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RESEARCH

Effects of dietary *Hericium erinaceus* extract on growth, nutrient utilization, hematology, expression of genes related immunity response, and disease resistance of Nile tilapia (*Oreochromis niloticus*)

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Abstract In recent years, there has been a growing focus on using herbal extracts as immune enhancers for aquatic species, replacing antibiotics. In the present study, the effects of dietary supplementation of *Hericium erinaceus* extract (HE) on growth, feed utilization, hematology, expression of immunity-related genes, and immune responses in Nile tilapia infected by *Streptococcus agalactiae* were examined. A total of 240 Nile tilapia with an average body weight of 17.28 ± 0.01 g were fed diets enriched with different levels of HE: 0 (HE0), 0.1 (HE0.1), 1.0 (HE1.0), and 5.0 (HE5.0) g/kg. The results showed that growth parameters, feed conversion ratio, and organosomatic indexes were not linearly or quadratically affected by HE supplementation. Fish fed HE0.1 and HE1.0 increased protein efficiency ratio and protein productive values with significant linear and quadratic effects of HE enrichment. In addition, dietary supplementation of HE quadratically increased whole-body protein content. Red blood cell, white blood

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cell, and hematocrit were linearly and quadratically increased by HE supplementation. HE also linearly and quadratically decreased LDL cholesterol and linearly decreased the total cholesterol levels. Stress markers, serum glucose, and cortisol levels were linearly and/or quadratically decreased in HE-fed fish. The relative mRNA expression of *tnf-α*, *il-1β*, *il-6*, and *il-10* were upregulated in the HE0.1 and HE1.0 groups, while dietary supplementation of HE significantly decreased *hsp70cb1* mRNA expression in all groups. After feeding dietary HE supplementation for 10 weeks, fish were intraperitoneally injected with pathogenic *S. agalactiae*. A high survival after challenge was found in all HE supplementation groups with the highest percent survival observed in the HE1.0 and HE5.0 groups. Our findings represent that supplementation of 1 g/kg of HE (HE1.0) could obtain the greatest effects on immunity and survival of Nile tilapia. In addition, the present study also showed that dietary supplementation of HE can improve protein utilization, hematology, expression of genes related to immunity, stress markers, and resistance of Nile tilapia against pathogenic bacterial infection.

Keywords Medical mushroom · Nile tilapia · Immunity · *Streptococcus agalactiae* · Stress

Introduction

The world's population is predicted to continue to increase up to 9.7 billion by 2050, leading to a significant increase in food consumption. Aquaculture activities have produced animal protein with expansion faster than other animal protein sectors for over two decades (FAO 2020, 2021). Intensive aquaculture has contributed to the development of aquaculture, but this system often exposes fish to stressful circumstances that stunt growth and make them more susceptible to diseases (Ciji et al. 2021; Dawood et al. 2018). However, the continued use of conventional antibiotics in aquaculture was constrained by a number of negative consequences of antibiotic residuals in the aquaculture products and their negative impacts on ecology (Liu et al. 2017). To enhance animal welfare and to alleviate stress-related economic loss, effective methods must be used to relieve fish from stress under intensive culture conditions (Dawood et al. 2020a, b; Lumsangkul et al. 2022). To protect fish from pathogenic illnesses, numerous techniques and preventative measures have been created, but only a small number of them have been successfully used. Dietary supplementation of immunostimulants is one such successful methods that strengthen disease tolerance and well-being of aquatic animals (Bilen et al. 2021; Ching et al. 2021; Dawood et al. 2020a, b; Khieokhajonkhet et al. 2023). Recently, many herbal plants, mushrooms, and their compounds have been used as immunostimulants for aquaculture due to their inexpensive, viable, sustainable, and eco-friendly nature.

Mushrooms have long been consumed as a nutritious diet and feed supplementation in aqua-feed due to their anti-inflammation, antioxidant, and immunomodulatory capacities (Liu et al. 2015; Prabawati et al. 2022; Wei et al. 2018). Medicinal mushrooms are rich in numerous bioactive compounds such as polysaccharides, mannans, hemicellulose, and β-glucan which generally augment innate and adaptive immunity, growth, and digestibility in aquatic species (Elumalai et al. 2020). Recent reports utilize several parts of mushroom (e.g., stalk waste, mycelia, and spent mushroom substrate) as a functional feed supplementation (Ching et al. 2021; Friedman 2015; Van Doan et al. 2017). Various physiological functions of mushrooms have been reported including facilitating nutrient absorption and digestion in fish and shrimp (Prabawati et al. 2022; Safari and Sarkheil 2018), improving growth and feed utilization by upregulation of gene-related digestive enzymes in Nile tilapia (Yilmaz et al. 2023), stimulating antioxidant capacity in hybrid tilapia (Wan-Mohtar et al. 2021), modulating intestinal immunity in a common carp (Hoseinifar et al. 2019), and exerting anti-inflammatory responses of *Ctenopharyngodon idella* (Hoseinifar et al. 2020). Several mushroom extracts and powders also stimulated immune status and antipathogenic virulent bacteria in fish (Gou et al. 2018; Harikrishnan et al. 2011a, b; Kim et al. 2012).

Hericium erinaceus, known as the lion's mane mushroom, belongs to the Hericiaceae family, and has widely been used in the cuisine and traditional medicine in China and Asian countries since its earliest history (Khan et al. 2013). *H. erinaceus* is a good source of protein, fat, cellulose, fibers, vitamins, minerals, and polysaccharides (Hou et al. 2022). In addition, *H. erinaceus* is a plentiful source of bioactive compounds

including tocopherol, vitamin C, sterol, phenolic compounds, antioxidant compounds, polysaccharides, and β-actin (Heleno et al. 2015; Wong et al. 2009; Yan et al. 2018). The polysaccharide made up of more than 10 monosaccharides is the primary active constituent of *H. erinaceus* present in mycelium broth and culture as well as in the fruiting body (Friedman 2015; He et al. 2017; Wang et al. 2014). Specifically, more than 20 and 12 derivatives of erinacine and hericenones are contained in *H. erinaceus*, which process medical and various biological activities such as nerved cell synthesis, hepatoprotective, antioxidative, antimicrobial, anti-inflammation, anti-tumor, and anti-cancer (Friedman 2015; Kuo et al. 2017). A significant body of research papers has illuminated the beneficial applications of *H. erinaceus* mushrooms in aquatic species. For example, the application of *H. erinaceus* powder demonstrated a notable enhancement in the immune response and disease resistance in Nile tilapia (Khieokhajonkhet et al. 2022), as well as in white shrimp (*Litopenaeus vannamei*) (Yeh et al. 2011). Currently, there exists two documented report HE utilization, ranging from 1×10^{-4} to 10 g/kg, as a feed additive for olive flounder (*Paralichthys olivaceus*) (Harikrishnan et al. 2011a, b) and zebra fish (*Danio rerio*) (Paola et al. 2021).

Nile tilapia (*Oreochromis niloticus*) is regarded as the world's second most cultured fish species that greatly contributes to global food security (FAO 2021). In the intensive aquaculture systems of Nile tilapia, bacterial, viral, and parasitic infections have hampered the cultivation of tilapia, exerting a negative impact on Nile tilapia production. Among these pathogenic microorganisms, *Streptococcus agalactiae* is frequently regarded as a serious virulent bacterial pathogen that causes a high mortality rate and generates significant economic losses on a global scale (Wang et al. 2020). Strategies to increase Nile tilapia production have become crucial as a result of the tremendous growth in its production. To our knowledge, there is presently no documented instance of utilizing dietary supplementation with HE as an immunostimulant for Nile tilapia, a significant species in the aquaculture. Moreover, only a restricted number of studies have chosen extraction methods in this context. The aim of the present study is, therefore, to investigate the effect of dietary HE on growth, feed utilization, hematology, expression of gene-related immunity, and stress markers in Nile tilapia. Furthermore, to confirm the potential of HE to enhance fish disease resistance, the *S. agalactiae* challenge experiment was also conducted.

Materials and methods

Hericium erinaceus extract preparation

The mushroom, *H. erinaceus*, was kindly provided from The Organic Sentang Hed mushroom farm in Phitsanulok, Thailand. Collected mushroom samples were washed with tap water and proceeded as previously done in our laboratory (Khieokhajonkhet et al. 2022). Briefly, the fresh mushroom was transversely cut with approximately 2–3 cm long and dried at 50 °C overnight. Dry mushroom sample was ground and sieved pass through a 40-mesh sieve (~ 0.42 mm) and stored in plastic bags at − 20 °C until used. Thereafter, dried mushroom powder of approximately 100 g was extracted under boiling water in 1000 mL of distilled water for 2 h in a water bath under continuous agitation. HE was then cooled down at room temperature and centrifuged at 10,000 × *g* for 10 min. The supernatant was subjected to evaporation and lyophilization using a Ratavapor R-210 Buchi Labortechnik rotary evaporation (Flawil, Switzerland) and Christ Beta 2–8 LD plus freeze dry (Martin Christ, Germany), respectively. The crude extract of the mushroom was stored at − 20 °C until used for feed formulation.

Experimental diets

All four experimental diets were designed by following the principles of isonitrogenous (300 g/kg crude protein) and isolipidic (70 g/kg crude lipid) diets. The basal group without HE supplementation, referred to as HE0, was used as a control diet. Since wheat flour contains the lowest crude protein and fat content, dietary HE supplementation was formulated by replacing 0.1, 1.0, and 5.0 g/kg of wheat flour with HE, and these experimental diets corresponded to HE0.1, HE1.0, and HE5.0, respectively (Table 1). The basal feed ingredients were purchased from a commercial feed distributor (Phraepan, Co, Ltd., Phitsanulok, Thailand).

Table 1 Feed formulation and proximate composition of the experimental diets (dry matter)

Items	HE0	HE0.1	HE1.0	HE5.0
Feed formula (g/kg)				
Fish meal	150	150	150	150
Soybean meal	350	350	350	350
Corn meal	185	185	185	185
Broken rice	77	77	77	77
Rice flour	30	30	30	30
Squid meal	40	40	40	40
Wheat flour	80	79.9	79	75
Hericium erinaceus extract	0	0.1	1	5
Soybean oil	50	50	50	50
Lysine	3	3	3	3
Methionine	5	5	5	5
Vitamin-mix[1]	10	10	10	10
Mineral-mix[2]	10	10	10	10
Vitamin C	5	5	5	5
Lecithin	5	5	5	5
Total	1000	1000	1000	1000
Composition (g/kg)				
Crude protein	299.1	305.2	309.7	306.5
Crude fat	80.1	75.6	79.4	79.0
Fiber	39.3	38.3	37.4	41.1
Ash	25.3	26.8	24.6	23.1
Dry matter	924.6	936.8	924.5	921.2
Gross energy (MJ kg^{-1})[3]	17.13	17.19	17.56	17.38

Annotations: Crude protein content of feed ingredients (%): fish meal, 54.0; soybean meal, 42.1; corn meal, 7.4; broken rice, 7.1; squid meal, 45.7; wheat flour, 5.0; Crude fat content of feed ingredients (%): fish meal, 8.8; soybean meal, 1.2; corn meal, 1.7; broken rice, 0.9; squid meal, 4.5; wheat flour, 0.2

[1]Vitamin-mix, Sun-Mix, Munkong Interfood, Co., Ltd., Nakorn Pratom, Thailand

[2]Mineral-mix, Minmin, Munkong Interfood, Co., Ltd., Nakorn Pratom, Thailand

[3]Gross energy value was calculated on the basis of combustion values of 23.6 kJ/g protein, 39.5 kJ/g lipid, and 17.2 kJ/g carbohydrate (NRC 2011)

All feed ingredients were sieved pass through the 40-mesh sieve and homogenized by following the target formulations in Table 1 using a kitchen mixer with a 20-kg capacity (C-B20G-A1, CKI Family, Nonthaburi, Thailand). After homogenization, soybean oil was added, and the mixture was blended for another 5 min for each inclusion. To form a dough, 35% of distilled water (v/w) was also added followed by the final mixing for 10 min. Diets were assembled using a meat mincer (ICK family Co. Ltd., Nonthaburi, Thailand) to obtain a pellet with a size approximately 2.0-mm diameter and cut into 2–3 mm long. Obtained feed pellets were then air-dried at 50 °C overnight and kept in airtight polyethylene bags at − 20 °C until used for chemical analysis and feeding trial.

Experimental conditions

Nile tilapia (total 400 fish, approximately 10.92 ± 0.57 g/fish) was purchased from a local hatchery and transported to The Aquatic Animal Feed Laboratory, Faculty of Agriculture Natural Resources and Environment, Naresuan University, Phitsanulok, Thailand. Fish were acclimatized in the experimental rearing system in the 500-L plastic tank capacity. Fish were fed twice daily until apparent satiation using a herbivorous commercial diet (320 g/kg crude protein, 40 g/kg crude lipid, and 40 g/kg crude fiber, NB distribution Co, Ltd., Ratchaburi, Thailand) for 3 weeks. Fish with an overall initial body weight (IBW, 17.28 ± 0.01 g/fish) were bulk weighed and randomly allocated at a density of 20 fish in each tank into 12 glass tanks with a capacity of 150 L (0.45 × 0.45 × 0.90 m), each of which was filled with 120 L of dechlorinated water under natural lightness and darkness (approximately 12-h lightness: 12-h darkness). Nile tilapia was fed on experimental diets ad libitum twice daily (8.30 and 17.30) for 10 weeks. Each tank was equipped with two submerged airstones with continuous aeration. Every morning, feces and debris were siphoned to maintain consistent cleanliness and hygiene in the experimental setup. Additionally, approximately two-thirds of the water volume in each tank were exchanged and replenished with dechlorinated water. Water quality was determined daily during the feeding trial; the pH range was 7.0–8.2; the temperature was 26.9–28.2 °C; and the dissolved oxygen was higher than 5.3 mg/L. These water quality parameters were measured using a digital pH meter (Mettler Toledo, USA) and a portable digital probe meter (Mettler Toledo, Ulm, Germany). All procedures were conducted in accordance with the animal ethics guidelines established by Naresuan University Animal Care and Use Committee (NUACUC), Center for Animal Research, Naresuan

University, Phitsanulok, Thailand, which permitted for the collection, handling, and rearing conditions of Nile tilapia.

Pathogenic bacterial challenge

S. agalactiae was kindly provided by the Fish Disease Laboratory, Phayao University (Phayao, Thailand). The pathogenic bacteria were prepared under hygienic guidelines following the previous description from our laboratory (Khieokhajonkhet et al. 2022). The final concentration of *S. agalactiae* used in the present study was 1.5×10^8 CFU/mL. At the beginning of the bacterial challenge, ten fish were randomly selected and injected with 0.1 mL of *S. agalactiae* in the intraperitoneal cavity. Injected fish were transferred to plastic tanks ($0.8 \times 0.4 \times 0.4$ m^3 containing 90 L). The number of dead fish was recorded and used to determine mortality and the relative percent of survival as described previously (Khieokhajonkhet et al. 2022).

Sample collection

Growth and feed utilization

At the end of the feeding trial, fish were fasted for 24 h and anesthetized with 30 mg/L clove oil solution (ethanol/clove oil, 9:1). Fish of each tank were bulk weighed to obtain the final body weight (FBW). Growth, feed utilization, and survival parameters were calculated by following formulas: Weight gain rate (WGR, %) = 100 × [(FBW − IBW)] / IBW; Specific growth rate (SGR, %/day) = 100 × [(Ln FBW − Ln IBW)] / days; Survival (%) = 100 × [(final survived fish number) / (initial fish number)]; Feed conversion ratio (FCR) = feed intake/weight gain; Protein efficiency ratio (PER) = wet weight gain / protein intake; Protein productive value (PPV, %) = (protein gain/ protein intake) × 100. Two fish from each tank ($n = 6$) were randomly selected to determine the individual weight (g) and total length (cm) to calculate the condition factor (K-value). Fish samples were then euthanized with an overdose of clove oil solution to dissect internal organs including the liver and visceral organs under hygienic conditions. These organs were then individually weighed to calculate the hepatosomatic index (HSI) and viscerosomatic index (VSI). Organosomatic indexes were calculated by the following formulas: K value g/cm^3 was calculated as (FBW / body length^3) × 100; HSI % and VSI % = (organ weight / FBW) × 100.

Chemical scrutiny

Gross energy content in the experimental diet was calculated according to the combustion values (NRC 2011). All experimental ingredients, diets, and initial and whole-body (two fish per tank, pooled sample, $n = 3$) fish samples were used to determine proximate composition according to the standard procedures (AOAC 1990). Dry matter content was determined after drying at 105 °C until constant weight. Ash content was analyzed according to the combustion method using a combustion in a muffle furnace (Carbolite ELF 11/14, Hope Valley, England) at 550 °C for 6 h. Crude protein content was determined by following the Kjeldahl method ($N \times 6.25$) using a semi-automatic Kjeldahl, Gerhardt Vapodest, 45 s (Königswinter, Germany). The crude lipid was analyzed with petroleum ether using the Soxhlet apparatus (Gerhardt, Germany). Crude fiber content was determined according to Yasumaru and Lemos (2014) using acid (H_2SO_4) and base (NaOH) digestions and incineration at 525 °C for 5 h using a muffle furnace.

Hematological and biochemical determination

Two fish from each tank were randomly sampled (pooled sample, $n = 3$) and anesthetized. Blood samples were hygienically collected from the caudal vein using a 1-mL syringe containing anticoagulation (10% EDTA). The collected blood samples were used to determine red blood cell (RBC, $\times 10^6$ cell/μL) and white blood cell (WBC, $\times 10^3$ cell/μL) using a Neubauer hemocytometer (Houston 1990). Hemoglobin (Hb, g/dL) content was determined according to Blaxhall and Daisley (1973) using a 540-nm spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). Hematocrit (Hct, %) was analyzed using DM1424 (DLAB, Beijing, China) by following the micro-hematocrit method. Blood glucose levels (mmol/L) were determined using the Accu-Chek Active Glucometer (Roche, Mannheim, Germany). Another two fish were also randomly collected to withdraw blood samples using no anticoagulations. Blood samples were stood on ice for 1 h and subjected to centrifugation at 3000 rpm at 4 °C for 15 min. The supernatant was used

to determine blood biochemistry. All serum biochemistry was determined as previously described in our laboratory by Khieokhajonkhet et al. (2022) using Cobas C311 automated analyzer for chemistry (Roche Diagnostics, Switzerland). Cortisol levels (ng/mL) were analyzed according to Brown et al. (2004) protocol using a Cobas C311 automatic biochemical analyzer, Roche Diagnostics (Switzerland).

RNA extraction and cDNA synthesis

Two individual Nile tilapia per tank were randomly selected ($n=6$ for each treatment), and approximately 2 g of liver tissue was hygienically collected from each fish, followed by storage in RNAlater (Amnion, Cambridgeshire, UK) at − 80 °C until used for total RNAs extraction. Total RNAs were extracted in liquid nitrogen using a pestle and mortar. Obtained fine powder was dissolved in 1 mL of QIAzol lysis reagent (Qiagen, Maryland, USA) and subsequently purified using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation. Purified RNAs were treated with DNase I (Thermo Fisher Scientific, Waltham, USA) to remove the potential genomic DNA contamination. Obtained total RNAs were assessed for purity and integrity using a microplate reader Synergy H1 Multi-Mode Reader (BioTek Instrument Inc., VT, USA) at 260/280 absorbance ratio and gel electrophoresis with 2% agarose gel, respectively. One microgram of total RNAs was reverse-transcribed with a RevertAid First-Strand Synthesis System (Thermo Scientific, Fermentas, USA) following the manufacturer's protocol. The cDNAs were treated with RNase H (Invitrogen) to remove contaminated RNAs for 20 min at 37 °C and stored at − 20 °C until used.

Quantitative real-time polychain reaction

Quantitative real-time PCR was performed with Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific, Lithuania) according to the manufacturer's protocol. All gene-specific primer sequences, tumor necrosis factor-α (*tnf-α*), interleukin-1β (*il-1β*), interleukin-6 (*il-6*), interleukin-10 (*il-10*), and heat shock protein70cB1 (*hsp70cb1*) were used, with *β-actin* used as a reference gene (Table 2). *β-actin* was chosen due to stable expression in Nile tilapia (Dawood et al. 2020a, b; Jian et al. 2023) and its common use across various conditions in several teleost species (Li et al. 2020; Yang et al. 2013). The PCR was performed in triplicate as reported previously (Khieokhajonkhet et al. 2022). The Ct values were determined by the comparative Ct method.

Statistical analysis

All dietary inclusion of HE was assigned by a completely randomized design. Prior to performing statistical analysis, the hypotheses of normality and homogeneity were tested. To determine whether the evaluated parameters were significantly impacted by HE supplementation ($P<0.05$), all data were subjected to an analysis of variance (ANOVA) using the

Table 2 Sequences of primers used in the present study

Genes	Primer sequences (5′-3′)	References
tnf-α	F-GCTGGAGGCCAATAAAATCA	Kayansamruaj et al. 2017
	R-CCTTCGTCAGTCTCCAGCTC	
il-1β	F-AAGATGAATTGTGGAGCTGTGTT	Zhi et al. 2018
	R-AAAAGCATCGACAGTATGTGAAAT	
il-6	F-ACAGAGGAGGCGGAGATG	Niu et al. 2019
	R-GCAGTGCTTCGGGATAGAG	
il-10	F-TGGAGGGCTTCCCCGTCAG	Niu et al. 2019
	R-CTGTCGGCAGAACCGTGTCC	
hsp70cb1	F-CTCCACCCGAATCCCCAAAA	NM_001279671.2
	R-TCGATACCCAGGGACAGAGG	
β-actin	F-TGGTGGGTATGGGTCAGAAAG	XM_003443127
	R-TGTTGGCTTTGGGGTTCA	

Abbreviations: *tnf-α*, tumor necrosis factor-α; *il-1β*, interleukin-1β; *il-6*, interleukin-6; *il-10*, interleukin-10; *hsp70cb1*, heat shock protein70cb1

statistical program SPSS (Version 17, Chicago, IL, USA). Data were then used to determine Dunnett's analysis for testing a significant difference between HE supplementing diets and a reference diet. To determine whether there were linear and/or quadratic effects on the response in the dependent variable to HE levels, orthogonal polynomial contrast was used.

Results

Growth, feed utilization, survival, and organosomatic indexes

During the feeding experiment, Nile tilapia accepted all experimental diets. In addition, the final weight of all diet groups was nearly 10 times higher than the initial body weight, being approximately 162–165 g/fish (Table 3), which indicates that all fish were in good condition. There were no significant linear or quadratic effects of HE on FBW, WGR, SGR, and FCR ($P > 0.05$). In addition, survival of Nile tilapia was 98.33% in all diet groups with no significant differences ($P > 0.05$). Among the feed utilization parameters, PER and PPV were linearly and quadratically increased with increasing dietary supplementation of HE ($P < 0.001$). In addition, dietary supplementation of HE0.1 and HE1.0 resulted in a significantly higher PER and PPV than the control group (Dunnett's test, $P < 0.05$). The organosomatic indexes were not significantly affected ($P > 0.05$) by dietary supplementation of HE (Table 4).

Chemical composition of whole-body Nile *tilapia*

Whole-body composition of Nile tilapia fed dietary supplementation of HE is illustrated in Table 5. Whole-body dry matter, crude lipid, and ash contents were not significantly affected by dietary HE supplementation ($P > 0.05$). However, Nile tilapia fed with HE supplementation showed a higher crude protein content with a quadratic effect ($P = 0.004$). Significant differences in whole-body crude protein were observed in HE0.1 and HE1.0 groups compared to the control group (Dunnett's test, $P < 0.05$).

Table 3 Growth performance and feed utilization of Nile tilapia fed dietary supplementation of *Hericium erinaceus* extract for 10 weeks

Items	HE0	HE0.1	HE1.0	HE5.0	*SEM*	Linear	Quadratic
IBW (g/fish)	17.28 ± 0.02	17.29 ± 0.01	17.28 ± 0.01	17.28 ± 0.02	0.001	0.233	0.282
FBW (g/fish)	162.26 ± 3.92	165.69 ± 5.15	164.19 ± 6.24	164.70 ± 8.32	37.501	0.723	0.691
WGR (%)	839.94 ± 22.58	858.27 ± 29.61	850.36 ± 36.69	853.30 ± 47.64	50.759	0.710	0.699
SGR (%/day)	3.20 ± 0.03	3.23 ± 0.04	3.22 ± 0.06	3.22 ± 0.07	0.003	0.750	0.685
Survival (%)	98.33 ± 2.89	98.33 ± 2.89	98.33 ± 2.89	98.33 ± 2.89	8.333	1.000	1.000
FCR	1.21 ± 0.04	1.26 ± 0.12	1.29 ± 0.02	1.35 ± 0.05	0.004	0.141	0.967
PER	2.50 ± 0.09	2.88 ± 0.06*	2.70 ± 0.07*	2.56 ± 0.06	0.005	< 0.001	< 0.001
PPV (%)	36.49 ± 0.73	38.73 ± 0.44*	37.84 ± 0.69*	36.83 ± 0.31	0.324	< 0.001	< 0.001

The values are the average of the three repetitions (mean ± SEM, $n = 3$ for growth, survival, and feed utilization). Asterisk in each row indicate significant difference of HE treatment groups versus a control group (Dunnett's test, $P < 0.05$)

Table 4 Organosomatic indexes of Nile tilapia fed dietary supplementation of *Hericium erinaceus* extracts for 10 weeks

Organosomatic indexes	HE0	HE0.1	HE1.0	HE5.0	*SEM*	Linear	Quadratic
K-value (g/cm^3)	4.10 ± 0.36	3.93 ± 0.22	4.14 ± 0.34	4.01 ± 0.40	0.117	0.936	0.897
HSI (%)	1.76 ± 0.36	1.54 ± 0.34	1.44 ± 0.21	1.56 ± 0.29	0.097	0.234	0.195
VSI (%)	8.04 ± 0.82	7.99 ± 0.65	8.24 ± 0.64	8.58 ± 0.87	0.570	0.194	0.533

The values are the average of the six repetitions (mean ± SEM, two fish per tanl was used to dertermin, $n = 6$)

Table 5 Whole-body composition of Nile tilapia fed dietary inclusion of *Hericium erinaceus* extract for 10 weeks

Items (g/kg)	HE0	HE0.1	HE1.0	HE5.0	*SEM*	Linear	Quadratic
Dry matter	245.9 ± 0.40	253.6 ± 0.55	251.8 ± 0.23	252.8 ± 0.37	0.167	0.063	0.130
Crude protein	128.1 ± 0.29	138.6 ± 0.51*	139.9 ± 0.71*	131.6 ± 0.47	0.269	0.324	0.004
Crude lipid	70.1 ± 0.14	71.4 ± 0.10	73.7 ± 0.05	73.5 ± 0.06	0.010	0.055	0.335
Ash	39.5 ± 0.03	40.9 ± 0.04	41.2 ± 0.10	41.0 ± 0.12	0.007	0.064	0.160

The values are the average of the three repetitions (mean ± SEM, two fish per tank, pooled sample, $n = 3$). Asterisk in each row indicate significant difference of HE treatment groups versus a control group (Dunnett's test, $P < 0.05$)

Hematology and biological characteristics

Hematological parameters showed significant linear and quadratic effects of HE on RBC, WBC, and Hct (Table 6). RBC, WBC, and Hct in all HE supplementation groups were significantly higher compared to the control group (Dunnett's test, $P < 0.05$). RBC and WBC were found to be the highest in the HE1.0 group, while the Hct level was the highest in the HE0.1 group. Hb levels were not significantly affected ($P > 0.05$) by dietary HE supplementation (Table 6). Dietary supplementation of HE did not linearly and quadratically influence the protein, albumin, AST, ALT, ALP, triglycerides, or HDL-cholesterol levels at the significance level used. On the other hand, cholesterol levels were linearly decreased by dietary HE supplementation ($P = 0.005$), and a significant difference was observed in the HE1.0 and HE5.0 groups compared to the control group (Dunnett's test, $P < 0.05$). LDL-cholesterol levels were linearly and quadratically decreased ($P = 0.010$; $P = 0.002$). All HE-supplemented groups showed a significant decrease in LDL-cholesterol levels compared to the control group (Dunnett's test, $P < 0.05$).

Blood glucose and cortisol levels

Dietary supplementation of HE quadratically decreased glucose levels ($P < 0.001$). The highest glucose level was observed in the control group (HE0), while the HE0.1 and HE1.0 groups showed significantly lower glucose levels than the control group (Table 7, Dunnett's test, $P < 0.05$). A similar tendency was observed

Table 6 Blood hematology and biochemistry of Nile tilapia fed different levels of *Hericium erinaceus* extract for 10 weeks

Items	HE0	HE0.1	HE1.0	HE5.0	*SEM*	Linear	Quadratic
Hematology							
RBC ($\times 10^6$cell/μl)	2.50 ± 0.06	2.67 ± 0.04*	2.75 ± 0.09*	2.68 ± 0.03*	0.003	0.003	0.007
WBC ($\times 10^3$cell/μl)	11.69 ± 1.18	15.73 ± 1.52*	16.92 ± 1.06*	16.24 ± 0.55*	1.091	0.003	0.007
Hct (%)	35.37 ± 0.51	37.74 ± 0.81*	37.45 ± 0.50*	37.23 ± 0.53*	0.363	0.010	0.006
Hb (g/dL)	13.23 ± 0.75	14.17 ± 0.46	14.12 ± 0.79	13.96 ± 0.12	0.359	0.204	0.147
Serum biochemistry							
Protein (g/dL)	2.61 ± 0.21	2.66 ± 0.15	2.77 ± 0.05	2.76 ± 0.06	0.045	0.655	0.889
Albumin (g/dL)	1.27 ± 0.05	1.26 ± 0.04	1.30 ± 0.02	1.36 ± 0.05	0.004	0.475	0.656
Globulin (g/dL)	1.34 ± 0.08	1.39 ± 0.13	1.47 ± 0.04	1.39 ± 0.01	0.001	0.969	0.116
AST (U/L)	17.55 ± 0.61	17.56 ± 0.82	18.06 ± 0.08	17.60 ± 0.84	0.439	0.777	0.639
ALT (U/L)	11.85 ± 2.90	12.21 ± 2.54	15.56 ± 0.79	11.56 ± 0.79	4.026	0.715	0.200
ALP (U/L)	17.61 ± 0.86	22.09 ± 4.37	20.56 ± 0.78	24.05 ± 1.35	5.567	0.076	0.780
Cholesterol (mg/dL)	196.18 ± 1.46	191.31 ± 1.65	162.82 ± 7.91*	176.18 ± 5.71*	24.979	0.005	0.061
Triglycerides (mg/dL)	149.77 ± 6.16	131.65 ± 3.61	147.37 ± 2.89	141.70 ± 4.96	20.988	0.590	0.127
HDL-cholesterol (mg/dL)	58.73 ± 0.97	64.16 ± 1.52	64.18 ± 2.78	61.18 ± 1.42	7.761	0.372	0.633
LDL-cholesterol (mg/dL)	108.92 ± 1.67	94.08 ± 4.33*	87.81 ± 2.26*	98.41 ± 0.43*	6.720	0.010	0.002

The values are the average of the three repetitions (mean ± SEM, two fish per tank were pooled, $n = 3$). Asterisk in each row indicate significant difference of HE treatment groups versus a control group (Dunnett's test, $P < 0.05$)

in cortisol levels with significant linear and quadratic effects ($P=0.023$; $P=0.028$), and the HE1.0 group showed a significantly lower cortisol level than the control group (Dunnett's test, $P>0.05$).

Expression of genes related immunity

The oral administration of HE significantly increased the expression of *tnf-α*, *il-1β*, *il-6*, and *il-10* mRNA in the Nile tilapia (Fig. 1A to D, respectively; Dunnett's

Table 7 Blood glucose and cortisol levels of Nile tilapia fed different levels of *Hericium erinaceus* extract for 10 weeks

Items	HE0	HE0.1	HE1.0	HE5.0	*SEM*	Linear	Quadratic
Serum biochemistry							
Glucose (mmol/dL)	36.79 ± 1.26	27.48 ± 3.71*	30.71 ± 2.18*	32.87 ± 1.44	5.573	0.132	< 0.001
Cortisol (mg/mL)	8.84 ± 0.18	7.58 ± 0.65	6.91 ± 0.10*	7.55 ± 0.41	0.159	0.023	0.028

The values are the average of the three repetitions (mean ± SEM, two fish per tank were pooled, $n=3$). Asterisk in each row indicate significant difference of HE treatment groups versus a control group (Dunnett's test, $P<0.05$)

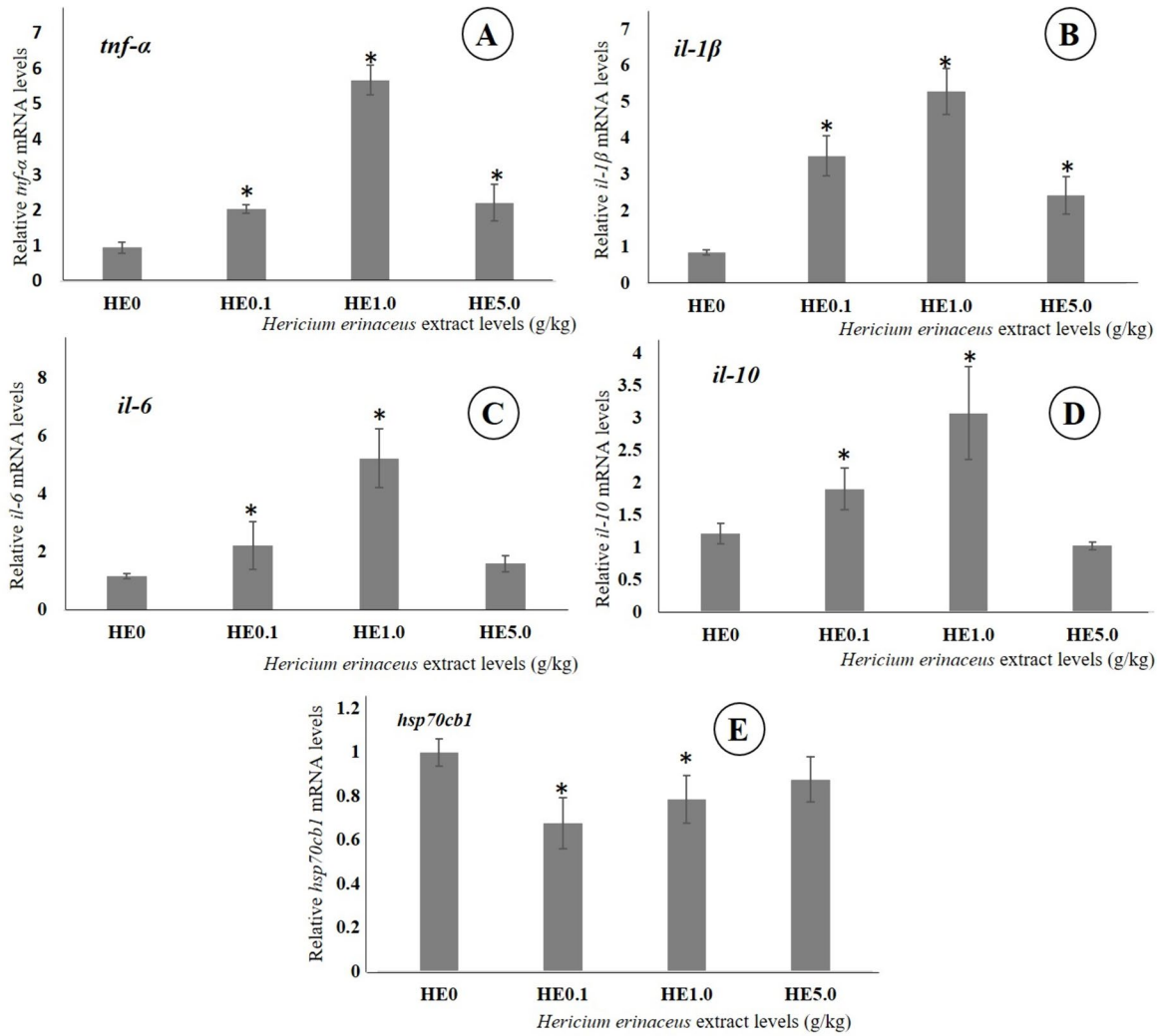


Fig. 1 Relative expression of tumor necrosis factor-α (*tnf-α*), interleukin-1β (*il-1β*), interleukin-6 (*il-6*), interleukin-10(*il-10*), and heat shock protein70cb1 (*hsp70cb1*) genes in the liver of Nile tilapia fed diets supplemented with different HE levels for 10 weeks. Values are presented as the mean ± S.D., and different letters denote the significant difference between treatments ($P<0.05$)

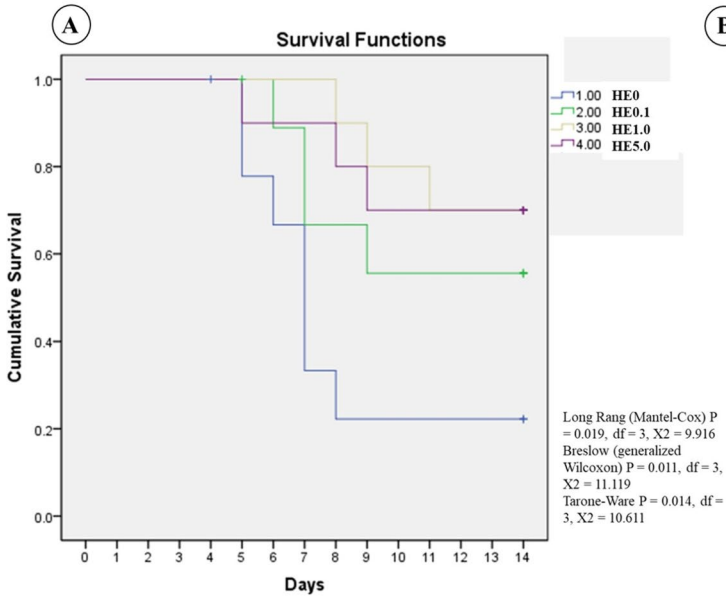
test, $P < 0.05$), and their expression levels were highest in the HE1.0 group. The highest expression of *hsp70cb1* mRNA was observed in the HE0 (control) group, followed by the HE5.0, HE1.0, and HE0.1 groups (Fig. 1E).

Streptococcus agalactiae challenge test

After feeding with dietary supplementation of HE for 10 weeks, Nile tilapia were intraperitoneally infected with *S. agalactiae*. The initial mortality of Nile tilapia was observed on day 5 in the control and HE5.0 groups, and higher mortality was observed in all groups thereafter except for the HE0.1 and HE1.0 groups (Fig. 2A). Meanwhile, the survival curves tended to stabilize after day 8 for the control group, day 9 for HE0.1 and HE5.0 groups, and day 11 for HE1.0 group. At the termination day, the cumulative survival of the dietary HE supplementation groups was higher than the control group (Fig. 2A). The higher relative percent of survival (RPS) was recorded in HE1.0 and HE5.0 (62.5%), followed by HE0.1 (50.0%) group (Fig. 2B).

Discussion

In aquaculture, functional feed ingredients represent a promising future. The immune system, particularly non-specific defense mechanisms, can be enhanced by the use of herbal immunostimulants as feed additives, exerting positive effects on growth, metabolism, and disease resistance (Barton 2002). In the present study, dietary supplementation of HE tended to increase in growth performance, although the difference did not reach the statistical significance ($P > 0.05$). These results are in agreement with previous studies in Nile tilapia fed with oyster mushroom (*Pleurotus pulmonarius*) stalk waste extract (Ching et al. 2021), rainbow trout fed with Artist's conk mushroom (*Ganoderma applanatum*) extract (Manayi et al. 2016), and yellowtail (*Seriola quinqueradiata*) fed with *Flammulina velutipes* mushroom extract (Bao et al. 2009). In the present study, dietary supplementation of HE linearly and quadratically increased PER and PPV ($P < 0.05$), suggesting that dietary supplementation of mushrooms improves protein utilization and its turnover in Nile tilapia (Lee et al. 2014). Some studies suggested that dietary supplementation of mushroom



B

Survival and relative percent survival (RPS, %)

Treatments	Survival (%)	RPS (%)
HE0	30.0	-
HE0.1	60.0	50.0
HE1.0	70.0	62.5
HE5.0	70.0	62.5

Fig. 2 Percent survival of Nile tilapia fed dietary supplementation of *Hericium erinaceus* extract after being challenged with *Streptococcus agalactiae* for 14 days. A Kaplan–Meier survival curve analysis of Nile tilapia after challenged with *Streptococcus agalactiae* for 14 days. B Survival (%) and relative percent survival (RPS, %) of Nile tilapia after challenged with *Streptococcus agalactiae* for 14 days

powder or its extract contains a high level of β-glucan that could promote growth and nutrient utilization in fish (Ahmed et al. 2017a; Ai et al. 2007).

The somatic or morphological indexes, K-value, HSI, and VSI, are used as bioindicators to determine individual nutritional and physiological conditions. In the present study, dietary supplementation of HE did not significantly affect K-value, HSI, or VSI in Nile tilapia. Katya et al. (2016) also found that dietary supplementation of fermented oyster mushrooms did not affect HSI in Amur catfish. Similarly, Nile tilapia fed HE powder also showed no significant difference in K-value, HSI, and VSI (Khieokhajonkhet et al. 2022). Previous studies showed that dietary inclusion of mushroom extracts significantly lowered organo-somatic indexes in many fish species (Ahmed et al. 2017a, 2017b; Pascual et al. 2017; Wan-Mohtar et al. 2021), possibly reflecting the diversity of mushroom and target fish species.

In the present study, whole-body dry matter, crude lipid, and ash contents were not significantly affected by dietary HE supplementation, but supplementation of HE quadratically increased crude protein content in the whole body. These results suggested that Nile tilapia could well utilize dietary protein more effectively, increasing the whole-body protein deposition after being administrated with HE. Supportive evidence was observed in the increased protein utilization parameters (PER and PPV). In accordance with these results, previous studies showed that supplementation of mushrooms significantly increased whole-body protein content in Amur catfish (*Silurus asotus*), rainbow trout, and Nile tilapia (Katya et al. 2016; Khieokhajonkhet et al. 2022; Pascual et al. 2017).

Monitoring fish health and nutritional metabolism using hematological markers is a valuable practice. It serves as an important tool for diagnosing disorders, determining nutrient levels, and assessing both hygienic conditions and overall fish health. RBCs, Hb, and Hct have been precisely identified as hematological markers that reflect the erythrocyte state and the oxygen-carrying capacity in fish (Houston 1990). It has been found that supplementing mushrooms to feed has a favorable impact on the hematological profiles of various fish species (Dawood et al. 2020a, b; Khieokhajonkhet et al. 2022). In the present study, dietary supplementation of HE linearly and quadratically increased RBC and Hct ($P < 0.05$) and slightly increased Hb. In addition, WBC also linearly and quadratically increased with supplementation of HE. White blood cell levels are regarded as a major indicator of fish immunological response (De Pedro et al. 2005). These results are consistent with previous studies on fish fed dietary supplementation of mushrooms, such as dietary supplementation of *Agaricus bisporus* powder (Harikrishnan et al. 2018), *Agaricus bisporus* polysaccharide extract (Harikrishnan et al. 2021), white button mushroom powder (Dawood et al. 2020a, b), *H. erinaceus* powder (Khieokhajonkhet et al. 2022), and *Pleurotus eryngii* powder (Safari & Sarkheil 2018). The results of the present study indicate that the dietary inclusion of HE could support non-specific immunity activation in Nile tilapia.

The general state of health and physiological stress in fish can be assessed by determining the serum biochemical parameters (Houston 1990). In the present study, serum protein, albumin, globulin, AST, ALT, ALP, HDL-cholesterol, and triglyceride levels were not significantly affected by dietary HE supplementation. Meanwhile, cholesterol and LDL-cholesterol levels decreased with increasing HE supplementation levels ($P < 0.05$). Previous studies also found a decrease in triglycerides, cholesterol, and LDL-cholesterol levels in Nile tilapia fed *H. erinaceus* powder (Khieokhajonkhet et al. 2022). These results suggest that dietary supplementation of HE does not fundamentally alter metabolic function and health, but improves lipid metabolism in Nile tilapia.

One of the objectives of supplementing feed additives to aquaculture feed is to enhance innate and adaptive immune responses (Dawood et al. 2018). Interleukins (*ils*) are the first group of cytokines that leucocytes express to control innate and adaptive immunological reactions in fish (Secombes 1996; Secombes et al. 2011). The *ils* have both pro- and anti-inflammatory effect properties in fish. *il-1β* and *tnf* play important roles in the modulation of innate immune responses and body responses against toxins and microbial agents (Yuan et al. 2008; Zou and Secombes 2016). In the early phase of infection, *tnf-α* is expressed and triggers the other cytokines genes related to inflammation including *il-1β*, *il-8*, and *tnf-α*, as well as genes related to antimicrobial responses (Roca and Ramakrishnan 2013; Zou and Secombes

2016). By activating target genes involved in cell growth, differentiation, apoptosis, and proliferation, *il-6* plays a crucial role in cell and tissue homeostasis and physiological functions (Hodge et al. 2005). In this study, we found that dietary supplementation of HE significantly upregulated *tnf-α*, *il-1β*, *il-6*, and *il-10* mRNA expression in all three feeding doses of HE with the highest level observed in the HE1.0 group. Recent studies also found upregulation of these genes in Nile tilapia after fed *H. erinaceus* powder (Khieokhajonkhet et al. 2022) and only *il-1β* and *tnf-α* genes in grass carp (*Ctenopharyngodon idella*) fed with *H. caput-medusae* polysaccharide extract (Gou et al. 2018). These results revealed an augmentation of fish resistance against pathogenic microbial infection (Gou et al. 2018; Khieokhajonkhet et al. 2022). *il-10* is an anti-inflammatory cytokine that strongly regulates inflammation responses by inhibiting the production of anti-inflammatory cytokines and activation of macrophages (Fiorentino et al. 1991; Zou and Secombes 2016). Previous studies also showed an upregulation of pro- and anti-inflammation cytokine genes in fish fed with herbal immunostimulants such as laurel-leaf cistus (*Cistus laurifolius*) extract (Bilen et al. 2021), dandelion (*Taraxacum officinale*) flower extract (Hosseini et al. 2021), and *Ginkgo biloba* leaf extract (Abdel-Latif et al. 2021). Together, these findings suggest that dietary supplementation of HE might boost expression of gene-related immunity and cytokine genes that potentially contribute to the survival in Nile tilapia.

Pathogenic bacteria are the most common pathogens in the intensive aquaculture system. *S. agalactiae* is a common pathogen that causes infectious diseases in farmed tilapia, resulting in high mortality, low flesh quality output, and significant economic losses (Amal and Zamri-Saad 2011). In the present study, supplementing the diet with HE at concentrations of 1.0 and 5.0 g/kg resulted in greater cumulative survival, relative survival, and RPS following a challenge with *S. agalactiae* compared to the remaining groups. The significant immunological effects of HE could be responsible for the favorable effects on fish survivability (supportive evidenced by elevation mRNA expression levels of *tnf-α*, *il-1β*, *il-6*, and *il-10* genes). Some active ingredients in HE such as bioactive erinacines and polysaccharides have been demonstrated to function against microbial activities of both non-resistant and antibiotic-resistant pathogenic bacteria (Friedman 2015). Kim et al. (2012) reported that polysaccharides extracted from HE could inhibit salmonella in mice. In the same manner, Ma et al. (2012) found that ergosterol oxide isolated from *H. erinaceus* could inhibit *Staphylococcus aureus*, *Bacillus megaterium*, *B. thuringiensis*, *B. subtilis*, and *Escherichia coli*. Similar results have been shown in grass carp (*Ctenopharyngodon idella*) fed supplementation of *H. caput-medusae* and *H. erinaceus* extracts at 0.4–10 g/kg which increased survival up to 70% after challenged with virulent pathogenic bacterial in olive flounder (*Paralichthys olivaceus*) and grass carp (*Ctenopharyngodon Idella*) (Gou et al. 2018; Harikrishnan et al. 2011a). In addition, dietary supplementation of *H. erinaceus* powder at levels ranging from 2 to 20 g/kg resulted in a substantial improvement in survival rates, increasing from 30 to 90% after being challenged with pathogenic bacterial in white shrimp (Yeh et al. 2011) and Nile tilapia (Khieokhajonkhet et al. 2022).

In farmed fish, especially those kept in settings of intense farming, handling or changes to water physicochemical characteristics are known to result in stress (Serradell et al. 2020). Stress urges an allostatic physiological response resulting in reestablished fish to homeostasis by the release of cortisol and glucose into the bloodstream to cope with stressful conditions, generate energy, and reduce the negative effects of stress (Barton 2002; Bonga 1997). In the current study, supplementation of HE in the diet for Nile tilapia linearly and quadratically decreased cortisol levels, and a quadratic effect was observed for glucose levels. These results suggest that the bioactive compounds in HE could reduce stress conditions in Nile tilapia. In line with these results, dietary supplementation of *H. erinaceus* powder significantly decreased glucose and cortisol levels in Nile tilapia (Khieokhajonkhet et al. 2022). Similarly, Nile tilapia fed with dietary supplementation of white bottom mushroom also significantly decreased serum glucose and cortisol in Nile tilapia (Dawood et al. 2020a, b). *hsp70cb1* is regarded to be a useful stress marker since it involves cell defense and repair on the fish body under stress circumstances (Yu et al. 2021). In the present study, HE0.1 and HE1.0 supplemented diets showed a significant difference *hsp70cb1* mRNA expression compared with a control group, suggesting that HE

could relieve stress in Nile tilapia. It is similar to the previous studies which also showed a significant difference in dietary supplementation of white button mushroom powder and *β-glucan* with a similar effect of *hsp70* expression in fish (Dawood et al. 2020a, b; Douxfils et al. 2017; Ji et al. 2017).

Conclusion

The present study showed that dietary administration of HE strongly improved disease resistance of Nile tilapia against *S. agalactiae* infection. Furthermore, HE supplementation also exerts pro—and anti-inflammatory cytokine effects by upregulating *tnf-α*, *il-1β*, *il-6*, and *il-10* mRNA gene expression. At the same time, dietary HE supplementation could relieve stress responses by reducing serum cortisol and glucose levels. Overall, the application of HE at 1.0 g/kg showed the most pronounced increase of RBC, WBC, disease resistance against *S. agalactiae* infection, and the expression of *tnf-α*, *il-1β*, *il-6*, and *il-10* genes. Taken together with these results, dietary supplementation of 1.0 g/kg of HE can be a useful application for the future Nile tilapia aquaculture.

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Author contribution Anurak Khieokhajonkhet: project administration, funding acquisition, methodology, conceptualization, formal analysis, investigation, and writing-original draft; Piluntasoot Suwannalers, Korntip Kannika, and Niran Aeksiri: formal analysis, investigation, and funding acquisition; Gen Kaneko: formal analysis, writing-original draft, and proofreading; Kumrop Ratanasut, Pattaraporn Tatsapong, Wilasinee Inyawilert, and Wutiporn Phromkunthong: conceptualization and proofreading. All the authors have read and approved the final version of the article.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics approval The protocol was also approved by the Naresuan University Animal Care and Use Committee (NUACUC), Naresuan University, Phitsanulok, Thailand (NUACUC No. AG-AQ0008/2564). Additionally, all procedures involving animals were also carried out following the guidelines and recommendations of the Institute of Animals for Scientific Purpose Development (IAD), the National Research Council of Thailand (NRCT), Bangkok, Thailand (License number U1/00704/2558).

Competing interests The authors declare no competing interests.

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