid contents were analysed using in-house methods aligned with AOAC (20th ed.) standards, specifically methods 950.46 (moisture), 981.10 (protein), 923.03 (ash), and 991.36 (lipid). Carbohydrate content was determined using an in-house method based on Pomeranz and Meloan (2000).

2.2.4 Fish and experimental design

The experimental design and overall workflow outlining the laboratory analyses are illustrated in Figure 1 and 2, respectively. A total of 350 healthy red hybrid tilapia (*Oreochromis* sp.), averaging 6.67 ± *0.07* g in initial weight, were obtained from a commercial supplier (Kam Sing Fish Farm, Selangor, Malaysia) and transferred to the Hatchery Unit at the Institute of Bioscience, Universiti Putra Malaysia (UPM). After arrival, the fish underwent a three-day acclimation period in 700 L fibreglass tanks equipped with a recirculating aquaculture system containing dechlorinated water. Throughout the acclimation period, the fish were observed for any signs of stress or disease before the commencement of the feeding trial. Additionally, 20 fish were randomly selected for screening to detect the presence of parasites or bacterial infections.

After the acclimation period, the fish were randomly distributed into two dietary groups: one group was fed a standard commercial diet, while the other received a microalgae-enriched feed containing *Chlorella vulgaris* at a 2.5% inclusion rate. The feeding trial lasted for seven weeks. To facilitate the different experimental assessments, 120 fish from each group were allocated to eight glass tanks (15 fish per tank) for a bacterial challenge study, 30 fish were assigned to three glass tanks (10 fish per tank) for growth performance evaluation, and the remaining 15 fish (five fish per tank) were designated for organ sampling and real-time PCR (qPCR) analysis.

Water quality parameters were regularly monitored and maintained within optimal ranges throughout the study. The temperature was maintained between 28°C and 30°C, with dissolved oxygen levels of 5–6 mg/L, a pH range of 7.2–7.7, and ammonia-nitrogen concentrations below 0.01 mg/L, all of which supported optimal fish health and growth.

2.2.5 Growth performance

The fish from the control and treatment groups were fed with either commercial feed (Group 1) or microalgae-supplemented diet (2.5% inclusion of *C. vulgaris,* Group 2), respectively, for seven weeks at 5% of their total body weight, two times daily at 7.00 a.m. and 4.00 p.m. The growth performance parameters for the seven weeks of feeding trials were calculated us

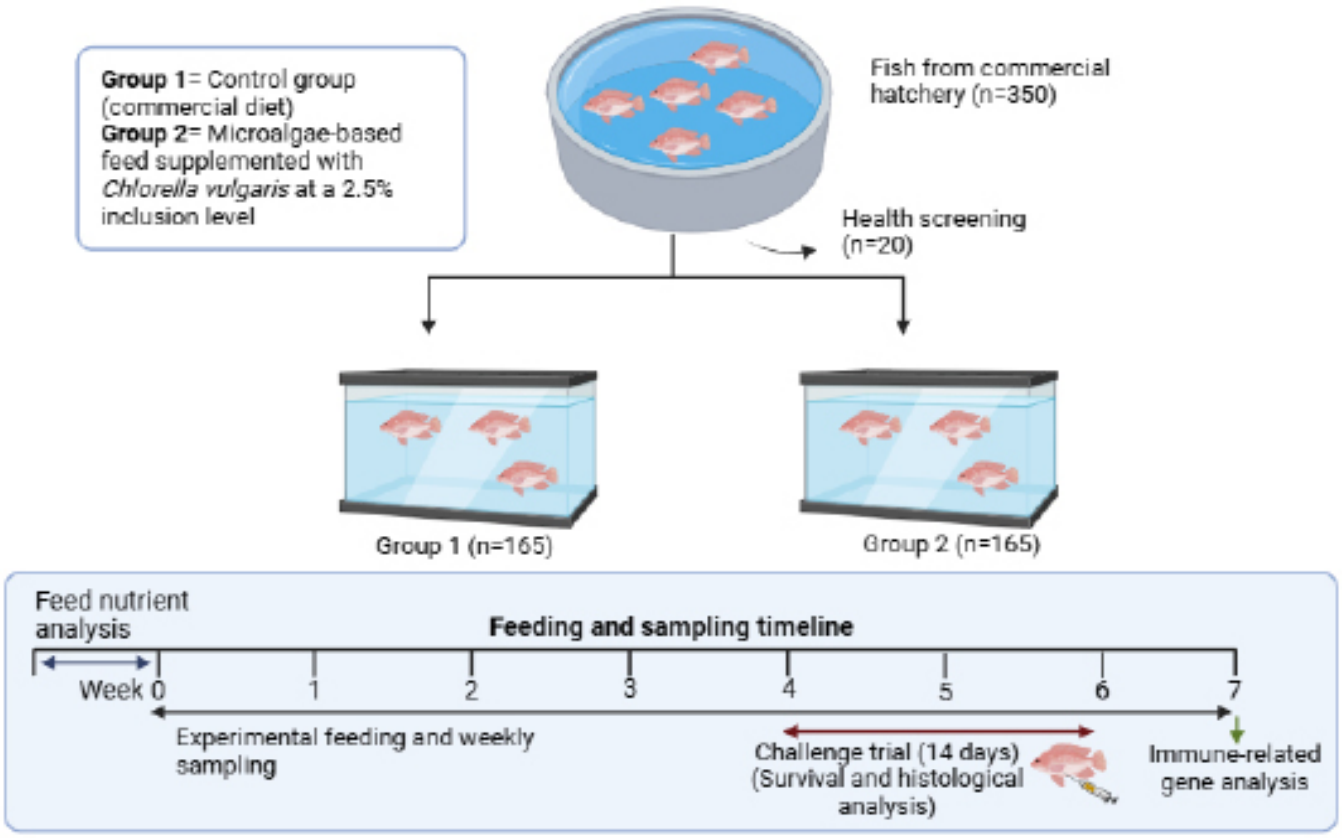

Figure 1. Schematic representation of the experimental design.

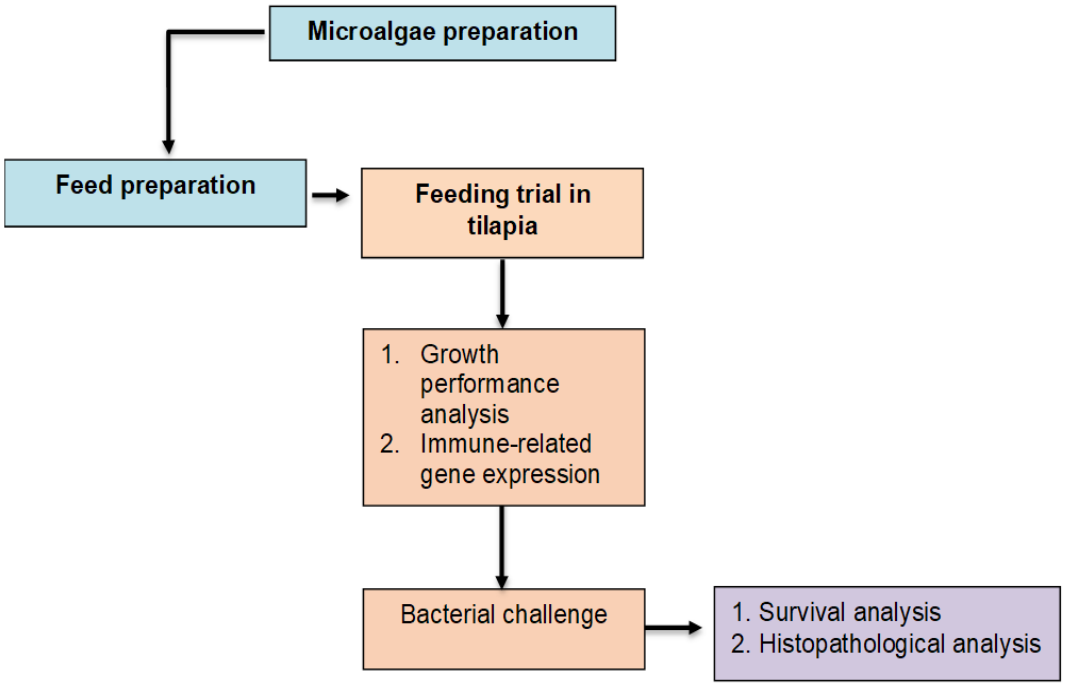

Figure 2. The overall workflow.

ing the following equations (Munni *et al*., 2023):

$$\text{Weight gained, WG (g)} = Wf - Wi \quad \text{..... (i)}$$

$$\text{Specific growth rate, SGR (\%/day)} = \frac{Ln\,(Wf) - Ln\,(Wi)}{Feeding\ duration\ (days)} \times 10 \quad \text{..... (ii)}$$

$$\text{Feed conversion ratio, FCR} = \frac{Total\ amount\ of\ feed\ consumed\ (g)}{Weight\ gained\ of\ fish\ (g)} \quad \text{..... (iii)}$$

Where :

W_i = the initial body weight of the fish (g) at the start of the feeding trial

W_f = the final body weight (g) at the end of the trial.

2.2.6 Bacterial challenge for protective efficacy analysis

To evaluate the protective effect of the *Chlorella vulgaris*-supplemented diet, a bacterial challenge was performed at week 4 of the feeding trial. The challenge involved intraperitoneal (i.p.) injection of *Streptococcus agalactiae* and *Aeromonas hydrophila* at concentrations of 1.8×10^3 CFU/mL and 2.0×10^4 CFU/mL, respectively, following the method described by Ali *et al*. (2023). The trial was conducted in triplicate, with three tanks designated for each bacterial pathogen. An additional tank was maintained as a negative control, in which fish received an i.p. injection of 0.1 mL sterile phosphate-buffered saline (PBS; Sigma-Aldrich, USA) instead of bacterial suspension. Following infection, fish were monitored daily for 14 days to record clinical signs, behavioural abnormalities, and mortality.

2.2.7 Histopathological examination

For histopathological analysis, tissue samples from the brain, gut, spleen, and liver were collected both before and after the bacterial challenge. These samples were immediately preserved in 10% neutral-buffered formalin for at least 48 hours to preserve tissue integrity. Thereafter, the samples were processed following standard histopathological procedures, which included embedding in paraffin, sectioning at a thickness of 4 µm, and proceeding with hematoxylin and eosin (H&E) staining. The stained tissue sections were analysed using a light microscope (RB30 Phase Contrast Microscope, USA) at magnifications between 40× and 400× to evaluate histopathological alterations and lesion formation associated with dietary treatment and bacterial challenge.

2.2.8 Quantitative real-time PCR (qPCR)

To evaluate immune responses, tissue samples were collected from the head kidney, spleen, and gut before and after the feeding trial. Total RNA was extracted using Trizol reagent and stored at -30°C for future analysis. Complementary DNA (cDNA) was then synthesised from the isolated RNA using the One-Script Hot cDNA Synthesis Kit, following the manufacturer's instructions, and preserved at -30°C for subsequent analyses. Real-time quantitative PCR was performed using the SYBR Green-based Quantinova RT-PCR kit on a Qiagen Rotor-Gene Q Real-Time PCR system (Qiagen, Hilden, Germany). The primers used in this study are listed in Table 1. The relative mRNA expression was determined using the Livak $2^{-\Delta\Delta CT}$ method, as described by Monir *et al*. (2021).

Table 1. List of primers targeting immune-related genes employed in quantitative real-time PCR (qPCR) assays

Immune-related genes	Primer sequences (5' – 3')	Amplicon size (bp)	Role	Reference
il-1β	F: CAAGGATGACGACAAGCCAACC R: AGCGGACAGACATGAGAGTGC	149	Pro-inflammatory gene	(Qiang *et al*., 2016)
C-type lysozyme	F: AAGGGAAGCAGCAGCAGTTGTG R: CGTCCATGCCGTTAGCCTTGAG	151	Innate defense against pathogen	(Qiang *et al*., 2016)
TNF-α	F: GGAAGCAGCTCCACTCTGATGA R: CACAGCGTGTCTCCTTCGTTCA	137	Resistance against infection and cancer	(Qiang *et al*., 2016)
MHC I	F: TTCTCACCAACAATGACGGG R: AGGGATGATCAGGGAGAAGG	188	Molecule presenting antigen for $CD8^+$	(Yao *et al*., 2019)
MHC II	F: AGTGTGGGGAAGTTTGTTGGAT R: ATGGTGACTGGAGAGAGGCG	207	Molecule presenting antigen for $CD4^+$	(Yao *et al*., 2019)
B-actin (housekeeping)	F: CCACACAGTGCCCATCTACGA R: CCACGCTCTGTCAGGATCTTCA	111	Housekeeping gene	(Qiang *et al*., 2016)

2.3 Analysis Data

Data were compiled using Microsoft Excel (Microsoft, Redmond, WA, USA) and analysed with IBM SPSS Statistics version 26 (SPSS Inc., Chicago, IL, USA). An independent t-test was employed to compare growth performance parameters and the relative expression levels of immune-related genes between the control group and the *Chlorella vulgaris*-supplemented diet group. Differences were considered statistically significant at $P < 0.05$.

3. Results and Discussion

3.1 Results

3.1.1 Feed quality analysis

The proximate composition of the commercial feed (Star Feed, Klang, Malaysia) and the microalgae-supplemented diet differed significantly ($P < 0.05$), except for moisture content (Table 2). The microalgae-supplemented diet contained significantly higher levels of crude protein, crude lipid, ash, and carbohydrates than the commercial feed ($P < 0.05$). The freeze-dried *Chlorella vulgaris* used in this study contained a high level of crude protein (54.7%), contributing to the elevated protein content observed in the supplemented diet.

3.1.2 Growth performance

The growth and feed performance results are summarised in Figure 3 and Table 3. After seven weeks, fish fed the microalgae-supplemented diet exhibited significantly higher growth performance ($P < 0.05$). Compared to those fed the commercial feed, the microalgae-supplemented diet resulted in a 17% increase in final weight, a 41% higher weight gain, and a 23% higher specific growth rate. Feed performance also improved, with a 29% lower feed conversion ratio.

3.1.3 Efficacy of microalgae-supplemented diet

The protective efficacy of microalgae-supplemented diet was evaluated by bacterial laboratory challenge via intraperitoneal (i.p) injection (Table 4). The negative control in the non-challenged group achieved 100% survivability. Mortalities were detected at 24 hours post-infection and lasted up to two weeks. The percentage of survival (%) of fish fed with microalgae-supplemented diet challenged with *S. agalactiae* was 68.89 ± 1.52%, significantly higher ($P < 0.05$) than the RPS of fish fed with commercialised feed (28.89 ± 0.57%). Following *A. hydrophila* infection, the RPS of fish fed with microalgae-supplemented diet was also significantly higher (88.89 ± 3.00%,

Table 2. Proximate composition of commercial feed and feed supplemented with microalgae. Significant differences compared to the control diet are indicated by asterisks ($P < 0.05$)

Composition (%)	Microalgae, *C. vulgaris* powder	Feed	
		Control	Microalgae-supplemented diet
Crude protein	54.7	30.85 ± 3.27	47.94 ± *2.19**
Crude lipid	0.3	7.06 ± 0.95	7.23 ± 0.17*
Ash	5.1	12.02 ± 1.49	17.18 ± 2.79*
Moisture	7.2	9.09 ± 1.77	10.00 ± 1.74
Carbohydrate	32.7	15.67 ± *0.5*	24.64 ± *2.15**

Table 3. Growth performance parameters of red hybrid tilapia (n = 30 per treatment) fed either a commercial diet or a microalgae-supplemented diet. Significant differences from the control group are indicated by asterisks ($P < 0.05$)

Growth parameters	Feed	
	Control	Microalgae–supplemented diet
Initial weight (g)	6.67 ± *0.07*	6.67 ± 0.07
Final weight (g)	13.67 ± *0.33*	16.00 ± *1.00**
Weight gain (g)	6.63 ± *0.33*	9.33 ± *1.00**
SGR (% day^{-1})	1.45 ± *0.42*	1.78 ± *0.21**
FCR (g/g)	2.96 ± *0.15*	2.10 ± *0.05**

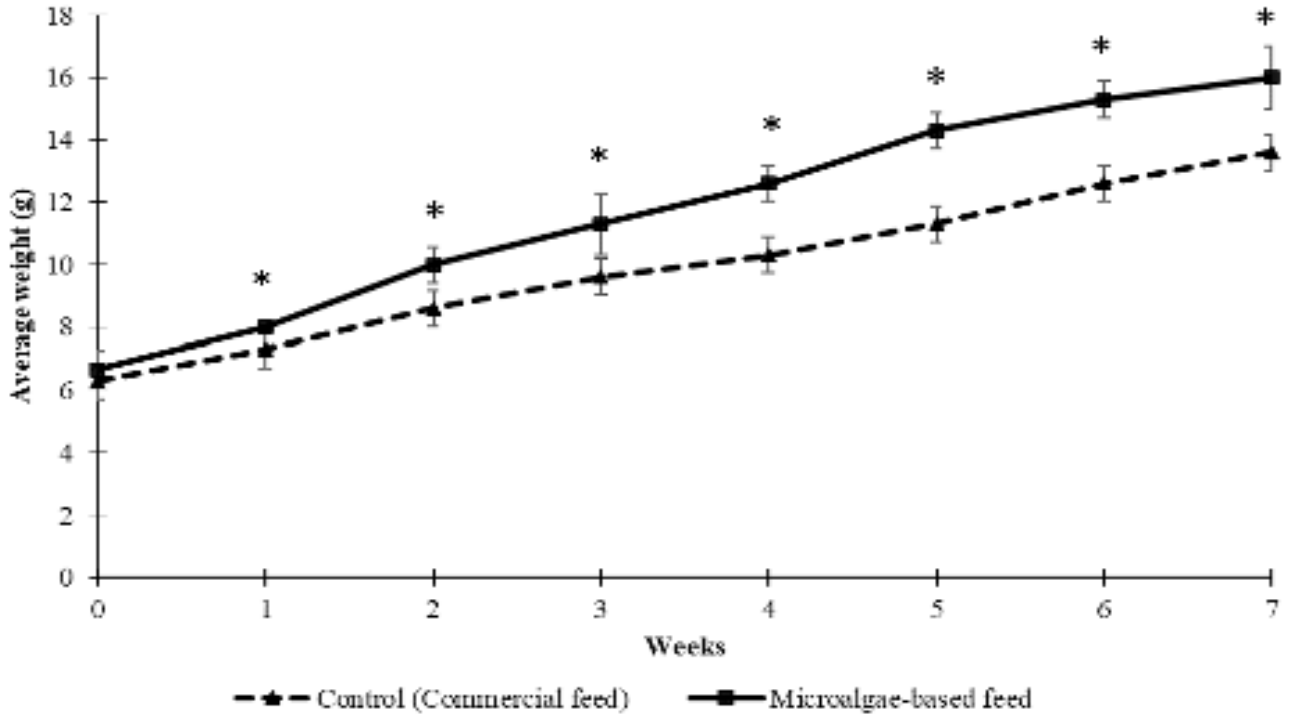

Figure 3. Growth performance of red hybrid tilapia based on total body weight (mean ± SE, n = 30) after seven weeks of feeding trial. Significant differences compared to the control group are denoted by asterisks (P < 0.05).

Table 4. Cross-protective efficacy of different diet groups against *Streptococcus agalactiae* and *Aeromonas hydrophila* in red hybrid tilapia. Significant differences compared to the control group are marked with asterisks (*P* < 0.05)

Pathogen	Challenge dose (CFU/mL)	Fish group	Dead/ total	Mortality (%)	Survival (%)
Streptococcus agalactiae	1.8 x 10^3	Control	32/ 45	71	28.89 ± *0.57*
		Microalgae–supplemented diet	14/ 45	31	68.89 ± *1.52**
Aeromonas hydrophila	2.0 x 10^4	Control	27/ 45	60	40.00 ± *1.00*
		Microalgae–supplemented diet	5/ 45	10	88.89 ± *3.00**
PBS	1x	Control	0/ 30	0	100 ± *0.00*
		Microalgae–supplemented diet	0/ 30	0	100 ± *0.00*

P < 0.05) than those fed with commercialised feed (40.00 ± 1.00%).

3.1.4 Histopathological examination

Histopathological analysis revealed varying degrees of lesions in all challenged fish than to the non-infected control (Figures 4A, 4D, 4G, 4J, 5A, 5D, 5G, and 5J). Generally, both *S. agalactiae* and *A. hydrophila* caused similar lesions, but the severity was generally lower in tilapia fed with microalgae-supplemented diet.

In brain cross-sections, fish in the control group (commercial feed) showed thickened meninges due to neutrophil infiltration, spongiosis, and neuronal degeneration in *A. hydrophila*-infected fish (Figure 5B). In contrast, no neutrophil infiltration was observed in the microalgae-supplemented diet group (Figures 4C and 5C). Gut cross-sections showed no noticeable differences between control and microalgae-supplemented diet groups after infection with either pathogen (Figure 4E, 4F, 5E, and 5F). However, liver cross-sections in the control group showed mild congestion following *S. agalactiae* infection (Figure 4H) and after *A. hydrophila* infection (Figure 5H). In the microalgae-supplemented diet group, the liver appeared normal after *S. agalactiae* infection (Figure 4I) but exhibited mild congestion after *A. hydrophila* infection (Figure 4I). Spleen cross-sections in the control group showed haemorrhages and melanomacrophage accumulation (Figures 4K and 5K). In the microalgae-supplemented diet group,

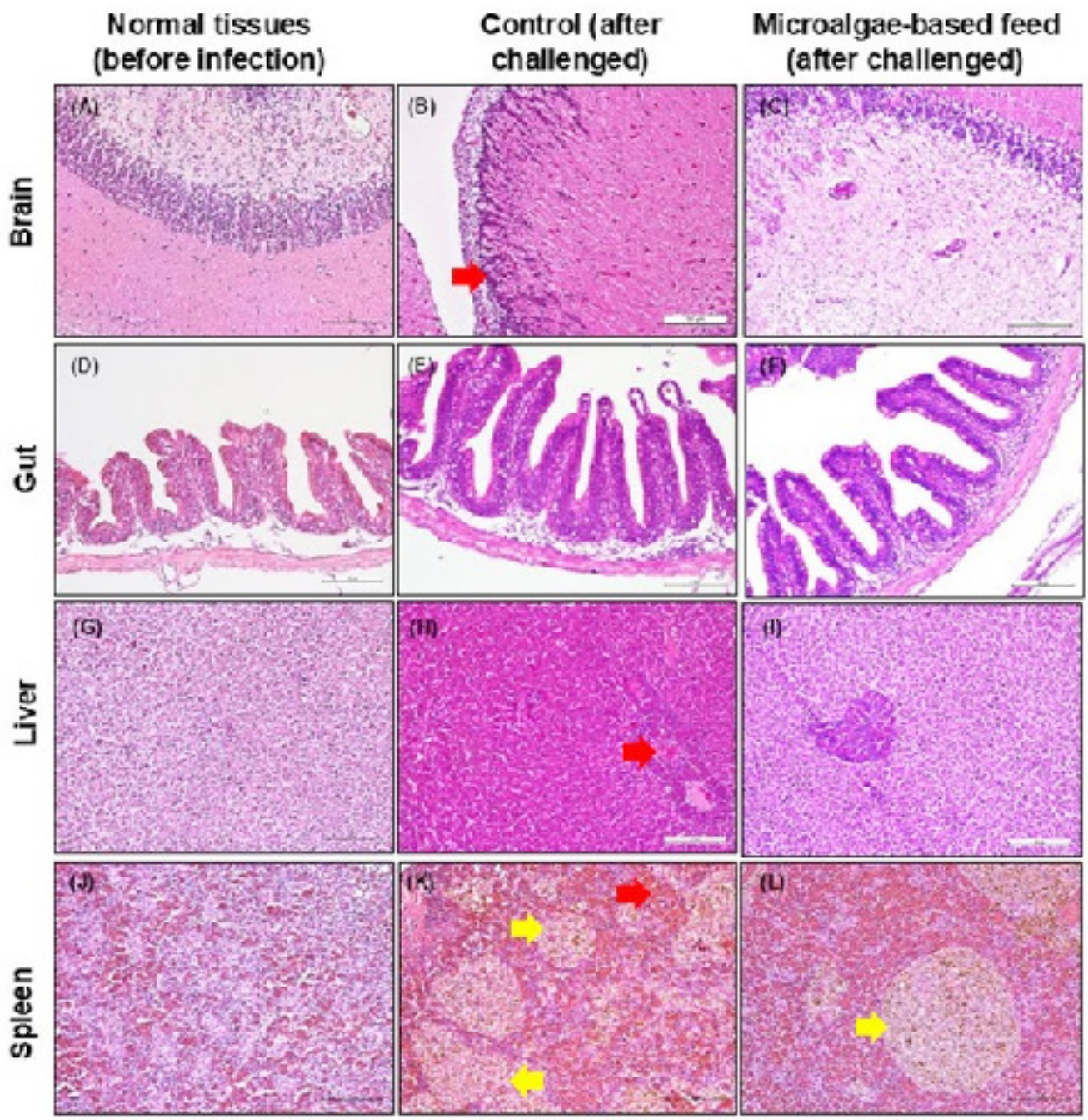

Figure 4. Histopathological examination of various organs from red hybrid tilapia before and after *Streptococcus agalactiae* challenge. (A) Normal brain section, stained with H&E, magnification 20×, scale bar = 50 µm. (B) Brain section from fish fed the control diet, displaying meningeal thickening (red arrow), H&E, 20×, scale bar = 50 µm. (C) Brain section from fish fed the microalgae–supplemented diet post-challenge, H&E, 20×, scale bar = 50 µm. (D) Normal intestinal section, H&E, 20×, scale bar = 50 µm. (E) Intestinal section of control fish post-challenge, H&E, 20×, scale bar = 50 µm. (F) Intestinal section of fish fed the microalgae-supplemented diet after challenge, H&E, 20×, scale bar = 50 µm. (G) Normal liver section, H&E, 20×, scale bar = 50 µm. (H) Liver section from fish fed the control diet showing mild vascular congestion (red arrow), H&E, 20×, scale bar = 50 µm. (I) Liver section from fish fed the microalgae-supplemented diet post-challenge, H&E, 20×, scale bar = 50 µm. (J) Normal spleen section, H&E, 20×, scale bar = 50 µm. (K) Spleen section of fish fed the commercial diet, indicating haemorrhage (red arrow) and melanomacrophage accumulation (yellow arrow), H&E, 20×, scale bar = 50 µm. (L) Spleen section from fish fed the microalgae-supplemented diet post-challenge, showing melanomacrophage accumulation (yellow arrow), H&E, 20×, scale bar = 50 µm.

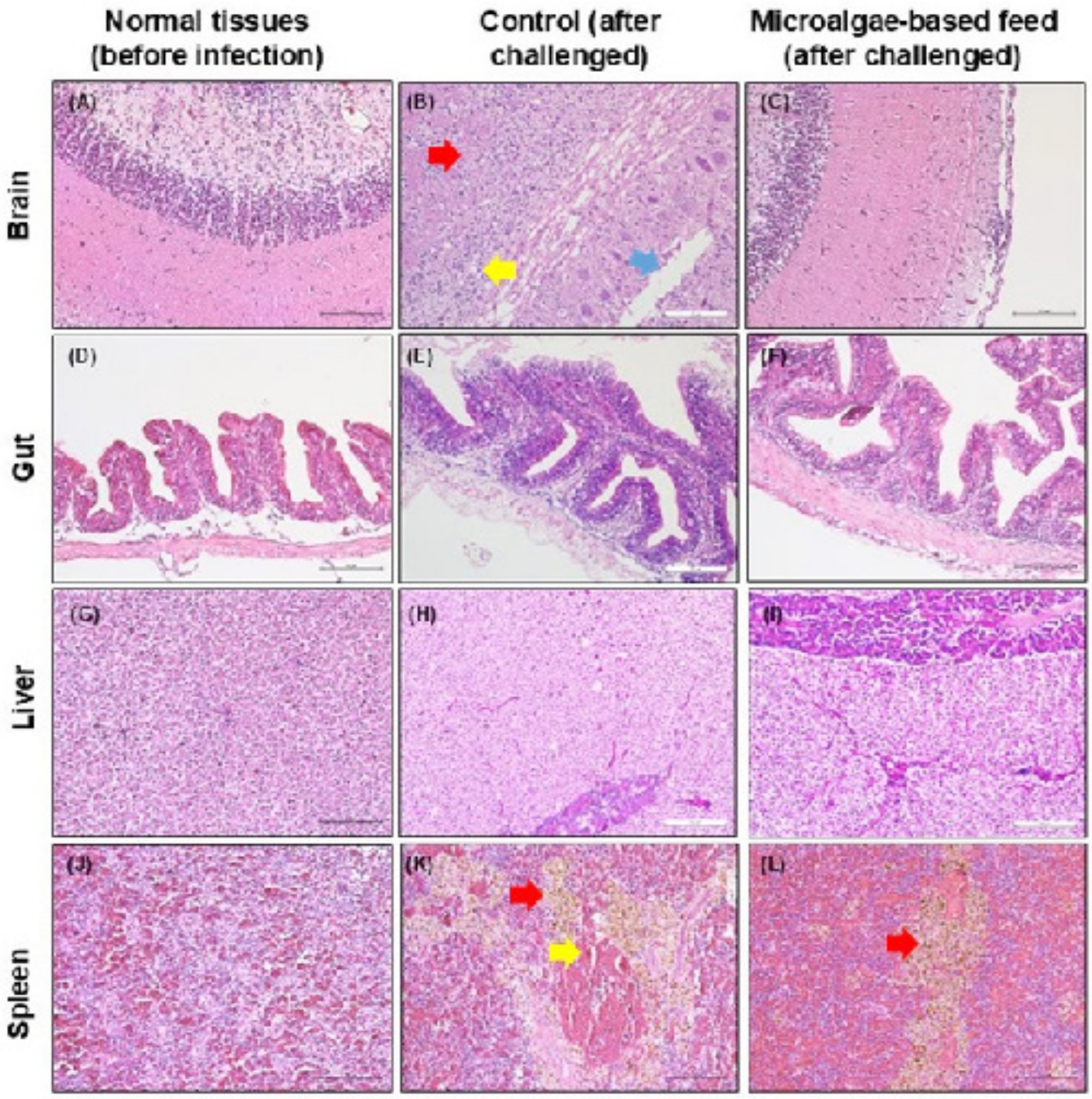

Figure 5. Histopathological assessment of red hybrid tilapia organs before and after *Aeromonas hydrophila* challenge. (A) Normal brain tissue, stained with H&E, magnification 20×, scale bar = 50 µm. (B) Brain section from fish receiving the commercial diet, displaying meningeal thickening (red arrow), spongiosis (yellow arrow), and neuronal degeneration (blue arrow), H&E, 20×, scale bar = 50 µm. (C) Brain tissue from fish fed with a microalgae-supplemented diet post-challenge, H&E, 20×, scale bar = 50 µm. (D) Normal intestinal cross-section, H&E, 20×, scale bar = 50 µm. (E) Intestinal tissue from fish fed with commercial feed following challenge, H&E, 20×, scale bar = 50 µm. (F) Intestinal section from fish fed with microalgae-supplemented diet post-challenge, H&E, 20×, scale bar = 50 µm. (G) Healthy liver section, H&E, 20×, scale bar = 50 µm. (H) Liver tissue from fish receiving commercial feed post-challenge, H&E, 20×, scale bar = 50 µm. (I) Liver section from fish fed the microalgae-supplemented diet after challenge, H&E, 20×, scale bar = 50 µm. (J) Normal spleen section, H&E, 20×, scale bar = 50 µm. (K) Spleen tissue of fish fed the commercial diet, exhibiting haemorrhages (yellow arrow) and accumulation of melanomacrophages (red arrow), H&E, 20×, scale bar = 50 µm. (L) Spleen section from fish fed with the microalgae-supplemented diet post-challenge, showing melanomacrophage accumulation (red arrow), H&E, 20×, scale bar = 50 µm.

melanomacrophage accumulation was present, but no haemorrhages were observed (Figure 4L and 5L).

3.1.5 Immune-related genes expression

The transcriptional profiles of selected immune-related genes, including MHC I, MHC II, C-type lysozyme, TNF, and IL-1β, in the gut (A), spleen (B), and kidney (C) of red hybrid tilapia fed with either a commercial diet (control) or a microalgae-supplemented diet (treated) at Weeks 0 and 7 are illustrated in Figure 6. No significant differences ($P > 0.05$) in gene expression were detected between the control and treated groups at the start of the trial across all examined tissues. However, after 7 weeks of feeding, fish receiving the microalgae-supplemented diet showed a significant upregulation ($P < 0.05$) of all evaluated genes in the gut compared to the control group (Figure 6A).

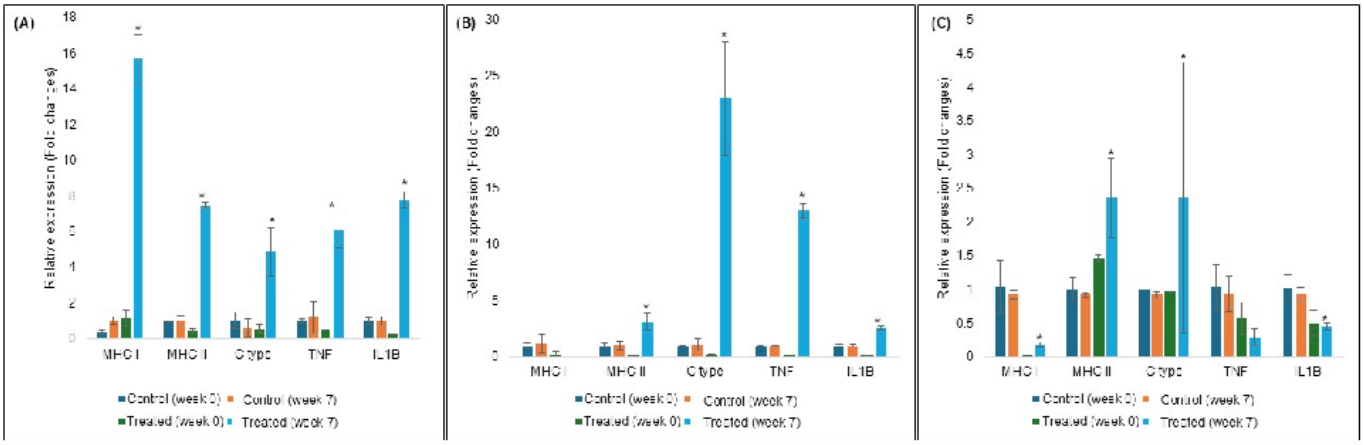

Figure 6. Relative expression of immune-related genes (MHC I, MHC II, C-type lysozyme, TNF, and IL-1β) in the gut (A), spleen (B), and kidney (C) of red hybrid tilapia following a 7-week feeding trial with either commercial feed (control) or microalgae-supplemented diet. The mRNA expression levels were normalised against the housekeeping gene β-actin, and the fold change was determined relative to the control group. Each sample was analysed in triplicate, and the results are expressed as mean ± standard error (SE). Asterisks (*) denote statistically significant differences ($P < 0.05$) compared to the control.

In the spleen, the expression of MHC II, C-type lysozyme, TNF, and IL-1β was significantly increased ($P < 0.05$) in the treated group. In contrast, MHC I expression remained similar to that of the control (Fig. 5B). A comparable trend was noted in the kidney, where the expression of MHC II and C-type lysozyme was significantly elevated ($P < 0.05$) in the microalgae-fed fish relative to the control (Figure 6C).

3.2 Discussion

3.2.1 Microalgae as immunostimulants in aquaculture

Microalgae have gained considerable attention as a sustainable alternative for fish feed due to their numerous nutritional and functional benefits. While many studies have explored immunostimulants as substitutes for antibiotics in aquaculture, research on the use of microalgae specifically as immunostimulants remains limited, particularly in tilapia, one of the most widely farmed fish species worldwide (Montoya-Camacho *et al*., 2019). In this study, *Chlorella vulgaris* was selected as an immunostimulant to enhance the immune response of red hybrid tilapia, as it is rich in essential amino acids and vitamins required for immune system function (Niccolai *et al*., 2019; Wang *et al*., 2024).

3.2.2 Nutritional enhancement of feed formulated with Chlorella vulgaris

The quality of feed plays a critical role in aquaculture production. A nutritionally balanced diet must provide adequate carbohydrates, proteins, and lipids to support optimal growth in aquatic organisms (Manam, 2023), as seen in commercially available feeds. While commercial feeds contain essential nutrients, their efficacy can be further improved by incorporating microalgae as a functional feed additive. In this study, *C. vulgaris* was incorporated at 2.5%, a level identified as optimal for commercial feed applications (Pantami *et al*., 2020). Proximate analysis revealed a notable increase in protein, lipid, and carbohydrate content after supplementation, with levels rising to 47.94 ± 2.19%, 17.23 ± 0.17%, and 24.64 ± 2.15%, respectively, compared to their pre-mixing values of 30.85 ± 3.27%, 7.06 ± 0.95%, and 15.67 ± 0.5%, respectively. These compositional changes are attributed to the high nutritional value of *C. vulgaris*, which is rich in protein, lipids, carbohydrates, vitamins, and minerals (Niccolai *et al*., 2019; Wang *et al*., 2024).

3.2.3 Growth performance and feed conversion efficiency

Numerous investigations have evaluated the impact of *Chlorella vulgaris* supplementation on the growth performance of various fish species, including Nile tilapia (Ferzola *et al.,* 2025) and Siberian sturgeon (Bayati *et al.,* 2025). The current study explicitly assessed the growth performance of red hybrid tilapia fed with a diet enriched with microalgae. Consistent with previous findings, the final weight of red hybrid tilapia fed with microalgae-supplemented diet was significantly higher (16.00 ± 1.00 g) than the control group (13.67 ± 0.33 g). Additionally, the feed conversion ratio (FCR) was lower in fish fed with microalgae-supplemented diet (2.100 ± 0.05 g/g) compared to the control group (2.956 ± 0.15 g/g), indicating improved feed efficiency. These results suggest that incorporating *C. vulgaris* into the diet enhances feed utilisation and growth performance.

Several mechanisms may explain the observed improvements. *C. vulgaris* contains a diverse array of growth-promoting components, including high-quality proteins, essential fatty acids, polysaccharides, vitamins, minerals, chlorophylls, carotenoids, and other bioactive metabolites (Abdel-Tawwab *et al.,* 2022; Mahmoud *et al.,* 2020). Bioactive compounds such as the S-nucleotide adenosyl peptide complex have been associated with growth enhancement in fish (Xu *et al.,* 2014). Additionally, *C. vulgaris* has been demonstrated to support the proliferation of probiotics in the fish gut (Huang *et al.,* 2023), thereby enhancing nutrient assimilation and digestive efficiency. The presence of β-glucans may also contribute indirectly to improved growth by strengthening physiological resilience (Abdel-Tawwab *et al*., 2022). Collectively, these nutritional and functional attributes support the role of *C. vulgaris* as an effective and practical functional feed additive capable of enhancing growth and overall performance in aquaculture species, including red hybrid tilapia.

3.2.4 Disease resistance and survival following bacterial challenge

Streptococcus and *Aeromonas* spp. are pathogenic bacteria that commonly cause diseases in freshwater fish, including red hybrid tilapia, posing a significant threat to the aquaculture industry in Malaysia (Azzam-Sayuti *et al*., 2021b; Basri *et al*., 2020). In this study, a challenge trial was conducted at week 4 of the feeding experiment to evaluate the protective effects of a diet supplemented with microalgae. This study found that the survival rate of fish fed a diet supplemented with microalgae was markedly higher than that of the control group. The beneficial effects of microalgae can be attributed to their rich composition of proteins, lipids, and carbohydrates, which are essential for enhancing fish health. Additionally, microalgae provide a well-balanced amino acid profile, further supporting immune function and overall resilience in fish (Nagappan *et al*., 2021; Niccolai *et al*., 2019).

Previous studies have demonstrated that *Chlorella vulgaris* can improve fish's innate and adaptive immune responses (Khani *et al*., 2017). This green microalga also contains various bioactive compounds, including polyunsaturated aldehydes, glycosides, terpenes, and chlorophyll-a derivatives, which exhibit antibacterial properties by inhibiting bacterial growth (Fihri *et al*., 2024). In addition, *C. vulgaris* contains phenolic compounds such as p-coumaric acid and ferulic acid, which are known for their antioxidant and antimicrobial properties and may further enhance the microalga's protective effects by reducing oxidative stress and suppressing pathogenic bacteria (Centikaya, 2025).

Together, these bioactive components may have contributed to the enhanced survival rates observed in the treated fish, underscoring the immunomodulatory potential of *C. vulgaris* as a functional feed additive in aquaculture.

3.2.5 Expression of immune-related genes

In this study, we analysed different targeted immune-related genes that play significant roles in the innate and adaptive response: MHC-I, MHC-II, C-type lysozyme, TNF-α, and IL-1β. Among these, MHC-II and C-type lysozyme were expressed in all targeted organs (gut, spleen and kidney), whereas in the spleen, only MHC-I was not detected. The Major Histocompatibility Complex I (MHC-I) plays a crucial role in inducing CD8+ T cells, contributing to innate immunity and controlling various bacterial infections (Berg and Forman, 2006; Witt, 2023). Meanwhile, Major Histocompatibility Complex II (MHC-II) is critical in innate and adaptive immunity by stimulating CD4+ T cells (Mangalam *et al*., 2006). CD4+ T cells play a crucial role in modulating the immune response by stimulating B lymphocytes, cytotoxic T cells, and cells of the innate immune system, as well as contributing to immune regulation and suppression (Luckheeram *et al*., 2012).

Interestingly, C-type lysozyme was highly expressed in all organs, likely due to its fundamental role in the innate immune response and antibacterial defence mechanisms (Ferraboschi *et al*., 2021). Studies have demonstrated that C-type lysozyme exhibits lytic activity against *Streptococcus agalactiae* (Biller *et al*., 2021). Notably, this gene is typically expressed

at low levels in unstimulated fish but is significantly upregulated during bacterial infections (Biller *et al*., 2021). The findings of this study suggest that dietary incorporation of *Chlorella vulgaris* may stimulate C-type lysozyme expression in tilapia, enhancing their immune preparedness even in the absence of infection.

Tumour necrosis factor-alpha (TNF-α) is a key cytokine involved in innate immune responses and is typically released during acute infections (Zawawi *et al*., 2023). It is mainly produced by macrophages and lymphocytes in reaction to cellular injury triggered by infections or malignant changes, although other cell types and tissues can also secrete TNF-α. As a pro-inflammatory mediator, TNF-α is essential in orchestrating immune responses and possesses both cytotoxic and cytostatic properties (Popko *et al*., 2010). As a pro-inflammatory cytokine, TNF-α plays a crucial role in immune responses and exhibits cytotoxic and cytostatic effects (Popko *et al*., 2010). TNF-α is typically upregulated in spleen lymphocytes as part of the adaptive immune response following bacterial infection (Li *et al*., 2021). However, this study observed TNF-α expression in the spleen and gut, suggesting that microalgae supplementation may induce TNF-α production without a pathogenic challenge. This expression may be attributed to immune stimulation by bioactive compounds in *C. vulgaris*, which could trigger TNF-α expression post-feeding with the microalgae-supplemented diet (Li *et al*., 2021).

Interleukin-1 beta (IL-1β), another pro-inflammatory cytokine, plays a key role in modulating the immune response by resetting the thermoregulatory centre, stimulating B cells, and co-stimulating T cell production (Delgado *et al*., 2003). IL-1β is also involved in the upregulation of other pro-inflammatory cytokines, including TNF-α, and these cytokines often function synergistically in immune and inflammatory responses (Li *et al*., 2023). The results of this study indicate that while TNF-α expression was predominantly upregulated in the spleen, IL-1β was expressed by monocytes, macrophages, and non-immune cells such as fibroblasts and endothelial cells (Zhang and An, 2009). This expression suggests that the immunomodulatory properties of *C. vulgaris* may influence multiple immune pathways, potentially enhancing disease resistance in tilapia.

3.2.6 Histopathological observations

Histopathological examination of the brain, liver, kidney, and spleen from both control and treated fish revealed significant pathological differences. *Streptococcus agalactiae* infection in the control group was associated with meningoencephalitis and haemorrhages in the brain, which are characteristic signs of streptococcosis (Zamri-Saad *et al*., 2010). Furthermore, severe histopathological alterations, including haemorrhages, congested blood vessels, and the accumulation of neutrophils and melanomacrophages, were observed in the internal organs of infected control fish. These pathological changes were consistent with previous findings in fish infected with *S. agalactiae* and *Aeromonas hydrophila* (AlYahya *et al*., 2018; Laith and Najiah, 2014; Laith *et al*., 2017).

In contrast, fish from the treated group exhibited milder pathological changes. Haemorrhages and vascular congestion were rarely observed, while a higher accumulation of melanomacrophages was noted, suggesting an active immune response in alignment with findings by Laith *et al*. (2017). Overall, the histopathological results were consistent with the survival rates observed in the challenge experiment, further supporting the hypothesis that feeding tilapia with microalgae-supplemented diet enhances their health and resistance to bacterial infections caused by *S. agalactiae* and *A. hydrophila*.

3.2.7 Overall implications and future perspectives

The use of *Chlorella vulgaris* as a dietary supplement in tilapia feed has demonstrated significant benefits. This study confirms that feeding tilapia with a microalgae-supplemented diet enhances their survival against common pathogenic bacteria, as evidenced by the upregulation of immune-related genes. In addition to boosting immune responses, *C. vulgaris* also promotes growth performance in tilapia. However, it remains uncertain whether *C. vulgaris* can similarly enhance the immune system of other fish species or whether different microalgae strains could provide comparable benefits for tilapia. Future studies should expand this work by evaluating multiple species and microalgal strains, optimizing inclusion levels, and incorporating analytical techniques such as FTIR to characterize the interaction of *C. vulgaris* compounds within the feed matrix and better elucidate the mechanisms underlying its beneficial effects.

4. Conclusion

This study demonstrates that incorporating *Chlorella vulgaris* into the diet of red hybrid tilapia enhances the expression of immune-related genes and significantly improves survival rates following infection with *Streptococcus agalactiae* and *Aeromonas hydrophila*. Dietary inclusion of microalgae also promoted growth performance, including increased growth rate, feed intake, and feed efficiency. These results suggest that *C. vulgaris* has significant potential as a sustainable and effective feed additive to support both the health and growth of farmed tilapia. Moving

forward, these findings can inform the development of commercial feed formulations incorporating *C. vulgaris*. Future studies should investigate optimal inclusion levels, long-term effects under commercial farming conditions, and the applicability of this approach across other aquaculture species.

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Authors' Contributions

The contribution of each author as follow, Mohamad, Syafiq-Aizat and Azzam-Sayuti; collected the data, drafted the manuscript, and designed the figures. Shaari, Salleh, Nazarudin and Ina Salwany; devised the main conceptual ideas and critical revision of the article. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

None of the authors have a conflict of interest to disclose.

Declaration of Artificial Intelligence (AI)

The author(s) acknowledge the use of ChatGPT and Grammarly to enhance grammatical correctness, spelling, and clarity in preparing this manuscript. All AI-generated content was rigorously reviewed, edited, and validated to ensure accuracy and originality. Full responsibility for the manuscript's final content rests with the authors.

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