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RESEARCH ARTICLE

Comparative Effects of Marine Microalgae-Supplemented Diets on Growth Performance, Survival and Immune-Related Gene Expression in Nile Tilapia (*Oreochromis niloticus*) Challenged with *Streptococcus agalactiae*

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ABSTRACT

This study evaluated the effects of dietary marine microalgae supplementation on growth performance, growth- and immune-related transcriptional responses, bacterial load and post-challenge survival in Nile tilapia (*Oreochromis niloticus*). Fish were fed a basal diet or diets supplemented with 5% (w/w) *Chaetoceros muelleri*, *Tetraselmis chuii*, or *Thalassiosira weissflogii* for 30 days. Growth performance and mRNA abundance of growth-related genes (*GH*, *IGF1* and *MSTN*) were assessed at the end of feeding. Fish were subsequently challenged with *Streptococcus agalactiae* (1×10^5CFU fish^{-1}). Immune-related genes (*hepcidin*, *IgM*, *IL-1β*, *MHC-II*, *TLR5* and *TLR9*) were quantified before challenge and at 1, 3 and 7 days post-infection (dpi) and bacterial load was determined at same post-challenge time points. Survival was monitored for 10 days. Fish fed *C. muelleri* and *T. chuii*-supplemented diets has significantly greater final body weight than controls, whereas average daily gain, feed conversion ratio and pre-challenge survival were unaffected. *T. chuii* increased *GH* and *IGF1* mRNA abundance in liver and muscle, while *MSTN* expression decreased in all supplemented groups. Following challenge, *C. muelleri* and *T. weissflogii* produced greater transcriptional responses in several immune-related genes, including *IgM*, *MHC-II*, *hepcidin*, *IL-1β* and *TLR9*. Bacterial load was significantly affected by dietary treatment and sampling time, with lower loads in microalgae-supplemented groups at 3 and 7 dpi. Post-challenge survival was significantly higher only in the *T. weissflogii* group. Overall, the three microalgae species induced distinct physiological, transcriptional and protective responses, supporting their further evaluation as targeted functional feed ingredients for Nile tilapia.

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INTRODUCTION

Nile tilapia (*Oreochromis niloticus*) is one of the most important freshwater aquaculture species worldwide due to its rapid growth and high reproductive capacity, with spawning occurring approximately every three months, making it a reliable food source (Barría *et al*., 2023; Deck *et al*., 2023). It is also valued as an affordable, high-quality protein source with good taste. Nile tilapia was first introduced to Thailand in 1965 by King Bhumibol Adulyadej (King Rama IX) to enhance protein and nutrient availability for rural communities.

Although *Oreochromis niloticus* is regarded as a relatively resistant species, infection with *Streptococcus agalactiae* can result in rapid and severe mortality in Nile tilapia (Ye *et al*., 2011), leading to substantial economic loss (Haenen *et al*., 2023). *Streptococcus agalactiae* belongs to Lancefield Group B Streptococcus (GBS) and is

categorized according to its surface antigen composition (Lancefield, 1934). To date, ten serotypes (Ia, Ib and II–IX) have been recognized, of which Ia, Ib, II and III are most frequently reported in tilapia infections (Gherardi *et al*., 2007; Slotved *et al*., 2007).

Dietary supplementation in aquafeeds is widely recognized as an effective approach to improving growth performance and enhancing immune responses in cultured fish species (Zlaugotne *et al*., 2022). Microorganisms (probiotics), yeasts, plant-based ingredients and microalgal feedstuffs are commonly used as feed additives in Nile tilapia diets due to their high levels of bioactive compounds which promote growth, stimulate immune responses and provide antioxidant properties (Hassaan *et al*., 2018; Han *et al*., 2019; Hassaan *et al*., 2019; Opiyo *et al*., 2019; Ahmad *et al*., 2020; Lumsangkul *et al*., 2022; Le Xuan *et al*., 2024; Zaid *et al*., 2025).

Marine microalgae species, including *Thalassiosira weissflogii*, *Chaetoceros* spp. and *Tetraselmis* spp. are increasingly used as dietary supplements in aquaculture. Their application has been particularly extensive in the culture of aquatic invertebrates, such as penaeid shrimps, crabs and bivalves, where they serve as valuable sources of nutrients and bioactive compounds. Marine microalgae are rich in essential fatty acids, especially the long-chain omega-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which have been associated with improved growth performance, immune function, antioxidant status and disease resistance in cultured aquatic species (Oniam *et al*., 2015; Southgate *et al*., 2017; Charoonnart *et al*., 2018; Etesami *et al*., 2020; Wu *et al*., 2026). In contrast, comparatively few studies have evaluated the potential of marine microalgae as functional feed ingredients for freshwater finfish species, including Nile tilapia (*Oreochromis niloticus*), one of the most important aquaculture species worldwide. Therefore, the present study investigated the effects of dietary supplementation with *Thalassiosira weissflogii*, *Chaetoceros muelleri* and *Tetraselmis chui* on growth performance and immune responses in Nile tilapia.

MATERIALS AND METHODS

Marine microalgae preparation and diet formulation: *Chaetoceros muelleri*, *Thalassiosira weissflogii* and *Tetraselmis chuii* were obtained from the Microalgal Laboratory, Walailak University. The microalgae were cultured in Guillard's F/2 medium and harvested at the stationary phase after 5 days. Biomass was freeze-dried, ground into powder and sieved (100μM) (Pooljun *et al*., 2022). A preliminary feeding trial evaluated dietary inclusion levels of 0, 2.5, 5.0 and 7.5% (wt/wt). Based on statistical analysis, the 5.0% inclusion level was selected for subsequent experiments because it resulted in highest final growth, while increasing supplementation to 7.5% did not provide a significant additional growth benefit. Microalgal powder was mixed with commercial feed (CP9931; crude protein ≥18%, lipid ≥3%). Pellets were air-dried, coated with 1.5% fish oil to reduce nutrient leaching (Guo *et al*., 2021) and stored at 4°C until use.

Experimental design and growth performance: A total of 300 juvenile Nile tilapia (*Oreochromis niloticus*) were randomly allocated to four dietary treatments: control (basal diet), *C. muelleri*, *T. chuii* and *T. weissflogii*. Each treatment consisted of five independent replicate 100L tanks with 15 fish per tank. Fish were maintained under continuous aeration and fed at 5% body weight day^{-1} for 30 days. Body weight was recorded at the beginning and end of the feeding trial to calculate final weight, average daily gain (ADG), feed conversion ratio (FCR) and survival rate. For growth-related gene expression analysis, liver and muscle tissues were sampled from two fish per tank across five replicate tanks per dietary treatment; gene expression values from two fish within each tank were averaged to yield a single value per tank (n=5 tanks per treatment) prior to statistical analysis.

***Streptococcus agalactiae* challenge:** A virulent strain of *Streptococcus agalactiae* serotype Ia was obtained from the Center of Excellence for Aquaculture Technology and Innovation and confirmed by PCR and molecular serotyping (Martinez *et al*., 2001; Imperi *et al*., 2010; Wongsathein *et al*., 2019) (Fig. 2A). The median lethal dose (LD_{50}) was determined by intraperitoneal injection of serially diluted *S. agalactiae* bacterial suspensions (Khunrang *et al*., 2023) and calculated using the Reed-Muench method based on cumulative mortality recorded over 10 days post-challenge (Reed and Muench, 1938). Based on the LD_{50} determination, a sublethal challenge dose of 1×10^5CFU fish^{-1} were selected for the infection experiment to induce bacterial infection while maintaining sufficient survival for immune response evaluation.

Following sample collection for growth-related gene expression analysis, the remaining fish were used for *S. agalactiae* challenge experiment. A total of 50 fish from each dietary treatment were randomly distributed into five replicate tanks (10 fish per tank). One replicate tank was injected with sterile saline as the control, while the remaining four replicate tanks were challenged with *S. agalactiae*. The challenged fish were further divided into two groups: a survival monitoring group and an immune gene expression analysis group. Two replicate tanks were monitored for clinical signs and mortality for 10 days post-infection (dpi). Mortality was recorded daily and bacterial infection in dead fish was confirmed by PCR analysis. The remaining two replicate tanks were used for immune gene expression analysis, Head kidney and spleen tissues were randomly collected from five fish per treatment at 1, 3 and 7 dpi, with individual fish serving as the replicate unit (n=5 fish per treatment per time point). Brain tissue was additionally collected from these same fish at each time point to determine bacterial load.

Gene expression analysis: Total RNA was extracted from liver, muscle, head kidney and spleen tissues using GeneZol reagent. RNA quality and concentration were determined spectrophotometrically and cDNA was synthesized using a commercial reverse-transcription kit. Quantitative real-time PCR (qRT-PCR) was performed to determine the expression of growth-related genes (*GH*, *IGF1* and *MSTN*) and immune-related genes (*hepcidin*, *IgM*, *MHC-II*, *IL-1β*, *TLR5* and *TLR9*). β-actin was used as the reference gene based on its established use in qRT-PCR studies of Nile tilapia (Huervana *et al*., 2022; Van Doan *et*

al., 2023; Khalafalaa *et al*., 2025) (Table 1). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Bacterial load quantification: Genomic DNA was extracted from brain tissue using DNeasy Blood & Tissue kit according to the manufacturer's instructions (QIAGEN) and DNA concentrations were standardized before qPCR. *S. agalactiae* DNA was quantified using the F1-IMOD primer set (Martinez *et al*., 2001) with SYBR Green detection (5×HOT FIREPOl qPCR Supermix; Solis Biodyne). A standard curve was generated from serial dilutions of *S. agalactiae* cultures with known concentration (CFU mL^{-1}). Sample Cq values were converted to CFU-equivalent quantities, normalized to tissue weight and expressed as $\log_{10}$ CFU equivalents g^{-1} of brain tissue.

Statistical analysis: Growth performance and growth-related gene expression data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test, with difference considered significant at $P<0.05$. For immune-related gene expression, ΔCq values were analyzed separately for each gene using a three-way factorial ANOVA with dietary treatment, tissue and sampling times as fixed factors, including all two-way and three interactions. Because gene expression is inherently tissue- and time-dependent, treatment effects were further examined within each tissue Í sampling time combination using one-way ANOVA followed by Tukey's multiple comparison test, to compare dietary treatment within the corresponding biological context. Differences were considered significant at $P<0.05$ and gene-expression data are presented as mean ± SD. Survival following bacterial challenge was analyzed using Kaplan–Meier survival curves and compared using the log-rank (Mantel–Cox) test, performed in GraphPad Prism 9. All analyses were performed using SPSS 22.0 except for the three-way factorial ANOVA, which was performed using Python.

RESULTS

Growth performance: Table 2 presents the growth performance and survival rate of the fish following the experimental trial. No significant differences ($P>0.05$) were observed in initial body weight or intermediate weight gain among treatments. Similarly, fish fed the control and *T. weissflogii*-supplemented diets showed no significant difference ($P=0.9950$) throughout the experimental period. However, fish fed diets supplemented with *C. muelleri* and *T. chuii* exhibited significantly higher body weight gain at day 30 compared to the control group ($P=0.0468$ and 0.0382, respectively). Although no significant differences in average daily gain (ADG) and feed conversion ratio (FCR) were found among treatments, fish fed *T. chuii*-supplemented diets tended to exhibit better growth and a low FCR than the other groups.

No significant differences ($P>0.05$) in survival rate were observed among treatments, with survival ranging from 85 to 90% at the end of the experiment. Overall, dietary supplementation with 5% microalgae improved growth performance without adversely affecting the survival rate of tilapia.

Table 1: Primer sequences, annealing temperatures, PCR amplification efficiencies, R^2 values and amplicon sizes of target genes analyzed in this study.

Genes	Primer sequence (5'→3')	Annealing temperature (°C)	Amplification efficiency (%)	R^2	Amplicon (bp)	Reference
Reference gene						
β-actin	F: GACCCACACAGTGCCCATCT R: TCTCGGCTGTGGTGGTGAA	58	104.5	1.000	140	Wang *et al*., 2015
Growth-related genes						
Growth hormone: *GH*	F: TCGGTTGTGTGTTTGGGCGTCTC R: GTGCAGGTGCGTGACTCTGTTGA	62	99.6	0.998	90	Qiang *et al*., 2012
Insulin-like growth factor 1: *IGF1*	F: TTGTCTGTGGAGAGCGAGGCTT R: CAGCTTTGGAAGCAGCACTCGT	60	98.9	0.999	103	Qiang *et al*., 2012
Myostatin: *MSTN*	F: GCATCTGTCTCAGATCGTGCT R: TGCCATCATTACAATTGTCTCCG	58	100	0.970	95	Aboukila *et al*., 2021
Immune-related genes						
Hepcidin	F: CGTTCAGTGTTGCAGTTGCAG R: GCAGCAAACTCCACAGATACC	60	99.9	0.996	242	Wuthisuthimethavee *et al*., 2025
Immunoglobulin M: *IgM*	F: GGGAAGATGAGGAAGGAAATGA R: GTTTTACCCCCCTGGTCCAT	58	99.6	0.994	120	Wang *et al*., 2015
Interleukin-1 beta: *IL-1β*	F: CAAGGATGACGACAAGCCAACC R: AGCGGACAGACATGAGAGTGC	60	100	0.991	149	Won *et al*., 2020
Major histocompatibility complex class II: *MHC-II*	F: TCAGCTGCACTCGTTCC R: CCAGCATCAACGTTCCTC	58	103	0.979	250	Chen *et al*., 2019
Toll-like receptor 5: *TLR5*	F: CAGGGTAAACATTTCACGCTTCT R: ACGCTTTGCCATGGGAACTTT	60	100	0.995	162	Basu *et al*., 2015
Toll-like receptor 9: *TLR9*	F: CCAGAGTCATTGGCTGGTGT R: CAGTACCACAGGTCCCAACC	60	99.8	0.997	158	Gao *et al*., 2023

Table 2: Effects of 5% dietary supplementation with *Chaetoceros muelleri*, *Tetraselmis chuii* and *Thalassiosira weissflogii* on body weight gain (BWG), average daily gain (ADG), feed conversion ratio (FCR) and survival over 30 days. Values are mean±SD; different superscripts indicate significant differences ($P<0.05$)

Treatment	Body weight gain (BWG: g)			Average daily gain (ADG: g/day)		Feed conversion ratio (FCR)		Survival rate (%)
	Initial (Day 0)	14 days	30 days	14 days	30 days	14 days	30 days	30 days
Control	2.698±0.58^a	4.460±1.24^a	6.848±2.22^a	0.126±0.023^a	0.138±0.040^a	1.38±0.320^a	1.29±0.560^a	90^a
C. muelleri	2.726±0.83^a	4.747±1.67^a	8.026±3.18^b	0.144±0.020^a	0.177±0.037^a	1.33±0.470^a	1.24±0.430^a	85^a
T. chuii	2.621±0.59^a	4.720±1.36^a	8.065±2.74^b	0.150±0.025^a	0.182±0.054^a	1.27±0.521^a	1.14±0.323^a	90^a
T. weissflogii	2.585±0.69^a	4.274±1.33^a	6.943±2.57^a	0.121±0.031^a	0.145±0.036^a	1.41±0.422^a	1.22±0.452^a	88^a

Growth-related gene expression: The mRNA expression levels of *GH*, *IGF1* and *MSTN* in the liver and muscle tissues of Nile tilapia were analyzed by real-time PCR after the feeding trials (Fig. 1). *GH* mRNA expression was significantly elevated in fish fed the *T. chuii*-supplemented diet, with higher expression observed in the muscle of all microalgae-fed groups compared to the control group (Fig. 1A; Fig. 1D). *IGF1* mRNA expression was significantly upregulated in the liver of fish fed the *T. chuii*-supplemented diet compared with the control group. In skeletal muscle, *IGF1* mRNA expression was significantly higher in fish receiving diets supplemented with *C. muelleri* and *T. chuii*, whereas no significant difference was observed in fish fed the *T. weissflogii*-supplemented diet relative to the control (Fig. 1B; Fig. 1E). *MSTN* mRNA was significantly downregulated in both liver and muscle tissues of fish fed microalgae-supplemented diets compared to the control group (Fig. 1C; Fig. 1F).

Immune-related gene expression: The mRNA expression levels of immune-related genes (*hepcidin, IgM, IL-1β, MHC-II, TLR5* and *TLR9*) in the kidneys and spleens of Nile tilapia were measured using quantitative RT-PCR (qRT-PCR). The specificity of the immune-related primers was evaluated through a melting curve analysis. The PCR amplification efficiencies of all primers ranged from 99.6 to 103% and the coefficients of determination (R^2) for standard curves were all greater than 0.99 (Table 1).

In the kidney after 30 days of feeding, *IgM* and *MHC-II* mRNA expression were significantly upregulated in fish fed diets supplemented with *C. muelleri* and *T. weissflogii* compared with the control group. In contrast, fish fed the *T. chuii*-supplemented diet exhibited no significant differences in the expression of either gene relative to the control. Following *S. agalactiae* challenge, *IgM* and *MHC-II* mRNA expression progressively increased from day 1 to day 7 post-infection, reaching peak levels at day 7 (Fig. 3). Prior to challenge (after 30 days of feeding), no significant differences were observed in the mRNA expression of *hepcidin*, *IL-1β*, or *TLR5* among the dietary treatments. In contrast, *TLR9* mRNA expression was significantly higher in fish fed the *T. weissflogii*-supplemented diet than in the control group. Following *S. agalactiae* challenge, *hepcidin*, *IL-1β* and *TLR9* mRNA expression was significantly upregulated in all microalgae-supplemented groups at 1day post-infection (dpi), followed by a gradual decline at 3 and 7 dpi. In contrast, *TLR5* expression remained relatively stable throughout the post-challenge period.

In the spleen after 30 days of feeding, *IgM* and *MHC-II* mRNA expression levels were significantly upregulated in fish fed the *C. muelleri*- and *T. weissflogii*-supplemented diets, whereas no significant differences were observed in fish receiving the *T. chuii*-supplemented diet compared with the control group. Following *S. agalactiae* challenge, *IgM* mRNA expression increased significantly in all microalgae-supplemented groups at 1 dpi and continued to rise through 7 dpi. No significant differences in *MHC-II* mRNA expression were detected among treatments at 1 dpi. However, at 3 dpi, fish fed the *C. muelleri*- and *T. weissflogii*-supplemented diets exhibited significantly higher *MHC-II* mRNA expression than those fed the *T. chuii*-supplemented diet, which did not differ from the control group. By 7 dpi, all microalgae-supplemented groups showed significantly higher *MHC-II* mRNA expression than the control group. Likewise, the mRNA expression of *hepcidin*, *IL-1β* and *TLR9* was significantly upregulated in the spleen of all microalgae fed groups at 1 dpi, followed by a gradual decline at 3 and 7 dpi (Fig. 4). In contrast, *TLR5* mRNA expression remained unchanged in both the kidney and spleen throughout the experimental period.

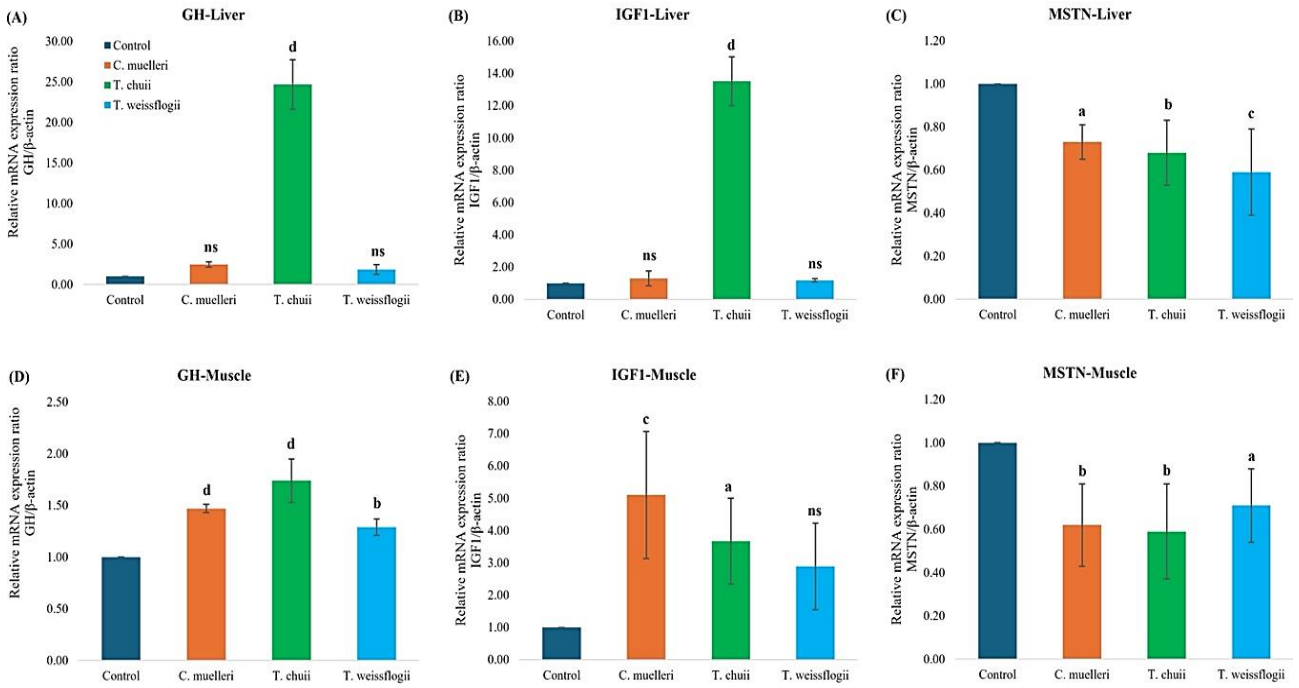


Fig. 1: Relative mRNA expression of *GH*, *IGF1* and *MSTN* in the liver (A-C) and muscle (D-F) of Nile tilapia fed diets supplemented with different marine microalgae compared with the control diet. Data are presented as the mean±SD (n=5). Significant differences compared with the control group are indicated by letters above the bars: a, P≤0.05; b, P≤0.01; c, P≤0.001; and d, P≤0.0001. "ns" indicates no significant difference.

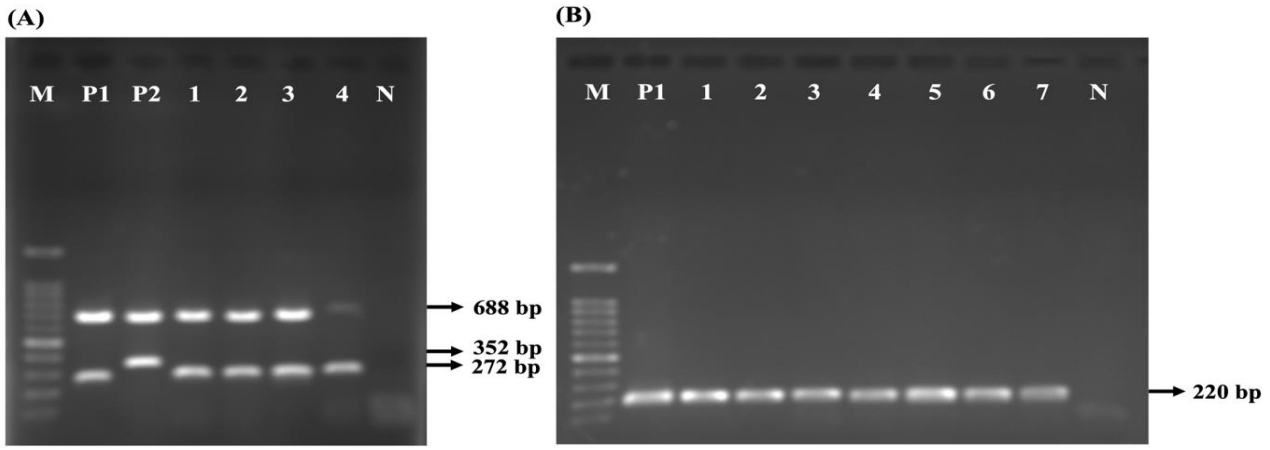


Fig. 2: Agarose gel electrophoresis analysis. (A) Multiplex PCR products used to determine the molecular serotypes of *Streptococcus agalactiae* based on their banding patterns. Lane M, 100-bp fluorescent DNA ladder (SOMOBIO Technology, Taiwan); lane P1, positive control for *S. agalactiae* serotype Ia with amplicons at 688 and 272 bp; lane P2, positive control for *S. agalactiae* serotype III, with amplicons at 688 and 352 bp; lanes 1–4, *S. agalactiae* isolates used in this study; lane N: negative control containing distilled water. (B) PCR products of *S. agalactiae* detected in pooled brain tissues from tilapia that died following bacterial challenge. Lane M, 100-bp fluorescent DNA ladder; lane P, positive control for *S. agalactiae*; lane 1, pooled brain tissue from the control group; lanes 2–3, pooled brain tissues from the *C. muelleri*-supplemented group; lanes 4–5, pooled brain tissues from the *Tetraselmis chuii*-supplemented group; lanes 6–7, pooled brain tissues from the *Thalassiosira weissflogii*-supplemented group; lane N, negative control containing distilled water.

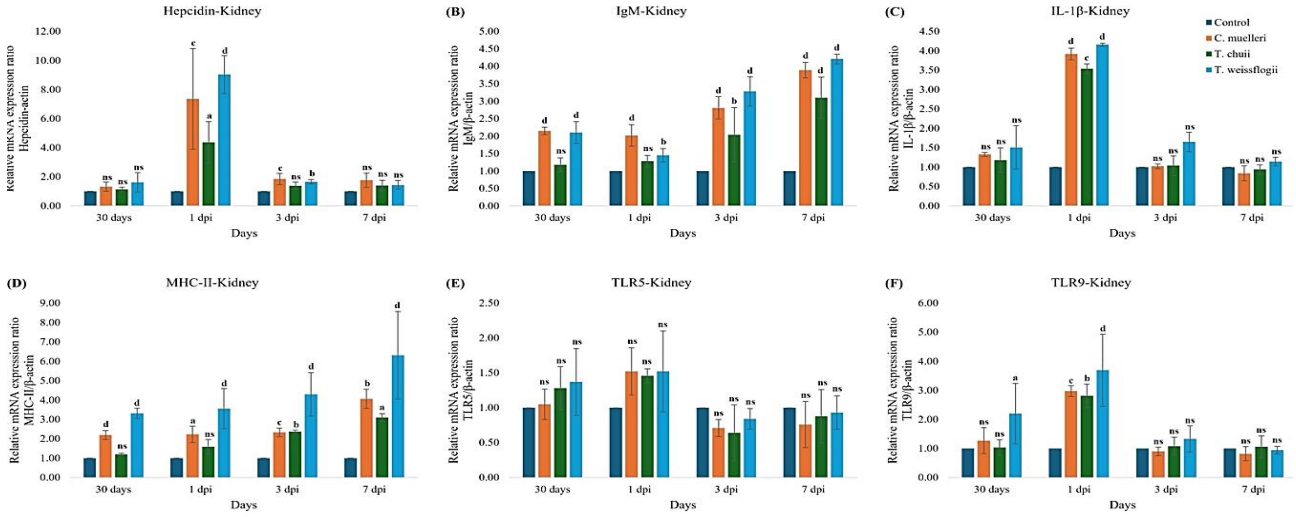


Fig. 3: Relative mRNA expression of *hepcidin, IgM, IL-1β, MHC-II, TLR5* and *TLR9* in the kidney of Nile tilapia fed diets supplemented with different marine microalgae compared with the control diet after 30 days of feeding and at 1, 3 and 7 days post-*Streptococcus agalactiae* infection (dpi). Data are presented as the mean±SD (n=5). Significant differences compared with the control group are indicated by letters above the bars: a, P≤ 0.05; b, P≤0.01; c, P≤0.001; and d, P≤0.0001. "ns" indicates no significant difference.

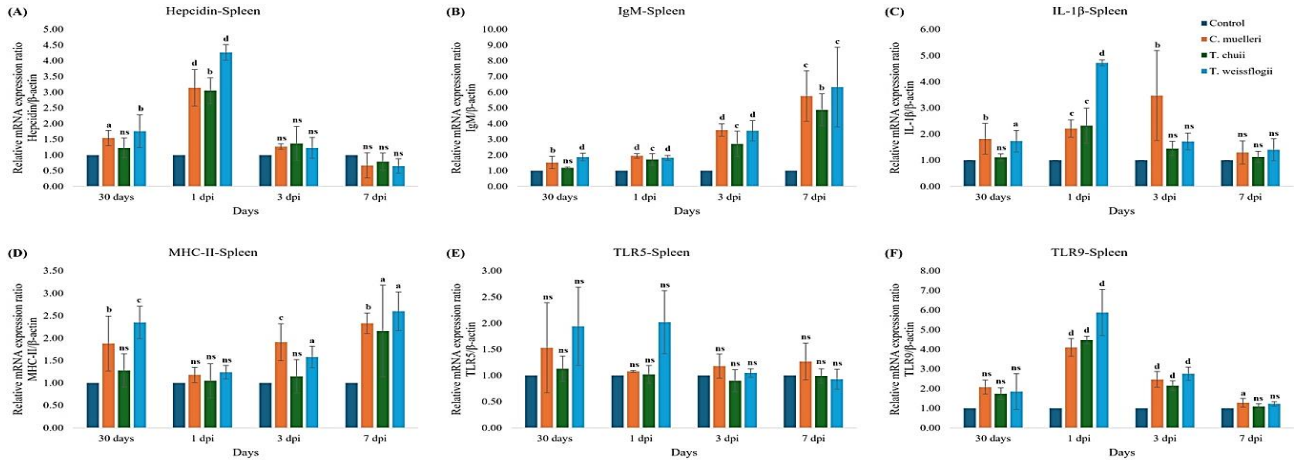


Fig. 4: Relative mRNA expression of *hepcidin, IgM, IL-1β, MHC-II, TLR5* and *TLR9* in the spleen of Nile tilapia fed diets supplemented with different marine microalgae compared with the control diet after 30 days of feeding and at 1, 3 and 7 days post-*Streptococcus agalactiae* infection (dpi). Data are presented as the mean±SD (n=5). Significant differences compared with the control group are indicated by letters above the bars: a, P≤0.05; b, P≤0.01; c, P≤0.001; and d, P≤0.0001. "ns" indicates no significant difference.

Three-way ANOVA of ΔCq values further showed significant overall effects of dietary treatment on *hepcidin, IgM, IL-1β, MHC-II* and *TLR9* mRNA expression, whereas no significant treatment effect was detected for *TLR5*. Tissue significantly affected all six genes, while sampling time significantly affects hepcidin, *IgM, MHC-II* and *TLR9* mRNA, but not *IL-1β* or *TLR5*. No significant treatment × tissue interaction was detected for any gene. A significant treatment × time interaction was observed only for hepcidin, whereas significant tissue × time interactions were detected for hepcidin, *IgM, IL-1β, MHC-II* and *TLR9*, but not *TLR5*. The treatment × tissue × time interaction was not significant for any of six immune-related genes examined (Table 3).

Table 3: F-values from three-way ANOVA testing the effects of dietary treatment, tissue, sampling time and their interactions on immune-related gene expression (ΔCq) in Nile tilapia

Source of variation	df	*hepcidin*	*IgM*	*IL-1β*	*MHC-II*	*TLR5*	*TLR9*
Treatment (Tr)	3	10.27^{***}	19.51^{***}	6.11^{***}	18.83^{***}	1.02^{ns}	5.31^{**}
Tissue (Ts)	1	18.90^{***}	27.40^{***}	31.70^{***}	204.24^{***}	9.12^{**}	88.40^{***}
Time (Tm)	3	130.27^{***}	71.63^{***}	2.55^{ns}	40.16^{***}	1.62^{ns}	24.30^{***}
Tr × Ts	3	1.44^{ns}	0.29^{ns}	0.64^{ns}	2.30^{ns}	0.71^{ns}	0.76^{ns}
Tr × Tm	9	4.13^{***}	1.28^{ns}	1.66^{ns}	0.64^{ns}	0.52^{ns}	1.16^{ns}
Ts × Tm	3	253.39^{***}	14.70^{***}	23.56^{***}	37.72^{***}	2.01^{ns}	30.75^{***}
Tr × Ts × Tm	9	0.37^{ns}	0.21^{ns}	0.65^{ns}	0.24^{ns}	0.29^{ns}	0.21^{ns}
Residual	128	-	-	-	-	-	-

Analysis was performed on ΔCq values (Cq target gene − Cq β-actin) for each gene separately, using a fully balanced three-way factorial ANOVA (treatment, 4 levels; tissue, 2 levels; time, 4 levels; n=5 fish per treatment × tissue × time combination). P<0.05; P<0.01; P< 0.001; ns, not significant (P≥0.05).

Brain bacterial load post-challenge: Brain bacterial load (expressed as log10-transformed starting quantity) was significantly affected by both dietary treatment and sampling time (P<0.001), whereas the treatment × time interaction was not statistically significant (P=0.124) (Table 4). At 1 dpi, bacterial load was significantly lower in the *T. weissflogii* group compared with the control and *C. muelleri* groups. At 3 and 7 dpi, the control group exhibited significantly higher bacterial loads than all microalgae-supplemented groups. Within dietary treatments, bacterial load decreased significantly from 1 to 7 dpi in the *C. muelleri* group, whereas the *T. weissflogii* group showed a transient increase at 3 dpi followed by a significant reduction at 7 dpi (Table 5).

Post-challenge survival and disease resistance: The bacterial challenge was monitored for 10 days post-infection (dpi). Mortality began at 1 dpi in fish challenged with *S. agalactiae* and continued until 6 dpi (Fig. 5). At the end of the challenge period, survival rates were 45, 70, 65 and 75% in the control, *C. muelleri*-, *T. chuii*- and *T. weissflogii*-supplemented groups, respectively. Survival in all marine microalgae-supplemented groups remained at or above 65% from 5 dpi until the end of the experiment. Kaplan-Meier survival analysis shows a significant difference between fish fed *T. weissflogii*-supplemented diet and the control groups (P=0.031). In contrast, survival did not differ significantly between the challenged control group and fish fed the *C. muelleri*- or *T. chuii*-supplemented diets (P=0.070 and 0.153, respectively).

Table 4: Two-way ANOVA of the effects of dietary treatment and sampling time on Streptococcus agalactiae bacterial load in the brain of Nile tilapia following challenge. Bacterial load was expressed as log10 CFU equivalents g-1 of brain tissue. Dietary treatment, sampling time and their interaction were included as fixed factors.

Effect	F	df	P-value
Dietary treatment	15.71	3, 48	<0.001
Sampling time	15.51	2, 48	<0.001
Treatment × time	1.78	6, 48	0.124

df values are presented as degrees of freedom for effect and error (df_{effect}-df_{error}). Bacterial load data were log10-transformed prior to statistical analysis. Statistical significance was set at P<0.05.

Table 5: Bacterial load of *Streptococcus agalactiae* in Nile tilapia at 1, 3 and 7 days post-infection (dpi) following bacterial challenge

Dietary group	1 dpi	3 dpi	7 dpi
Control	5.85 ± 0.49^{aAB}	6.63 ± 0.50^{aA}	5.40 ± 0.67^{aB}
C. muelleri	5.57 ± 0.40^{aA}	4.84 ± 1.29^{bAB}	3.70 ± 0.63^{bB}
T. chuii	4.84 ± 1.13^{abA}	4.99 ± 0.72^{bA}	4.11 ± 0.68^{bA}
T. weissflogii	4.12 ± 0.56^{bB}	5.14 ± 0.54^{bA}	3.47 ± 0.58^{bB}

Values are expressed as mean±SD of log10 starting quantity (SQ), n=5. Different lowercase superscript letters within the same column indicate significant differences among dietary groups at the same sampling time (P<0.05). Different uppercase superscript letters within the same row indicate significant differences among sampling times within the same dietary group (P<0.05), based on Tukey-adjusted pairwise comparisons.

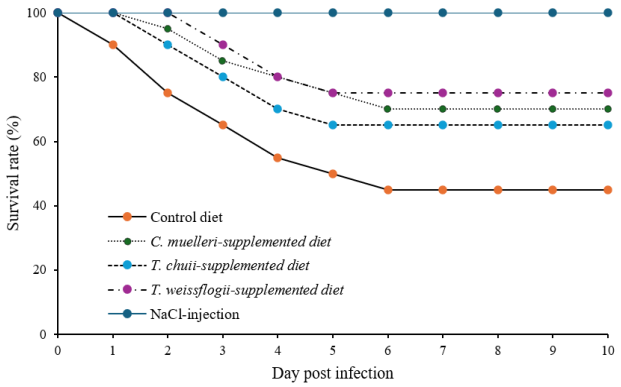


Fig. 5: Kaplan-Meier survival curve of Nile tilapia following challenge with virulent *Streptococcus agalactiae*. Survival was recorded daily for 10 days post-infection (dpi). Fish were challenged by intraperitoneal injection of 100 µL of bacterial suspension containing 1×10^5 CFU $fish^{-1}$ of *S. agalactiae* serotype Ia. *C. muelleri*-supplemented diet/Control diet (HR=2.350 (0.901-6.124), P=0.070). *T. chuii*-supplemented diet/Control diet (HR=1.904 (0.752-4.816), P=0.153); *T. weissflogii*-supplemented diet/Control diet (HR=2.893 (1.076-7.780), P=0.031)

Mortality was first recorded at 2 dpi in the *C. muelleri*- and *T. chuii*-supplemented groups and at 3 dpi in the *T. weissflogii*-supplemented group, compared with 1 dpi in the challenged control group. No mortality was observed in the negative-control group injected with sterile normal saline throughout the experimental period. Moreover, all dead fish from the challenged groups tested positive for *S. agalactiae* by PCR, confirming the presence of the challenge bacterium in the examined mortalities (Fig. 2B).

DISCUSSION

Dietary supplementation with microalgae at 5% (w/w) improved the growth performance of juvenile Nile tilapia (*Oreochromis niloticus*), with fish fed *C. muelleri* and *T. chuii* achieving significantly greater final body weights than the control. Although average daily gain (ADG) and feed conversion ratio (FCR) were not significantly affected, microalgae-fed fish showed numerically higher ADG and lower FCR; however, these trends should be

interpreted cautiously. Survival during the feeding trial was also unaffected by dietary treatment, indicating no apparent adverse effects at the tested inclusion level. These findings align with previous studies reporting beneficial growth-related responses to microalgal supplementation, including improved growth, survival and n-3 highly unsaturated fatty acid (HUFA) accumulation in common sole larvae fed *T. chuii* (Shawky *et al*., 2021), as well as enhanced growth performance in marine shrimp receiving *C. muelleri* and *T. weissflogii* (Pooljun *et al*., 2022; 2025). Microalgae contain various nutritional and bioactive constituents including proteins, essential amino acids, polyunsaturated fatty acids (PUFAs), vitamins, minerals, carbohydrates and pigments which support growth and metabolic functions in aquatic animals (Vallejos-Vidal *et al*., 2016; Dawood *et al*., 2017; Damaristelma de Jesús-Campos *et al*., 2020; Hernández-Sandoval *et al*., 2022; Conlon *et al*., 2024; Madyod *et al*., 2026). However, because the specific biochemical composition of the experimental microalgal biomass was not directly characterized in this study, the precise contribution of individual constituents remains unresolved and requires direct biochemical investigation.

The enhanced growth performance observed in this study was accompanied by alterations in the transcript abundance of key growth-related genes. Dietary supplementation, particularly with *Tetraselmis chuii*, significantly increased *GH* and *IGF1* mRNA levels in both liver and muscle tissues. These changes align with the upregulation of the GH–IGF axis, which plays a central role in regulating protein accretion, nutrient utilization and growth in teleost fish. Nevertheless, because protein concentrations and downstream receptor signaling were not evaluated, these increases in transcript abundance should be interpreted as potential axis modulation rather than direct evidence of functional activation. These findings consistent with previous work demonstrating that *Tetraselmis* sp. improves growth performance and digestive enzyme activities in Nile tilapia (Abdel-Tawwab *et al*., 2023), as well as reports of microalgae-based diets upregulating hepatic *GH* and *IGF1* expression in tilapia (Soliman *et al*., 2025) and *IGF-II* in shrimp (Sharawy *et al*., 2020). Conversely, *MSTN* mRNA abundance was significantly reduced in both liver and muscle tissues. Because MSTN serves as an established negative regulator of skeletal muscle development, diminished *MSTN* expression aligns with a profile of reduced growth-inhibitory signaling. Similar responses have been documented in Nile tilapia, zebrafish and channel catfish, where genetic disruption or suppression of *MSTN* was associated with enhanced body weight and muscle mass (Khalil *et al*., 2017; Wu *et al*., 2023; Liang *et al*., 2026). The concurrent upregulation of *GH* and *IGF1* transcripts alongside *MSTN* downregulation suggests coordinated transcriptional regulation of positive and negative growth pathways.

The successful establishment of *Streptococcus agalactiae* infection was confirmed by clinical signs, cumulative mortality and PCR validation, with mortality patterns during the first week post-infection matching historical challenge models in Nile tilapia (Abdel-Tawwab *et al*., 2023). Following the *S. agalactiae* challenge, dietary microalgae supplementation significantly reduced bacterial loads in brain tissue (notably at 3 and 7 days post-infection). However, Kaplan–Meier analysis demonstrated a statistically significant survival benefit exclusively in the *T. weissflogii* group, whereas differences observed for *C. muelleri* and *T. chuii* represented non-significant numerical trends. Discrepancies between these findings and previous studies reporting broader post-challenge survival improvements following microalgal supplementation (Abdel-Tawwab *et al*., 2023) may stem from variations in microalgal species, inclusion levels, feeding duration, pathogen strain, challenge dose, host condition, or overall experimental design**.**

Dietary microalgae supplementation also modulated the transcription of both innate and adaptive immune-related genes in Nile tilapia, with responses varying by species and tissue. Prior to the bacterial challenge, fish fed diets supplemented with *C. muelleri* and *T. weissflogii* exhibited higher mRNA levels of key immune-related genes including *MHC-II, IgM, hepcidin, IL-1β* and *TLR9* relative to the control group. These findings indicate distinct baseline transcriptional priming prior to pathogen exposure. (Vallejos-Vidal *et al*., 2016; Koch *et al*., 2021; Cheong *et al*., 2026; Madyod *et al*., 2026). Specifically, elevated *MHC-II* and *IgM* transcript levels align with antigen presentation and humoral immunity processes (Cerezuela *et al*., 2012), while increased expression of *TLR9*, *hepcidin* and *IL-1β* reflects early activation of pathogen recognition, antimicrobial defense, and inflammatory signaling pathways (Wang *et al*., 2015; Chen *et al*., 2021; Gao *et al*., 2023; Madyod *et al*., 2026). In contrast, *TLR5* transcript abundance remained unchanged prior to infection, consistent with its specific role in flagellin recognition. Following the *S. agalactiae* challenge, *TLR9*, *hepcidin* and *IL-1β* transcript levels rapidly increases, reflecting early innate immune engagement. The stronger induction of *TLR9* relative to *TLR5* aligns with the non-flagellated nature of *S. agalactiae*, suggesting a predominant transcriptional involvement of nucleic acid-sensing pathways over flagellin-recognition pathways during pathogen infection (Gao *et al*., 2023). Furthermore, *IgM* and *MHC-II* transcript abundance increased progressively from 1 to 7 days post-infection, reflecting a delayed transcriptional response of genes associated with adaptive immunity, consistent with previous observations in gibel carp infected with CyHV-2 (Gao *et al*., 2023).

Collectively, these results demonstrate that elevated mRNA abundance of immune-related genes and reduced brain bacterial DNA load do not automatically translate into enhanced functional immunity or overall host protection, which depends on complex interactions among pathogen virulence, challenge dose, nutritional status and effector protein activity. The three evaluated marine microalgae exhibited distinct, species-dependent biological response profiles: *T. chuii* predominantly enhanced growth-related responses through modulation of GH-IGF axis and *MSTN* expression, whereas *C. muelleri* and *T. weissflogii* elicited stronger immunomodulation responses. These differences demonstrate that three microalgae species are not functionally interchangeable feed ingredients. Instead, candidate species should be selected based on specific production objectives, such as growth promotion, immune enhancement, or a targeted combination of both.

Key methodological limitations of this study should be noted. First, immune gene expression and bacterial load were evaluated exclusively at the mRNA and DNA levels, respectively, without corresponding protein-level quantification or functional immune assays. Second, qRT-PCR normalization relied solely on β-actin (actb) without cross-validation against additional candidate reference genes across experimental conditions, despite its widespread use in Nile tilapia. Therefore, these transcriptional responses should be interpreted cautiously until confirmed by downstream functional and proteomic analyses.

Conclusions: Dietary supplementation with *C. muelleri*, *T. chuii* and *T. weissflogii* produced species-specific effects on growth performance and growth- and immune-related gene transcriptional responses in juvenile Nile tilapia. *Tetraselmis chuii* was associated with more produced changed in growth-related transcripts, whereas *C. muelleri* and *T. weissflogii* produced stronger transcriptional responses in several immune-related genes. Following *Streptococcus agalactiae* challenge, bacterial load in brain tissue was significantly affected by dietary treatment and sampling time, with lower bacterial loads observed in several microalgae-supplemented groups, particularly at 3 and 7 days post-infection. However, a significant improvement in post-challenge survival was observed only in the *T. weissflogii* group, whereas *C. muelleri* and *T. chuii* showed only non-significant numerical increases. These findings indicate that the three microalgae species are not functionally equivalent and highlight their distinct potential as dietary ingredients for Nile tilapia. Further studies integrating biochemical characterization, protein-level and functional immune measurements, large scale feeding trails and economic and environmental assessments will help define their practical application to sustainable aquaculture.

Ethical approval: All animal procedures were approved by the Animal Ethics Committee of Walailak University, Thailand (WU-ACUC-68022). Experiments involving Streptococcus agalactiae were conducted in accordance with Walailak University's biosafety regulations for scientific experiments (WU-IBC-68-025).

Authorship contribution statement: Conceptualization and design of study, CP, PJ; methodology, CP, NC, PK, TP, PD, PW; data curation, CP, NC, PJ; writing-original draft, CP, NC; writing review and editing, CP, PJ; supervision, IH; funding acquisition, CP. All authors have read and agreed to the published version of manuscript.

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REFERENCES

Abdel-Tawwab M, Khalil RH, Abdel-Razek N, *et al*., 2024. Dietary effects of microalga *Tetraselmis suecica* on growth, antioxidant-immune activity, inflammation cytokines and resistance of Nile tilapia fingerlings to *Aeromonas sobria* infection. Journal of Animal Physiology and Animal Nutrition 108(2):511-526.

Aboukila RS, Hemeda SAE, El Nahas AF, *et al*., 2021. Molecular characterization of *GHR1* gene and expression analysis of some growth-related genes in *Oreochromis niloticus*. Advances in Animal and Veterinary Sciences 9(7):1025-1033.

Ahmad MT, Shariff M, Md. Yusoff F, *et al*., 2020. Applications of microalga *Chlorella vulgaris* in aquaculture. Reviews in Aquaculture 12(1):328-346.

Barría A, Peñaloza C, Papadopoulou A, *et al*., 2023. Genetic differentiation following recent domestication events: A study of farmed Nile tilapia (*Oreochromis niloticus*) populations. Evolutionary Applications 16(6):1220-1235.

Basu M, Paichha M, Swain B, *et al*., 2015. Modulation of TLR2, TLR4, TLR5, NOD1 and NOD2 receptor gene expressions and their downstream signaling molecules following thermal stress in the Indian major Carp Catla (*Catla catla*).3 Biotech 5:1021-1030.

Cerezuela R, Guardiola FA, González P, *et al*., 2012. Effects of dietary *Bacillus subtilis*, *Tetraselmis chuii* and *Phaeodactylum tricornutum*, singularly or in combination, on the immune response and disease resistance of sea bream (*Sparus aurata* L.). Fish & Shellfish Immunology 33(2):342-349.

Charoonnart P, Purton S and Saksmerprome V, 2018. Applications of microalgal biotechnology for disease control in aquaculture. Biology:7(2): 24.

Chen X, Chi H, Xu B, *et al*., 2019. Oral bovine serum albumin immune-stimulating complexes improve the immune responses and resistance of large yellow croaker (*Pseudosciaena crocea*, Richardson, 1846) against *Vibrio alginolyticus*. Aquaculture Research 50:2802-2809.

Chen W, Luo L, Han D, *et al*., 2021. Effect of dietary supplementation with filamentous microalga *Tribonema ultriculosum* on growth performance, fillet quality and immunity of rainbow trout *Oncorhynchus mykiss*. Aquaculture Nutrition 27:1232-1243.

Cheong H, Christanus A, de Cruz CR, *et al*., 2026. *In-vitro* and *in-vivo* evaluation of *Chaetoceros* sp. immunomodulatory effects in red hybrid tilapia, *Oreochromis* spp. Biology 15(5):374.

Conlon T, Aranyos A, Luck T, *et al*., 2024. The effects of trophic mode and medium composition on the biochemical profile and antioxidant capacity of *Tetraselmis chuii* (CCAP 66/21B). Biocatalysis and Agricultural Biotechnology 61:103362

Damaristelma de Jesús-Campos, José Antonio López-Elías, Luis Ángel Medina-Juarez, *et al*., 2020. Chemical composition, fatty acid profile and molecular changes derived from nitrogen stress in the diatom *Chaetoceros muelleri*. Aquaculture Reports 16:100281.

Dawood MAO, Koshio S and Esteban MA, 2018. Beneficial roles of feed additives as immunostimulants in aquaculture: a review. Reviews in Aquaculture 10(4):950-974.

Deck CA, Salger SA, Reynolds HM, *et al*., 2023. Nutritional programming in Nile tilapia (*Oreochromis niloticus*): Effect of low dietary protein on growth and the intestinal microbiome and transcriptome. Plos One 18(10):e0292431

Etesami E, Jorjani S and Noroozi M. 2022. Improvement of *Thalassiosira weissflogii* as high valuable nutritional feed. Iranian Journal of Fisheries Sciences 21(1):15-32.

Gao JW, Hu YW, Xie M, *et al*., 2023. Alterations of plasma biochemical and immunological parameters and spatiotemporal expression of TLR2 and TLR9 in gibel carp (*Carassius auratus gibelio*) after CyHV-2 infection. Pathogens 12(11):1329.

Gherardi G, Imperi M, Baldassarri L, *et al*., 2007. Molecular epidemiology and distribution of serotypes, surface proteins and antibiotic resistance among group B streptococci in Italy. Journal of Clinical Microbiology 45(9):2909-2916.

Guo J, Davis R, Starkey C, *et al*., 2021. Efficacy of various coated materials to prevent nutrient leaching for Pacific white shrimp *Litopenaeus vannamei* commercial diets. Journal of the World Aquaculture Society 52(1):195-203.

Haenen OLM, Dong HT, Hoai TD, *et al*., 2023. Bacterial diseases of tilapia, their zoonotic potential and risk of antimicrobial resistance. Reviews in Aquaculture 15(Suppl.1):154-185.

Han P, Lu Q, Fan LL, *et al*., 2019. A review on the use of microalgae for sustainable aquaculture. Applied Sciences 9(11):2377.

Hassaan MS, El-Sayed AIM, Soltan MA, *et al*., 2019. Partial dietary fish meal replacement with cotton seed meal and supplementation with exogenous protease alters growth, feed performance, hematological indices and associated gene expression markers (GH, IGF-I) for Nile tilapia, *Oreochromis niloticus*. Aquaculture 503:282-292.

Hassaan, MS, Mahmoud SA, Jarmolowicz S, *et al*., 2018. Effects of dietary baker's yeast extract on the growth, blood indices and histology of Nile tilapia (*Oreochromis niloticus* L.) fingerlings. Aquaculture Nutrition 24:1709-1717.

Hernández-Sandoval FE, Del Ángel-Rodríguez JA, Núñez-Vázquez EJ, *et al*., 2022. Effects on cell growth, lipid and biochemical composition of *Thalassiosira weissflogii* (Bacillariophyceae) cultured under two Nitrogen sources. Applied Sciences 12(3):961.

Huervana FH, Dionela CS, de la Torre EDS, *et al*., 2022. Utilization of marine diatom *Thalassiosira weissflogii* as a feed additive in seawater-tolerant Nile tilapia (*Oreochromis niloticus*, Linnaeus 1758) strain. Frontiers in Sustainable Food Systems Sustain. 6:1052951.

Imperi M, Pataracchia M, Alfarone G, *et al*., 2010. A multiplex PCR assay for the direct identification of the capsular type (Ia to IX) of *Streptococcus agalactiae*. Journal of Microbiological Methods 80(2):212-214.

Khalafalaa M, Shehab SM, Aboraya MH, *et al*., 2025. Herbal essential oils improve growth, antioxidant response and gene expression in Nile tilapia fingerlings. Frontiers in Veterinary Science 12:1620632.

Khalil K, Elayat M, Khalifa E, *et al*., 2017. Generation of *Myostatin* gene-edited channel catfish (*Ictalurus punctatus*) via zygote injection of CRISPR/Cas9 system. Scientific Reports 7:7301.

Khunrang T, Pooljun C and Wuthisuthimethavee S, 2023. Correlation of *Streptococcus agalactiae* concentration on immune system and effective dose of inactivated vaccine for Chitralada 3 strain Nile tilapia (*Oreochromis niloticus*) in Thailand. BMC Veterinary Research 19(1):267.

Koch JFA, de Oliveira CAF and Zanuzzo FS, 2021. Dietary β-glucan (MacroGard®) improves innate immune responses and disease resistance in Nile tilapia regardless of the administration period. Fish & Shellfish Immunology 112:56-63.

Lancefield RC, 1934. A serological differentiation of specific types of bovine hemolytic streptococci (group B). Journal of Experimental Medicine59(4):441-458.

Le Xuan C, Vu Linh N, Wannavijit S, *et al*., 2024. Enhancing growth, immunity and gene expression in Nile Tilapia (*Oreochromis niloticus*) through dietary supplementation with avocado (*Persea americana*) seed powder. Aquaculture Reports 39: 102432.

Liang M, Xu Z, Long L, *et al*., 2026. Enhanced muscle growth via DNA methylation editing of mstnb exon in zebrafish. Aquaculture and Fisheries 11(5):893-899.

Livak KJ and Schmittgen TD, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25(4):402-408.

Lumsangkul C, Linh NV, Chaiwan F, *et al*., 2022. Dietary treatment of Nile tilapia (*Oreochromis niloticus*) with aquatic fern *(Azolla caroliniana)* improves growth performance, immunological response and disease resistance against *Streptococcus agalactiae* cultured in bio-floc system. Aquaculture Reports 24:101114.

Madyod S, Pholmai S, Prombanchong T, *et al*., 2026. Dietary β-glucans from the marine diatom *Chaetoceros muelleri* enhances immune responses and resistance of Nile tilapia to Tilapia Lake virus infection. Journal of Fish Diseases 49(9): e70184.

Martinez G, Harel J and Gottschalk M, 2001. Specific detection by PCR of Streptococcus agalactiae in milk. Canadian Journal of Veterinary Research 65(1):68-72.

Oniam V, Arkronrat W and Mohamed NB, 2015. Effect of feeding frequency and various shelter of blue swimming crab larvae, *Portunus pelagicus* (Linnaeus, 1758). Songkhlanakarin Journal of Science and Technology 37(2):129-134.

Opiyo MA, Jumbe J, Ngugi CC, *et al*., 2019. Dietary administration of probiotics modulates non-specific immunity and gut microbiota of Nile tilapia (*Oreochromis niloticus*) cultured in low input ponds. International Journal of Veterinary Science and Medicine 7(1):1-9.

Pooljun C, Jariyapong P, Wongtawan T, *et al*., 2022. Effect of feeding different types of β-glucans derived from two marine diatoms (*Chaetoceros muelleri and Thalassiosira weissflogii*) on growth performance and immunity of banana shrimp (*Penaeus merguiensis*). Fish & Shellfish Immunology 130:512-519.

Pooljun C, Jariyapong P, Laksana-Aut P, *et al*., 2025. Enhancing Pacific white shrimp immunity against *Vibrio parahaemolyticus* through β-glucans supplementation from marine diatoms. Veterinary World 18(4):1047-1058.

Qiang J, Yang H, Wang H, *et al*., 2012. Interactive effects of temperature-dietary protein level on somatotropic gene expression and its interrelationship with growth in juvenile GIFT tilapia *Oreochromis niloticus.* Aquaculture 364-365:263-271.

Reed LJ and Muench H, 1938. A simple method of estimating fifty percent endpoints. American Journal of Hygiene 27(3):493-497.

Sharawy ZZ, Ashour, M, Abbas, E, *et al*., 2020. Effects of dietary marine microalgae, *Tetraselmis suecica*, on production, gene expression, protein markers and bacterial count of Pacific white shrimp *Litopenaeus vannamei*. Aquaculture Research 51:2216-2228.

Slotved HC, Kong F, Lambertsen L, *et al*., 2007. A proposed new *Streptococcus agalactiae* serotype, serotype IX. Journal of Clinical Microbiology 45(9):2929-2936.

Soliman RE, Ateya A, Abou-El-Atta M, *et al*., 2025. Microalgae *Pediastrum boryanum* enhances growth and modulates physiology and transcriptome in Nile tilapia. Scientific Reports 15(1):21713.

Southgate PC, Braley RD and Milita T. 2017. Ingestion and digestion of micro-algae concentrates by veliger larvae of the giant clam, *Tridacna noae*. Aquaculture 473:443-448.

Vallejos-Vidal E, Reyes-Lopez F, Teles M, *et al*., 2016. The response of fish to immunostimulant diets. Fish & Shellfish Immunology 56:34-69.

Van Doan H, Wangkahart E, Thaimuangphol W, *et al*., 2023. Effects of *Bacillus* spp. mixture on growth, immune responses, expression of immune-related genes and resistance of Nile tilapia against *Streptococcus agalactiae* infection. Probiotics and Antimicrobial Proteins 15:363-378.

Wang JL, Meng XL, Lu RH, *et al*., 2015. Effects of *Rehmannia glutinosa* on growth performance, immunological parameters and disease resistance to *Aeromonas hydrophila* in common carp (*Cyprinus carpio* L.). Aquaculture 435:293-300.

Wang E, Wang K, Chen, D, *et al*., 2015. Evaluation and selection of appropriate reference genes for real-time quantitative PCR analysis of gene expression in Nile Tilapia (*Oreochromis niloticus*) during Vaccination and Infection. International Journal of Molecular Sciences 16(5):9998-10015.

Won S, Hamidoghli A, Choi W, *et al*., 2020. Effects of *Bacillus subtilis* WB60 and *Lactococcus lactis* on Growth, Immune Responses, Histology and Gene Expression in Nile Tilapia, *Oreochromis niloticus*. Microorganisms 8(1):67.

Wongsathein D, Raksri S, Urit T, *et al*., 2019. Experimental *Streptococcus agalactiae* infection in Nile tilapia (*Oreochromis niloticus*) via different routes. Veterinary Integrative Sciences 17(3):233-243.

Wu Y, Wu T, Yang L, *et al*., 2023. Generation of fast growth Nile tilapia (*Oreochromis niloticus*) by myostatin gene mutation. Aquaculture 562:738762.

Wu Y, Zhai Y, Li X, *et al*., 2026. Effects of mixed microalgae on larval metamorphosis of the Pacific white shrimp *Penaeus vannamei*: Integrated analysis of digestion, antioxidant capacity and transcriptome. Aquaculture 612:743136.

Wuthisuthimethavee S, Khunrang T, Pooljun C, *et al*., 2025. Immunoglobulin gene expression as a biomarker for Streptococcosis resistance in Nile tilapia (*Oreochromis niloticus*). Fish & Shellfish Immunology 159:110199.

Ye X, Li J, Lu M, *et al*., 2011. Identification and molecular typing of *Streptococcus agalactiae* isolated from pond-cultured tilapia in China. Fisheries Science 77:623-632.

Zlaugotne B, Pubule J and Blumberga D, 2022. Advantages and disadvantages of using more sustainable ingredients in fish feed. Heliyon 8(9):e10527.