

The effects of dietary tributyrin in low fishmeal diets on the growth performance and health status of oriental river prawn (*Macrobrachium nipponense*)

2026 Volume 3, Article number: e027

<https://doi.org/10.48130/animadv-0026-0012>

Received: 7 January 2026

Revised: 22 March 2026

Accepted: 24 April 2026

Published online: 8 October 2026

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Abstract

To assess the effects of soybean meal (SBM) substitution for fishmeal (FM) and the tributyrin (TB) supplementation on the growth and health status of *Macrobrachium nipponense*, 900 prawns (initial weight 0.38 ± 0.01 g) were randomly assigned into six groups with three replicates. Six isonitrogenous (40.3% crude protein) and isolipidic (8.7% crude lipid) diets were formulated: positive control (PC: 30% FM, 22% SBM), negative control (NC: 25% FM, 30% SBM), and NC supplemented with 0.05% (TB1), 0.1% (TB2), 0.2% (TB3), or 0.4% TB (TB4). After 8 weeks, growth performance indicators showed no significant differences ($p > 0.05$). Digestive enzyme analysis revealed that lipase activity in TB2-TB4 was significantly higher than that of PC and NC ($p < 0.05$). Compared with the PC and NC groups, the T2-T4 group exhibited significantly reduced malondialdehyde (MDA) content and markedly elevated superoxide dismutase (SOD) activity ($p < 0.05$). Total antioxidant capacity (T-AOC) of hemolymph was substantially greater in the TB2-TB4 groups than in the NC group ($p < 0.05$). The gene expression levels of heat shock protein 70 (HSP70) in TB1-TB3 and toll-like receptors (TLRs) in TB2-TB3 were significantly upregulated compared to the PC and NC groups ($p < 0.05$). In the bacterial challenge test, resistance to *Vibrio cholerae* was significantly enhanced in the groups fed diets supplemented with TB, and the estimated optimum TB requirement was 0.26%, based on second-degree polynomial regression analysis. In conclusion, substituting 5% FM with SBM had no adverse effects, whereas supplementing TB at an optimal level of 0.26% in low-FM diets significantly enhanced key health parameters in *M. nipponense*. This study supports the use of TB as a viable functional additive to improve health outcomes in aquatic animals.

Keywords: Low fish meal, *Macrobrachium nipponense*, Tributyrin, *Vibrio cholerae*

Citation: Liu X, Yossief GS, Zhu L, Liu B, Sun C, et al. 2026. The effects of dietary tributyrin in low fishmeal diets on the growth performance and health status of oriental river prawn (*Macrobrachium nipponense*). *Animal Advances* 3: e027 <https://doi.org/10.48130/animadv-0026-0012>

Introduction

Globally, capture fisheries and aquaculture collectively supply nearly 20% of the per capita animal protein for over 3.1 billion people, excluding subsistence fishing and illegal trade^[1]. Despite their vital contribution to global food security and nutritional value as animal protein sources, shrimp and fish cultivation face significant challenges, among which feed cost is paramount^[2]. Aquafeed pricing was principally determined by ingredient sourcing and nutritional composition^[3], with fishmeal (FM) being a major cost driver. FM serves as the predominant protein source in aquafeeds due to its superior nutritional profile, which includes high protein content, balanced essential amino acids, essential fatty acids, and rich mineral and vitamin levels^[4]. Although FM remains vital for aquafeed formulation owing to these unique characteristics, its price fluctuates unpredictably. Furthermore, FM production has declined due to fishing restrictions^[5]. Consequently, finding sustainable FM alternatives that maintain shrimp growth and health represents a critical priority for the sector.

Given the demonstrated importance of identifying sustainable FM alternatives for the long-term viability of aquaculture^[6], soybean meal

(SBM) has emerged as a leading replacement candidate due to its high protein content (45%–48%), favorable amino acid profile, low cost, and wide availability. Successful partial FM substitution with SBM has been reported in diets for amberjack (*Seriola dumerili*)^[7] and Nile tilapia (*Oreochromis niloticus*)^[8]. However, replacing FM with 36% or 48% SBM in the diet of olive flounder (*Paralichthys olivaceus*) significantly impairs growth performance and muscle quality^[9]. A major limitation of SBM is its content of antinutritional factors (ANFs)—including phytates, hemagglutinins, lectins, saponins, and oligosaccharides^[10]—which can suppress growth and compromise immunity in aquatic species. For example, 25%–75% FM replacement with SBM significantly reduced growth and metabolic capacity in *Litopenaeus vannamei*^[11]. To mitigate these adverse effects, recent studies indicate that functional additives can enhance SBM utilization. Taurine supplementation, for instance, improved 20%–60% FM replacement potential by SBM in largemouth bass (*Micropterus salmoides*) diets^[12].

Therefore, developing alternative feed additives to promote SBM substitution for FM in aquaculture has always been a top priority^[13]. Butyrate derivatives have emerged as promising functional additives,

with tributyrin (TB, a triacylglycerol analogue esterifying glycerol with three butyrate molecules) showing particular potential^[14]. To verify TB's ability to counteract the negative effects of ANFs, TB was added to a low-FM diet for the study. Empirical studies have confirmed TB's efficacy in aquatic species, demonstrating enhanced growth performance and gut health^[15,16]. The addition of 0.1% TB was also found to significantly alleviate intestinal damage due to substitution in a study of FM replacement by SBM^[17].

Oriental river prawn (*M. nipponense*), which belongs to the family Palaemonidae, holds substantial economic value in Chinese aquaculture^[18]. The global production was estimated to reach about 237,100 tons, and a market value approaching 2.8 billion USD^[19]. In light of its documented physiological roles, TB emerges as a promising feed additive^[20]. Therefore, we evaluated the impact of TB on growth, digestive and antioxidant capacity, and non-specific immunity in FM-replaced *M. nipponense*. This study identifies the optimal level of TB supplementation for FM replacement, providing critical data to facilitate its adoption as a functional aquafeed additive.

Materials and methods

Diet preparation and experimental design

Six isonitrogenous (40.3% crude protein) and isolipidic (8.7% crude lipid) diets were formulated for this experiment using conventional feed ingredients, with FM and SBM as the primary protein sources, adhering to the nutritional requirements for *M. nipponense*^[21]. The positive control (PC) diet consisted of 30% FM and 22% SBM, whereas the negative control (NC) diet contained 25% FM and 30% SBM. Four experimental diets were then created by supplementing the NC diet with TB at 0.05%, 0.1%, 0.2%, and 0.4%, respectively. TB is obtained from Shanghai Menon Biotechnology Co., Ltd (Shanghai, China) and has a 50% purity.

Diet preparation involved finely grinding macro-ingredients, sieving through a 60-mesh sieve, and homogenizing with micro-ingredients using an electric mixer. Water and oil were incorporated during mixing. The mixture was processed into pellets via a twin-screw extruder (Model: 1.0 mm die pelletizer; Guangzhou, China), dried to ~10% moisture, and then vacuum-packed and frozen at -20 °C until use. Complete ingredient formulations with percentage inclusion rates are detailed in Table 1.

Experimental prawns and management

Healthy juvenile *M. nipponense* of uniform age and size (initial weight: 0.38 ± 0.01 g) were obtained from the Dapu breeding station (Wuxi, China). Before the trial, prawns were acclimatized and fed a commercial diet (Wuxi Tongwei Feedstuffs Co., Ltd, China) to apparent satiation twice daily. Nine hundred juveniles were then randomly stocked into each of 18 of the same model (1,000 L) tanks (three tanks per diet, 50 prawns per tank). Prawns were hand-fed their respective diets to apparent satiation three times daily (08:00, 12:00, and 16:00) at an initial rate of 5%–8% body weight for 8 weeks. Daily feed rations were adjusted based on consumption and mortality. Residual feed and feces were siphoned out 2 h post-feeding. Water exchange (20%–30%) occurred weekly. Environmental conditions were maintained as follows: natural photoperiod, water temperature 25–27 °C, pH 7.5–8.0, dissolved oxygen 6.0–8.0 mg/L, ammonia nitrogen < 0.1 mg/L, and nitrate < 0.01 mg/L. Water quality parameters were monitored using a Sunpu Test assay kit (Beijing Sunpu Biochem. Tech. Co., Ltd) according to the manufacturer's protocol.

Table 1. Formulation and proximate composition of the experimental diets (% dry matter).

Ingredients	Diets					
	PC	NC	TB1	TB2	TB3	TB4
FM ¹	30.00	25.00	25.00	25.00	25.00	25.00
SBM ¹	22.00	30.00	30.00	30.00	30.00	30.00
Rapeseed meal ²	10.00	10.00	10.00	10.00	10.00	10.00
Shrimp meal ²	6.00	6.00	6.00	6.00	6.00	6.00
Squid paste ²	3.00	3.00	3.00	3.00	3.00	3.00
α-Starch	20.48	17.48	17.43	17.38	17.28	17.08
Oil (fish 1:soy 1) ³	1.50	1.50	1.50	1.50	1.50	1.50
Granulesten ²	1.50	1.50	1.50	1.50	1.50	1.50
Cholesterol ⁴	1.00	1.00	1.00	1.00	1.00	1.00
Monocalcium phosphate ⁴	0.50	0.50	0.50	0.50	0.50	0.50
Premix ⁵	2.00	2.00	2.00	2.00	2.00	2.00
Vitamin C ⁵	1.00	1.00	1.00	1.00	1.00	1.00
Choline chloride ⁴	0.50	0.50	0.50	0.50	0.50	0.50
Ecdysone ²	0.50	0.50	0.50	0.50	0.50	0.50
DMPT ²	0.02	0.02	0.02	0.02	0.02	0.02
Tributyrin 50% ⁶			0.05	0.10	0.20	0.40
Total	100	100	100	100	100	100
Proximate composition (dry matter basis)						
Crude protein	40.31	40.36	40.35	40.35	40.34	40.31
Crude lipid	8.79	8.71	8.71	8.71	8.71	8.71
Ash	11.00	10.26	10.26	10.26	10.26	10.26

Note: ¹ FM (fish meal), obtained from Wuxi Tongwei feedstuffs Co., Ltd, Wuxi, China, crude protein 61.3%, crude lipid 8.3%; SBM (soybean meal) obtained from Wuxi Tongwei feedstuffs Co., Ltd, Wuxi, China, crude protein 44.7% (db), crude lipid 1.1% (db); ² provided by Tongwei Co., Ltd (Wuxi, China); ³ oil consisted of a mixture of fish oil and soybean oil with a 1:1 ratio; ⁴ provided by Wuxi Hanove Animal Health Products Co., Ltd (Wuxi, China); ⁵ vitamin C and mineral premix were produced for shrimps by Wuxi Hanove Animal Health Products; ⁶ 50% efficiency, obtained from Shanghai Menon Biotechnology Co., Ltd (Shanghai, China).

Sample collection

The feeding trial lasted approximately 8 weeks, and all groups were treated under similar conditions. On the final day, a 24-h fasting period was implemented to clear the digestive tract before sample collection. Then, the prawns in every tank were counted and weighed to calculate growth performance. Nine prawns were then selected from each treatment group and dissected to collect samples. The separately collected intestines were cooled down to a frozen state using liquid nitrogen and then immediately stored at -80 °C for subsequent enzymatic activity laboratory analysis. Three samples from each experimental diet were taken for feed proximate chemical composition analysis.

Growth performance parameters

Growth performance, body index, and feed utilization parameters were determined as follows:

$$\text{Weight gain rate (WGR, \%)} = 100 \times \frac{W_t - W_i}{W_i}$$

$$\text{Specific growth rate (SGR, \%/day)} = 100 \times \frac{\ln W_t - \ln W_i}{\text{Experimental days}}$$

$$\text{Feed conversion ratio (FCR)} = 100 \times \frac{\text{Feed intake}}{W_t - W_i}$$

$$\text{Survival rate (SR, \%)} = 100 \times \frac{\text{Final number of prawns}}{\text{Initial number of prawns}}$$

$$\text{Condition factor (CF, g/cm}^3\text{)} = 100 \times \frac{W_t}{L_t^3}$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{Wt} - \text{Wi}}{\text{Protein intake}}$$

where g is gram, Wi is initial body weight, Wt is final body weight, Lt is final body length, and cm is centimeter.

Diet proximate chemical composition analysis

The proximate composition (crude lipid, crude protein, and ash) of the six experimental diets was analyzed according to the official methods of the Association of Official Analytical Chemists International. Then the samples were ground into powder using a mortar and pestle for further analysis. After acid digestion, the Kjeldahl method was used to measure the nitrogen ($\text{N} \times 6.25$) content to determine the crude protein content. As a Soxhlet extractor was used to determine the lipid content by extracting ether, the ash content of the diet was determined by combustion of the samples at 550 °C in a muffle furnace for 5 h.

Digestive enzyme assays

Total protein (A045-2-2), lipase (A054-1-1), and amylase (C016-2-1) activities were determined in intestinal tissue stored at -80 °C using respective assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) strictly adhering to the manufacturer's protocols.

Hemolymph and hepatopancreatic antioxidant enzymatic capacity assay

The specific procedures for hemolymph collection, anticoagulant preparation, and hepatopancreas homogenisation should be carried out in accordance with Liu et al.^[22]. Then, the activities of MDA (A003-1-2), CAT (A007-1-1), SOD (A001-3-2), T-AOC (A015-1-2), and NO (A012-1-2) were measured using a commercial test kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), following the manufacturer's protocol.

Bacterial challenge test

On the final day of the feeding trial experiment, 13 prawns from each tank were randomly taken and used for the pathogenic bacterium challenge test against *Vibrio cholerae* (*V. cholerae*). The *V. cholerae* was obtained from the College of Animal Science and Technology, Yangzhou University, China. *V. cholerae* (GXFL1-X) was activated and cultured in an LB liquid medium at 28 °C with 180 rpm shaking for 18 h. Bacterial concentration was quantified via the plate count method, followed by adjustment to target bacterial suspensions using sterile saline. The randomly selected prawns were injected intramuscularly with 10 µL in the second abdominal segment from the head at a concentration of 1×10^8 CFU/mL and reared in small containers at a controlled

temperature (25 ± 1 °C) for 96 h. Mortalities in each treatment group were recorded at 6, 12, 24, 48, 72, and 96 h, and the dead prawns were removed promptly.

RNA extraction, cDNA synthesis, and real-time PCR analysis

The mRNA expression levels of heat shock proteins 70 (*HSP70*), immune deficiency (*IMD*), *Relish*, Toll-like receptors (*TLRs*), *dorsal*, and β -actin (Table 2) were determined in two major steps as mentioned in the study by Zhao et al.^[23]. All primers were designed via Primer-BLAST using cDNA sequences from our transcriptome library and were commercially produced by Shanghai Biocolor BioScience & Technology Company (Shanghai, China).

Statistical analysis

The SPSS for Windows v25.0 was used for statistical analysis of the data. Using one-way analysis of variance, all the collected data from various parameters were subjected to a homogeneity test, and significant effects on the groups were determined by multiple range test analysis of Tukey's to see whether significant differences existed among the means of the treatment groups. All the data were expressed as a mean $\pm$ standard error of the mean ($M \pm \text{SEM}$).

Results

Growth performance

Results for the growth performance parameters of the experiment are provided in Table 3. In the 56-d feeding trial experiment, all the prawns fed with different treatments appeared to be healthy, and the survival rates (SR) were in the range of 86% to 92%, with no significant differences among the groups ($p > 0.05$). Likewise, in the indices final body weight (FBW), WGR, SGR, CF, PER, and FCR, there were no significant differences among all the groups ($p > 0.05$). In general, growth performance and feed utilization indices of *M. nipponense* were not affected by reducing FM content from 30% to 25%. Furthermore, supplementation of TB in low FM diets also has no significant effect on all the indices ($p > 0.05$).

Digestive enzymatic activities

The digestive enzymatic activities tested in the intestine are provided in Fig. 1. No significant difference was observed in the amylase among the groups ($p > 0.05$). Concerning lipase activities, there was no significant

Table 2. Real-time PCR primers.

Gene	Primer sequences (5'-3')	Product length (bp)	Amplification efficiency	Tm (°C)	XXX	Ref.
<i>TLRs</i>	(F) CGACCTCCACGACAACAAGA (R) AAAGTTCCTGCACCAATGCG	186	97.23%	59.97 59.69	MH178390	(Gu et al., ^[24] 2017)
<i>Dorsal</i>	(F) TACGACCAACGGACAAGAGC (R) CGCATTGTTGCTGTTCCCA	116	93.37%	60.04 59.97	OM066734	(Song et al., ^[25] 2020)
<i>IMD</i>	(F) GGCACCAAGCCTTCTTTTCAG (R) ATATCCTTCGGGTGCGATTTTC	164	126.81%	60.00 58.23		(Li et al., ^[26] 2018)
<i>Relish</i>	(F) CGGGAAGTTTGGACGGCATA (R) TCGTTTAAGGCTGTCTGGCA	198	100.26%	60.39 59.61	MZ466551	(Song et al., ^[25] 2020)
<i>HSP70</i>	(F) CTTTGAGGTGGCTGGATGGAA (R) GCAGTTCGGTGAATTTGGCA	112	100.54%	60.27 60.27	KC460343	(Song et al., ^[25] 2020)
β -actin	(F) GTGCCCATCTACAGGGTTA (R) CGTCAGGGAGCTCGTAAGAC	247	110.27%	58.88 59.90	MH540111	(Luo et al., ^[27] 2018)

Note: the mRNA sequences for each gene were obtained from *M. nipponense* hepatopancreas transcriptome sequencing database of the Aquatic Disease and Nutrition Laboratory of Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences. Primers for RT-PCR were designed using Primer 5.0.

Table 3. Growth performance and feed utilization of *M. nipponense* fed with diets of partially replaced fishmeal and supplemented with different levels of tributyrin.

Parameters	Diets					
	PC	NC	TB1	TB2	TB3	TB4
IBW (g)	0.39 ± 0.00	0.38 ± 0.00	0.38 ± 0.00	0.38 ± 0.00	0.38 ± 0.00	0.38 ± 0.00
FBW (g)	0.69 ± 0.02	0.67 ± 0.02	0.67 ± 0.02	0.68 ± 0.02	0.68 ± 0.01	0.68 ± 0.01
WGR (%)	79.71 ± 3.05	74.04 ± 3.70	75.93 ± 6.77	76.17 ± 3.95	77.61 ± 2.36	76.57 ± 3.44
SGR (%/day)	1.15 ± 0.03	1.09 ± 0.04	1.11 ± 0.07	1.11 ± 0.04	1.13 ± 0.03	1.11 ± 0.04
SR (%)	86.00 ± 1.15	89.33 ± 3.53	92.00 ± 3.06	88.00 ± 4.00	90.67 ± 0.67	87.33 ± 4.37
FCR	2.52 ± 0.13	2.70 ± 0.20	2.55 ± 0.16	2.54 ± 0.17	2.34 ± 0.12	2.68 ± 0.27
FI (g)	2.44 ± 0.02	2.44 ± 0.09	2.60 ± 0.03	2.67 ± 0.09	2.78 ± 0.14	2.77 ± 0.19
PER	0.92 ± 0.05	0.87 ± 0.07	0.92 ± 0.06	0.91 ± 0.06	1.00 ± 0.05	0.88 ± 0.10
CF (g/cm ³)	1.18 ± 0.01	1.18 ± 0.02	1.16 ± 0.00	1.15 ± 0.01	1.13 ± 0.05	1.13 ± 0.02

Note: values are presented as mean ± SEM ($n = 3$). Different letters indicate significant differences between treatments ($p < 0.05$). IBW: initial body weight; FBW: final body weight; WGR: weight gain rate; SGR: specific growth rate; SR: survival rate; FCR: feed conversion ratio; FI: feed intake; PER: protein efficiency ratio; CF: condition factor.

difference in PC, NC, and TB1. However, it has substantially increased in the groups fed with higher TB concentrations ($p < 0.05$). Lipase activities significantly rose as dietary TB levels increased up to 0.2% and remained at a plateau up to the concentration of 0.4% ($p < 0.05$).

Hemolymph antioxidant enzymatic capacity

Hemolymph antioxidant enzyme activity results are presented in Fig. 2. Although MDA levels did not differ significantly ($p > 0.05$) between prawns fed the PC and NC diets, MDA was significantly reduced ($p < 0.05$) in all TB-supplemented diets. In contrast to MDA, CAT activity was unaffected by TB supplementation ($p > 0.05$) observed among dietary treatments.

SOD activity and T-AOC did not differ significantly ($p > 0.05$) between the NC and PC groups. However, SOD activity was significantly higher ($p < 0.05$) in the TB2 and TB3 groups compared to all other experimental groups. T-AOC was significantly elevated ($p < 0.05$) in the TB2, TB3, and TB4 groups relative to the NC group. Furthermore, the TB4 group exhibited significantly higher T-AOC ($p < 0.05$) than the PC group.

Hepatopancreatic antioxidant enzyme capacity

Hepatopancreatic antioxidant enzyme activity results are presented in Fig. 3. MDA levels did not differ significantly ($p > 0.05$) between the PC and NC groups. However, MDA levels were significantly lower ($p < 0.05$) in the TB2, TB3, and TB4 groups compared to both the PC and NC groups. NO levels remained unaffected by dietary treatments ($p > 0.05$). No statistically significant variation in SOD activity was observed between the two control groups (PC vs NC) ($p > 0.05$), whereas SOD activity was significantly elevated in the TB2, TB3, and TB4 groups relative to both control groups ($p < 0.05$).

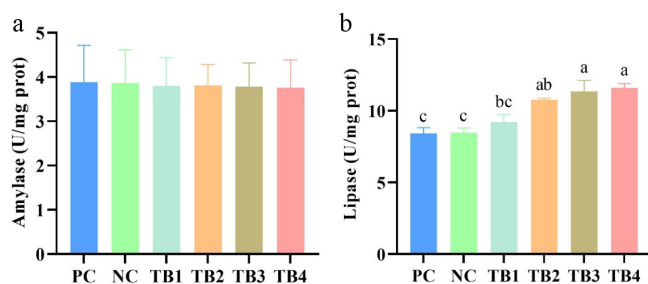


Fig. 1 The effect of substitution of fish meal and addition of different levels of tributyrin on intestinal enzymatic activities of *M. nipponense*. Note: (a) amylase; (b) lipase. Values are presented as mean ± SEM ($n = 9$). Different letters indicate significant differences between treatments ($p < 0.05$).

Intestinal immune-related gene expression

Relative intestinal gene expression of *HSP70*, *IMD*, *Relish*, *TLRs*, and *Dorsal* in *M. nipponense* fed TB-supplemented diets for 8 weeks is presented in Fig. 4. *HSP70* expression showed no significant difference ($p > 0.05$) between PC and NC control groups. However, TB1, TB2, and TB3 groups exhibited significantly elevated *HSP70* expression ($p < 0.05$) relative to PC, NC, and TB4 groups. *Dorsal*, *IMD*, and *Relish* expression remained unaffected across all dietary treatments ($p > 0.05$). *TLRs* expression did not differ significantly ($p > 0.05$) between NC and PC groups but was significantly upregulated ($p < 0.05$) in TB2 and TB3 groups compared to both controls.

Bacterial challenge test

The result of the bacterial challenge test of *M. nipponense* fed with diets of different TB supplementation levels is presented in Fig. 5. Statistical analysis indicated no significant disparity in cumulative survival between the control groups ($p > 0.05$). Prawns fed the TB3 diet exhibited significantly higher survival compared to both control groups ($p < 0.05$). The second-degree polynomial regression analysis based on survival rate or resistance of *M. nipponense* against *V. cholerae* and different

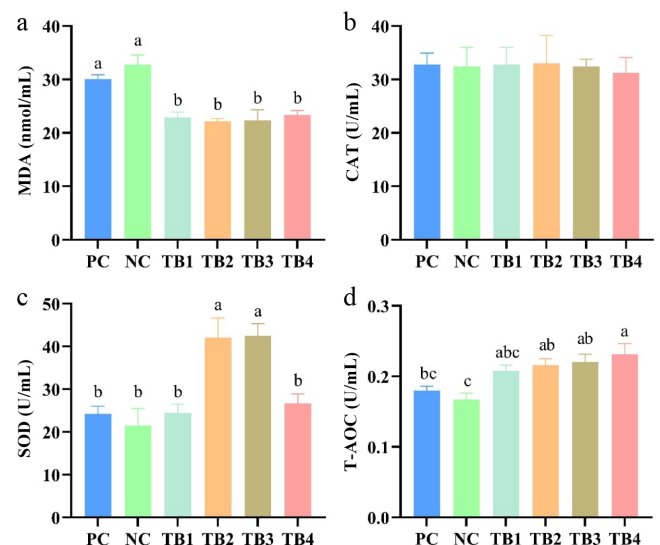


Fig. 2 The effect of substitution of fish meal and addition of different levels of tributyrin on antioxidant enzymatic capacity in hemolymph of *M. nipponense*. Note: (a) malondialdehyde (MDA); (b) catalase (CAT); (c) superoxide dismutase (SOD); (d) total antioxidant capacity (T-AOC). Values are presented as mean ± SEM ($n = 9$). Different letters indicate significant differences between treatments ($p < 0.05$).

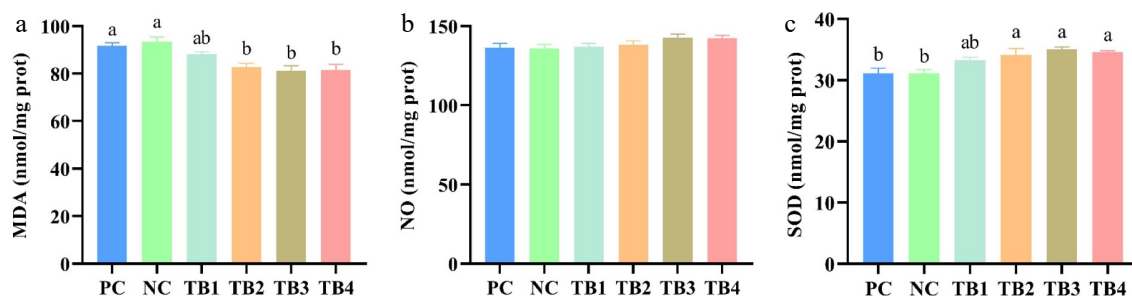


Fig. 3 The effect of substitution of fish meal and addition of different levels of tributyrin on antioxidant enzymatic capacity in hepatopancreas of *M. nipponense*. Note: (a) malondialdehyde (MDA); (b) superoxide dismutase (SOD); (c) nitrous oxide (NO). Values are presented as mean $\pm$ SEM ($n = 9$). Different letters indicate significant differences between treatments ($p < 0.05$).

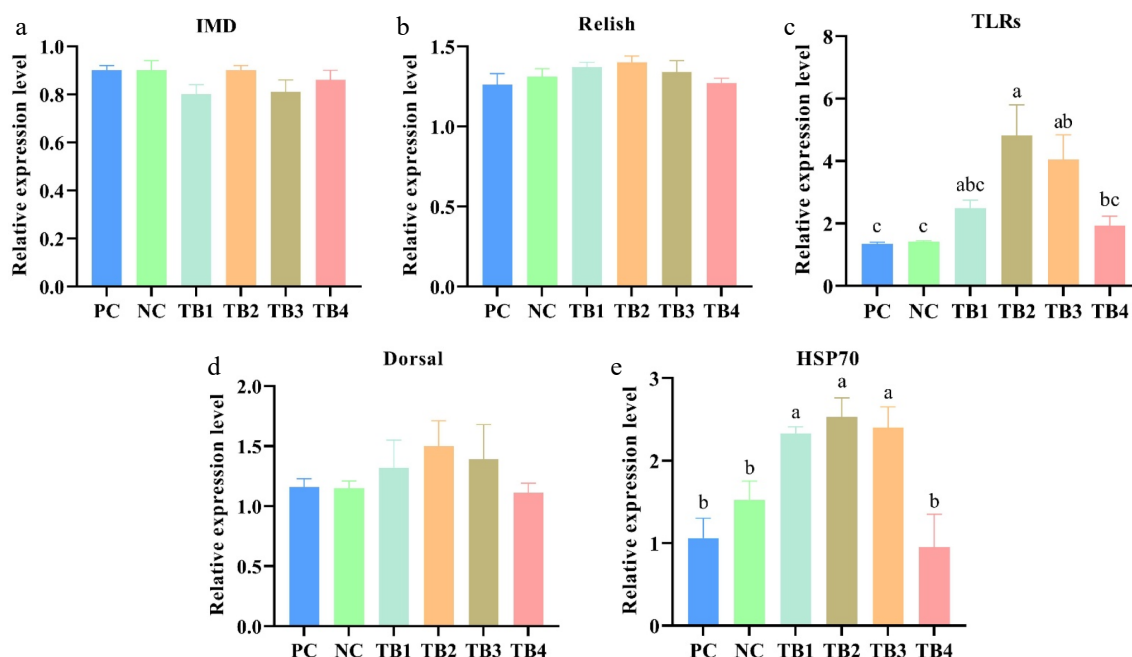


Fig. 4 The effect of substitution of fish meal and addition of different levels of tributyrin on immune related genes expression in hepatopancreas of *M. nipponense*. Note: (a) Immune deficiency (IMD); (b) Relish; (c) Toll-like receptors (TLRs); (d) Dorsal; (e) Heat shock protein 70 (HSP70). Values are presented as mean $\pm$ SEM ($n = 9$). Different letters indicate significant differences between treatments ($p < 0.05$).

supplementation levels estimated that the optimum TB requirement is 0.26% (Fig. 5c).

Discussion

FM, a critical but increasingly constrained aquafeed ingredient due to pricing and sustainability concerns, has prompted extensive research into alternative protein sources. Substantial evidence indicates that SBM substitution for FM often reduces growth performance in aquatic species, though dietary additives may mitigate these effects^[28]. In the present study, replacing 5% FM with SBM (reducing total FM to 25%) with supplemental TB at 0.05%–0.4% elicited no significant differences in growth performance across treatments. This aligns with findings in *Litopenaeus vannamei*, where 20%–40% FM replacement with SBM yielded comparable WGR, specific growth rate (SGR), and protein efficiency ratio (PER)^[29]. The reason for this may be closely related to quality and nutritional value, but sometimes it varies according to soybean composition. Besides the source, processing procedures also play an important role^[3].

TB, a butyrate prodrug, has gained prominence in nutritional science for enhancing nutrient bioavailability and intestinal health in

aquatic species^[30]. However, the absence of growth enhancement in the current study contrasts with positive responses observed in juvenile black sea bream (*Acanthopagrus schlegelii*)^[15], juvenile yellow drum (*Nibea albiflora*)^[17], and *Litopenaeus vannamei*^[31]. Notably, null growth effects align with findings in *L. vannamei* fed 50% SBM diets^[32]. This interspecies variation likely reflects divergent digestive physiology and feeding behaviors. Butyrate liberation from TB exhibits compartment-specific kinetics: minimal during gastric digestion but extensive in the intestinal segment^[33]. This site-specific metabolism enhances intestinal utilization efficiency over gastric absorption^[34], potentially explaining the observed growth response disparities and warranting further mechanistic investigation.

Digestive enzyme activity in decapod crustaceans—particularly trypsin, lipase, and amylase—critically influences nutrient assimilation and has been extensively studied^[35]. Unlike esterases (which hydrolyze simple alcohol esters), lipases require interfacial activation for full catalytic efficiency and specifically cleave glycerol esters. In *M. nipponense*, dietary TB significantly elevated lipase activity, suggesting TB stimulates lipase production to facilitate butyrate release, thereby enhancing nutrient absorption and health. This aligns with observations in *Larimichthys crocea*, where TB supplementation

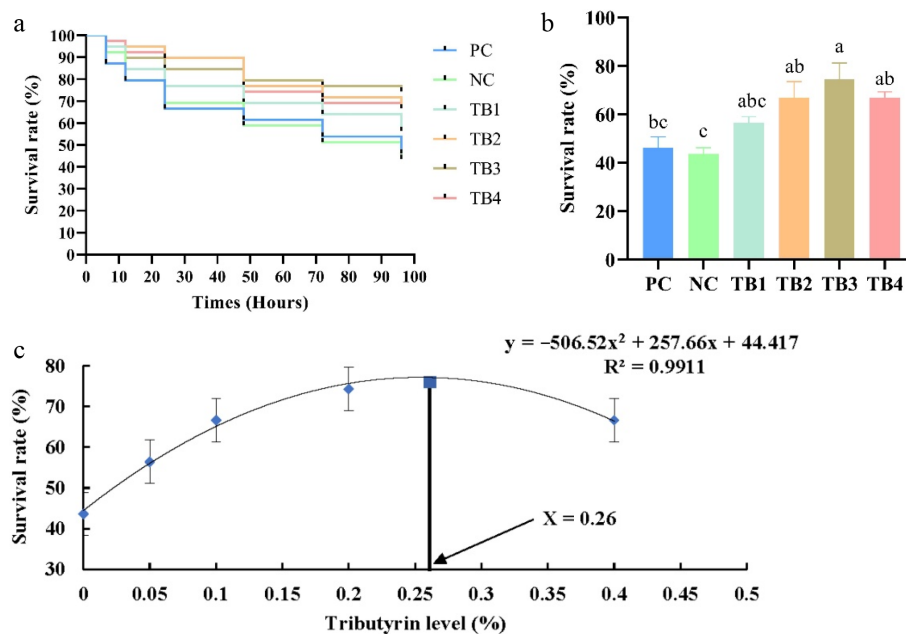


Fig. 5 Effect of dietary tributyrin on the survival rate of *M. nipponense* against *Vibrio cholerae* (2×10^7 CFU/mL). Note: (a) the survival rate of *M. nipponense* at different time periods after injection of bacteria; (b) the survival rate of *M. nipponense* after 96 h of injection of bacteria; (c) the optimum level of ferulic acid supplementation based on second-order polynomial analysis on the survival rate of *M. nipponense* after 96 h of bacterial challenge. Values are presented as mean $\pm$ SEM ($n = 39$). Different letters indicate significant differences between treatments ($p < 0.05$).

increased lipase activity^[36]. Conversely, TB supplementation did not significantly affect amylase activity—consistent with the absence of growth or feed efficiency differences among treatments. While short-chain fatty acids (SCFAs) generally enhance digestive enzyme activity in aquaculture^[37], such effects depend on rearing conditions. During the rearing time, if shrimps were not exposed to any stress in a clean water system with low density and good water quality and provided with ideal growth conditions, a significant difference might not be observed^[38]. In our study, all the treatment groups of *M. nipponense* were not exposed to any stress, and the result correlates with this statement.

The antioxidant enzymatic system protects organisms from oxidative damage by neutralizing reactive oxygen species (ROS) and maintaining cellular homeostasis^[39]. In crustaceans, key antioxidant enzymes—including SOD, CAT, and T-AOC—collectively mitigate oxidative stress^[40]. MDA, a lipid peroxidation biomarker, reliably indicates oxidative injury severity^[41]. In this study, MDA levels did not differ significantly between positive and NC groups. However, TB-supplemented diets significantly reduced MDA concentrations in hemolymph and hepatopancreas tissues. This aligns with findings in *L. vannamei*, where TB lowered MDA levels^[31]. In crustaceans, SOD scavenges excess ROS during pathogen challenge, maintaining cellular metabolic homeostasis^[42]. Analysis confirmed the absence of significant differences in SOD activity after replacing 5% FM with SBM, whereas the addition of 0.1% vs 0.2% of TB resulted in a significant increase in SOD activity in haemolymph and hepatopancreas, which was similar to the results of the pike crab study, where the addition of moderate amounts of TB prominently increased the activities of T-SOD, polyphenol oxidase, nitric oxide, acid phosphatase, and alkaline phosphatase^[43]. While plant-protein substitution can compromise aquatic animal health, TB supplementation mitigates oxidative damage by enhancing antioxidant and immune capacity^[15].

The T-AOC represents a comprehensive measure of an organism's ability to counteract oxidative stress^[44]. In this study, T-AOC did not differ significantly between PC and NC groups. However, TB2, TB3,

and TB4 groups exhibited substantially elevated T-AOC relative to NC. Relevant studies have found that dietary TB supplementation elevates intestinal T-AOC and SOD activities and concurrently lowers MDA and protein carbonylation contents in prawns^[45]. Similarly, the addition of moderate amounts of TB was also found to improve antioxidant capacity in a grass carp study^[46]. These findings collectively indicate that the addition of a moderate amount of TB improves the antioxidant capacity of *M. nipponense* by increasing SOD, T-AOC content, and reducing MDA levels after the replacement of 5% FM with SBM.

Nitric oxide (NO)—an evolutionarily conserved signaling molecule—mediates critical physiological functions in aquatic species, including neuromodulation, cardiovascular regulation, host defense, and developmental processes^[47]. It further contributes to cellular homeostasis and oxidative stress mitigation^[48]. In this study, NO levels did not differ significantly among dietary groups, indicating that TB supplementation does not primarily exert immunomodulatory or antioxidant effects through NO-dependent pathways. Given the unresolved mechanisms underlying TB's bioactivity, we conducted quantitative PCR analysis of immune-related genes and pathogenic challenge assays to elucidate alternative pathways.

Crustaceans, including *M. nipponense*, rely primarily on innate immunity to combat microbial pathogens through activation of cellular and humoral immune genes^[49]. Within this system, heat shock protein 70 (*HSP70*) serves as a critical molecular chaperone with dual functions: facilitating cytoprotection in gut epithelial cells and mitigating inflammatory-oxidative stress *in vivo* and *in vitro*^[50]. While *HSP70* expression did not differ significantly between PC and NC groups, the TB1, TB2, and TB3 groups exhibited markedly upregulated *HSP70* compared to both controls. This aligns with findings in juvenile common carp (*Cyprinus carpio*), where microencapsulated sodium butyrate enhanced intestinal *HSP70* expression^[51]. Collectively, these results indicate that optimal TB supplementation enhances non-specific immunity in *M. nipponense* through *HSP70* induction.

The IMD and Toll pathways constitute the primary regulatory axes governing invertebrate immune responses^[52]. Extensive studies confirm their critical roles in pathogen defense and stress adaptation in crustaceans^[53], where these evolutionarily conserved pathways orchestrate antimicrobial peptide and immune effector expression to eliminate infections^[54]. In this study, 5% FM substitution with SBM did not alter *IMD* or *TLRs* expression. However, 0.1%–0.2% TB supplementation significantly upregulated *TLRs* expression relative to both the PC and NC groups, while *IMD* remained unaffected. This aligns with findings in shrimp administered *Clostridium butyricum* (a butyrate-producing anaerobe), where *TLRs* expression increased significantly^[55]. It was hypothesized that triglycerides are hydrolysed in the intestine by lipase, releasing butyric acid (the main active ingredient) and glycerol, which acts as a signalling molecule to enhance the body's non-specific immunity by activating immune receptor *TLRs*.

Upon pathogen invasion, pattern recognition receptors (e.g., *TLRs* or *IMD*) detect microbial ligands and initiate intracellular signaling cascades that ultimately activate the NF- κ B transcription factor in the nucleus^[56]. The NF- κ B transcription factor family, comprising members such as *Relish* (*IMD* pathway) and *Dorsal/Dif* (*Toll* pathway), plays pivotal roles in governing immune and inflammatory responses^[57,58]. While in this study there was no significant difference in the expression of *Dorsal* and *Relish* among the groups, the possible explanation is that the regulation of *TLRs* expression is not completely dependent on a single transcription factor, *Dorsal*, and that it is coupled with the lack of significant expression of the genes in the *IMD-Relish* pathway. To further investigate the effect of TB on non-specific immunity, a bacterial challenge test was performed.

V. cholerae, a pathogenic bacterium affecting both fish and humans, naturally inhabits brackish and estuarine environments^[59]. Different studies have demonstrated that SCFAs and their salts contribute to disease mitigation in aquaculture by enhancing host health via significant antimicrobial effects^[60]. Propionate, butyrate, and acetate salts, which have similar characteristics to TB, exhibited strong inhibitory capacity against *Vibrio* spp. in *Litopenaeus vannamei*^[61]. In the present study, the bacterial challenge test revealed that there was no significant difference in survival between the PC and NC groups. However, TB2, TB3, and TB4 survival was significantly higher than that of the NC group. Similar results were found in the study of *Litopenaeus vannamei*, where dietary supplementation with an appropriate amount of TB significantly increased the survival rate against *Vibrio harveyi*^[14]. As per the second-order polynomial regression analysis between the survival percentage and TB supplementation levels, the optimum level for *M. nipponense* was estimated to be 0.26%. Similar results were found in the study of juvenile black sea bream (*Acanthopagrus schlegelii*), where the optimum addition of TB was 0.24% based on the second-order polynomial regression analysis of the SGR^[15], and likewise in the study of juvenile grass carp (*Ctenopharyngodon idellus*), where the optimum addition of butyric acid was found to be 0.2%^[62]. These results demonstrate that supplementation with 0.2%–0.4% TB significantly enhances pathogen resistance, likely through immunomodulatory mechanisms.

Conclusions

In conclusion, this study investigated the effects of SBM replacement of FM and the addition of TB on the growth performance, digestive enzymes, antioxidant capacity, and non-specific immunity of *M. nipponense*. Interestingly, the replacement of FM by SBM at 5% did not cause significant adverse effects on *M. nipponense*. The addition of 0.1%–0.4% TB significantly enhances lipase activity and promotes digestive capacity, thereby improving antioxidant properties and

non-specific immunity. Through bacterial challenge assays and second-order polynomial regression analysis, the optimal TB inclusion level was determined to be 0.26% in *M. nipponense* diets. These findings provided critical data supporting the useful information for the small-scale replacement of FM by SBM and the development of TB as an immunostimulant for *M. nipponense*.

Ethical statements

All procedures were reviewed and preapproved by the Animal Care and Use Committee of Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, identification number: LAECFFRC-2021-06-30, approval date: 30 June 2021. The research followed the 'Replacement, Reduction, and Refinement' principles to minimize harm to animals.

Author contributions

The authors confirm their contributions to the paper as follows: methodology, formal analysis, writing – original draft, data curation: Liu X; investigation, validation: Yossief GS, Liu B; supervision, writing – review and editing: Zhu L, Zheng X; resources, investigation, visualization: Jing X*, Yu Y; funding acquisition, writing – review and editing, project administration: Sun C, Liu B*. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Acknowledgments

This work was supported by the China Agriculture Research System of MOF and MARA (CARS-48); the Central Public-Interest Scientific Institution Basal Research Fund, CAFS (No. 2025XT0801); the Central Public-Interest Scientific Institution Basal Research Fund, CAFS (2023TD63) and the '333 High Level Talent Project in Key Industry' of Jiangsu Province.

Conflict of interest

The authors declare they have no conflict of interest.

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