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Effect of replacement of fish meal by meat and bone meal and poultry by-product meal in diets on the growth and immune response of *Macrobrachium nipponense*

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Abstract

The potential use of poultry by-product meal (PBM) and meat and bone meal (MBM) as alternative dietary protein sources for juvenile *Macrobrachium nipponense* was studied by a 70-day growth trial. Triplicate groups of *M. nipponense* (initial body weight: 0.37 g) were fed at 20.7–22.4 °C on each of the five isoenergetic and isonitrogenous diets (protein content about 38%) with different replacement of fish meal by MBM or PBM. The control diet used white fish meal as the sole protein source, the other four diets were prepared with 15% or 50% fish meal protein substituted by either MBM (MBM_{15}, MBM_{50}) or PBM (PBM_{15}, PBM_{50}). The results showed that replacement of fish meal by MBM in diets did not affect growth performance of *M. nipponense* ($P > 0.05$), while specific growth rate in PBM_{15} was significantly higher than that in other groups ($P < 0.05$). Survival rates of shrimp fed with MBM_{15} diet were significantly higher than that in other groups ($P < 0.05$). No significant differences in immunological parameters, including total haemocyte count (THC), phenoloxidase activity (PO) and respiratory burst (O_2^-), were observed between the shrimps that were fed five experimental diets, and all determined immunological parameters in control groups were slightly higher than those in replacement groups. In conclusion, either MBM or PBM investigated could replace up to 50% fish meal protein in diets for *M. nipponense*.

Keywords: Meat and bone meal; Poultry by-product meal; Growth; Immune response; *Macrobrachium nipponense*

1. Introduction

Macrobrachium nipponense is an economically important freshwater shrimp. It is widely reared in P.R. China, Japan and other South-East Asian countries. In recent years, the farming production of *M. nipponense* increased quickly in P.R. China. This fast development partly relied on the increased

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production of the formulated diets for this species. In previous studies, the dietary protein requirement of juvenile *M. nipponense* was determined to be about 38% of the diet [1]. High-grade white fish meal was traditionally used as a major or even the sole protein source in the commercial diets, which highly increased its production cost. Thus, it is imperative to reduce feed cost by exploring cheaper alternative protein sources in the diets.

Meat and bone meal (MBM) and poultry by-product meal (PBM) are two potential alternative protein sources because of their high protein content (45–65%) and low cost compared to fish meal, and have been successfully used for replacement of fish meal in diets of many aquatic animals [2].

In the context of researching alternative protein sources for aquatic animals, growth performance, feed utilization and whole body composition were the main parameters for evaluation. Dietary changes often cause no grossly observable signs, but they may severely influence the organism's health status, which would not emerge from nutritional parameters. The effect of different proteins on immune function should also be taken into consideration, but this has been seldom addressed. Bransden et al. [3] reported that the replacement of fish meal by dehulled lupin meal or hydrolysed poultry feather meal had no effect on immune function in Atlantic salmon *Salmo salar* L. However, an effect of dietary inclusion level of soybean meal on immunological parameters of Atlantic salmon was observed [4]. Similar results were reported in rainbow trout *Oncorhynchus mykiss* that serological and non-specific defense mechanisms increased when fed with soybean based diets compared to the fish meal based control diets [5]. In channel catfish *Ictalurus punctatus*, immune parameters including total cell count, red blood cell count and hemoglobin content were significantly affected by the interaction between dietary levels of cottonseed (replacing soybean meal in diet) and iron supplementation [6]. Obviously, changes of immune parameters must be considered as important criteria for evaluating the nutritive value of alternative proteins for fish meal [3]. However, no relevant study has been previously conducted in shrimp.

Invertebrate animals lack true antibodies and have to rely solely on innate immune mechanisms. To evaluate the immune ability of the shrimp, the three immune parameters most widely assayed are total haemocyte count (THC), phenoloxidase (PO) activity and respiratory burst or superoxide anion (O_2^-) production [7,8]. The circulating haemocyte or total haemocyte count (THC) of decapod crustaceans plays an important role in regulating the physiological functions and varies with intrinsic or extrinsic factors [9]. The prophenoloxidase (proPO) system is activated by several microbial polysaccharides from fungal cell walls [10]. The activity of phenoloxidase has been detected in a wide range of crustaceans [11]. The production of superoxide anion known as respiratory burst plays an important role in microbicidal activity [12]. Song and Hsieh [13] first demonstrated a respiratory burst in *Penaeus monodon* haemocytes. It has been reported in haemocytes of *Penaeus stylirostris* [14], *Penaeus vannamei* [15], *Macrobrachium rosenbergii* [16] and *Litopenaeus vannamei* [17].

The present study was performed to evaluate the effect of replacement of fish meal by meat and bone meal and poultry by-product meal in diets on the growth and immune response of *M. nipponense*.

2. Materials and methods

2.1. Experimental diets

MBM and PBM were supplied by the Asian Regional Office of the National Renderers Association (NRA), USA. All the dietary ingredients were analyzed for chemical composition (Table 1) prior to the formulation of diets. White fish meal was used in the control diet as the only protein source, and the other four diets were prepared to contain 15% and 50% MBM (MBM_{15}, MBM_{50}) or PBM protein (PBM_{15}, PBM_{50}). All diets were formulated to be approximately isoenergetic and isonitrogenous (38% dietary protein) (Table 2). The diets were made into sinking pellets using a pellet machine, oven-dried at 60 °C and stored at 4 °C.

Table 1
Proximate composition of the ingredients used in the experimental diets (g 100 g^{-1} dry matter)

	White fish meal (USA) (FM)	Poultry by-product meal (NRA, USA) (PBM)	Meat and bone meal (NRA, USA) (MBM)
Protein	63.65	60.84	55.02
Lipid	10.06	18.99	13.60
Ash	21.42	12.82	25.34
Moisture	2.03	2.98	2.10

2.2. Shrimp, experimental conditions and feeding

The experiment was conducted in an indoor recirculation system comprising 15 circular plastic tanks with flat bottoms (water volume: 90 L). Each tank was provided with continuous aeration. During the experiment, water quality was monitored weekly. Water temperature ranged from 20.7 to 22.4 °C and pH from 7.1 to 7.3. Photoperiod was 12D:12L with the light period from 0800 to 2000 h. Dissolved oxygen was more than 7.2 mg L^{-1}, ammonia-N was less than 0.5 mg L^{-1}, and residual chloride was less than 0.01 mg L^{-1}.

Juvenile *M. nipponense* in the intermolt stage were obtained from the Liangzihu hatchery (Hubei, P.R. China) and then stocked in the experimental system for acclimation. The shrimps were fed the control diet for two weeks and then an equal mixture of five experimental diets for one week prior to the experiment. At the start of the experiment, all the shrimps were deprived of food for one day. Forty shrimps were randomly selected, weighed and stocked into each tank. Three tanks were randomly assigned to each diet. Initial body weight of the shrimp was about 0.37 g. Shrimps were fed four times a day at about 0800, 1100, 1400 and 1600 h to satiation, seven days per week. In each tank, uneaten feed and fecal pellets in the water were siphoned off every day, and about 70% of the water was exchanged weekly to maintain water quality.

The growth trial lasted for 70 days. At the end of the trial, the shrimps from each tank were bulk weighed after one day of food deprivation. Growth and survival rate were calculated. After the final weighing, shrimps were randomly taken from each tank. Haemolymph (100 μl) was withdrawn from the ventral sinus of several shrimps in the same tank into a 1 ml sterile syringe (25 gauge) containing 0.9 ml ice-cold anticoagulant solution (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6) [18]. Haemolymph was then placed in 1.5 ml Eppendorf tubes for THC, O_2^- and PO activity determination. For each parameter, two analyses were performed in each tank.

2.3. Chemical analyses

Dry matter was determined by drying to constant weight at 105 °C [19]. Nitrogen was determined by the semi-Kjeldahl's method, and protein content was calculated from the nitrogen content multiplied by 6.25. Lipid was determined by chloroform/methanol extraction [20], ash by combustion at 550 °C, and energy content by bomb calorimetry (Phillipson microbomb calorimeter, Gentry Instruments Inc., Aiken, USA). Dietary Ca^{2+} and Mg^{2+} content were analyzed by inductively coupled plasma (ICP) spectrophotometry (IRIS Advantage, USA) at the Analysis Centre of Hubei Academy of Agricultural Sciences, P.R. China. At least two measurements were made for each sample.

2.4. Immune parameters

Three immune parameters including THC, PO activity and O_2^- were assayed. A drop of the anticoagulant–haemolymph mixture was placed on a haemocytometer and the THC was counted under an inverted phase microscope with 40× magnification (Olympus, BH-2).

Table 2
Formulation and chemical composition of the five different diets

Ingredients	Control	MBM_{15}	MBM_{50}	PBM_{15}	PBM_{50}
Fish meal (USA)	57.00	48.45	28.50	48.45	28.50
MBM (NRA, USA)	0.00	9.89	32.97	0.00	0.00
PBM (NRA, USA)	0.00	0.00	0.00	8.95	29.82
Cornstarch	15.00	15.00	15.00	15.00	15.00
Combined oil[a]	2.79	2.31	1.18	1.96	0.00
Cholesterol	0.50	0.50	0.50	0.50	0.50
Soybean lecithin	2.00	2.00	2.00	2.00	2.00
CMC	3.00	3.00	3.00	3.00	3.00
Cellulose	19.71	14.13	12.13	15.42	16.46
Vitamin premix[b]	1.50	1.50	1.50	1.50	1.50
Choline	0.20	0.20	0.20	0.20	0.20
Vitamin C	0.02	0.02	0.02	0.02	0.02
Mineral premix[c]	3.00	3.00	3.00	3.00	3.00
Chemical composition (*in dry matter*)					
Dry matter (%)	93.00	93.63	93.77	93.48	93.50
Protein (%)	38.30	38.46	38.81	38.97	38.95
Lipid (%)	9.76	10.45	9.12	9.70	9.64
Ash (%)	16.85	17.93	19.12	16.52	16.19
Energy (kJ/g)	17.22	17.75	18.19	17.29	18.98
Ca^{2+} (%)	4.35	4.35	5.16	4.09	3.93
Mg^{2+} (%)	0.19	0.19	0.20	0.19	0.18

[a] Fish oil:corn oil (7:3).

[b] Vitamin premix (mg g^{-1} premix): vitamin A, 1.0; vitamin D_3, 0.63; vitamin E, 10; vitamin K_3, 1.8; niacin, 5; riboflavin, 2.63; pyridoxine, 1; aminobenzoic acid, 5; thiamin, 0.75; D-calcium pantothenate, 5; biotin, 0.75; folic acid, 0.19; vitamin B_{12}, 0.15; inositol, 60. α-cellulose, 905.75.

[c] Mineral premix (mg g^{-1} premix): KCl, 28; $MgSO_4 \cdot 7H_2O$, 100; NaH_2PO_4, 215; KH_2PO_4, 100; $Ca(H_2PO_4)_2 \cdot H_2O$, 265; $CaCO_3$, 105; $C_6H_{10}CaO_6 \cdot 5H_2O$, 165; $FeC_6H_5O_7 \cdot 5H_2O$, 12; $ZnSO_4 \cdot 7H_2O$, 4.76; $MnSO_4 \cdot H_2O$, 1.07; $AlCl_3 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, 0.24; $CoCl_2 \cdot 6H_2O$, 1.4; KI, 0.23. α-cellulose, 2.15.

The methods reported by Cheng and Chen [16] with slight modification were used to assay PO and O_2^-. Phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA). The diluted haemolymph was centrifuged (Beckman, Allegra™ 64R) at 300×g at 4 °C for 10 min, the supernatant fluid was discarded and the pellet was rinsed, re-suspended gently in 1 ml cacodylate–citrate buffer (sodium cacodylate 0.01 M, sodium chloride 0.45 M, trisodium citrate 0.10 M, pH 7.0) and then centrifuged again. The pellet was then re-suspended with 200 μl cacodylate buffer (sodium cacodylate 0.01 M, sodium chloride 0.45 M, calcium chloride 0.01 M, magnesium chloride 0.26 M, pH 7.0). Aliquots (100 μl) were incubated with 50 μl of trypsin (1 mg ml^{-1}), which served as an elicitor, for 10 min at 25–26 °C. Fifty microliters of L-DOPA was then added, followed by 800 μl of cacodylate buffer 5 min later. The optical density at 490 nm was measured using a Unico™ UV-2000 spectrophotometer (Shanghai, P.R. China). The control solution which consisted of 100 μl cell suspension, 50 μl cacodylate buffer (to replace the trypsin) and 50 μl L-DOPA was used for the background phenoloxidase activity in all test conditions. The background phenoloxidase activity optical density values were in the range of 0.02–0.09. The phenoloxidase activity optical density value of shrimp was expressed as dopachrome formation per 50 μl haemolymph.

Respiratory burst of haemocytes was quantified using the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion (O_2^-). Briefly, 100 μl haemolymph in anticoagulant solution was deposited in triplicate in flat-bottomed 96-well microtitre plates previously coated with 100 μl poly-L-lysine solution (0.2%) to improve cell adhesion. Microplates were cytocentrifuged (Hettich, Universal-16,

Germany) at 300×g for 15 min. Plasma was removed and then 100 μl zymosan (0.1% in Hank's solution minus phenol red, Sigma) was added to react for 30 min at room temperature. Zymosan was discarded and the haemocytes were washed three times with 100 μl Hank's solution and stained with 100 μl NBT solution (0.3%) for 30 min at room temperature. The staining reaction was terminated by removing the NBT solution and adding 0.1 ml 100% methanol. After washing three times with 10 μl 70% methanol, the haemocytes were air-dried. Formazan was dissolved by adding 120 μl 2 M KOH and 140 μl dimethyl sulfoxide. The optical density at 630 nm was measured in triplicate using a microplate reader (Bio-RAD, Model 550). Respiratory burst was expressed as NBT-reduction per 10 μl haemolymph.

2.5. Statistical analysis

One-way analysis of variance was used to test the effects of the diets. Duncan's procedure was used for multiple comparisons. Differences were regarded as significant when $P < 0.05$.

3. Results

3.1. Growth performance and survival

Final body weight (FBW), specific growth rate (SGR), and survival of the shrimp are shown in Table 3. Replacement of fish meal by MBM in diets did not affect growth performance of shrimp ($P > 0.05$), while SGR in PBM_{15} was significantly higher than that in other groups ($P < 0.05$). Survival rates of the shrimps fed with MBM_{15} diet were significantly higher than that in other groups ($P < 0.05$).

3.2. Immune parameters

THC in haemolymph of *M. nipponense* fed five diets is shown in Fig. 1. No significant differences in THC were observed in the shrimp fed different diets ($P > 0.05$). The means (±SE) of THC varied from $18.00 \pm 4.79 \times 10^4$ to $27.50 \pm 3.18 \times 10^4$ cells ml^{-1}.

The highest PO activity values were observed in the control group, but it is not significantly different from the others (Fig. 2). The phenoloxidase activity varied from 0.05 to 0.14.

No significant difference in respiratory burst was observed among the shrimp fed different diets ($P > 0.05$) (Fig. 3). The respiratory burst of shrimp varied from 0.01 to 0.04 expressed as NBT-reduction per 10 μl haemolymph.

Table 3
Growth performance of *M. nipponense* fed experimental diets for 70 days (mean±SE, $n = 3$)

Diets	Control	MBM_{15}	MBM_{50}	PBM_{15}	PBM_{50}
IBW (g)	0.36 ± 0.00	0.37 ± 0.01	0.37 ± 0.01	0.38 ± 0.01	0.38 ± 0.00
FBW (g)	0.60 ± 0.06^{a}	0.56 ± 0.02^{a}	0.62 ± 0.01^{ab}	0.70 ± 0.01^{b}	0.59 ± 0.01^{a}
SGR (%)	0.70 ± 0.15^{a}	0.59 ± 0.06^{a}	0.74 ± 0.06^{a}	0.88 ± 0.05^{b}	0.63 ± 0.03^{a}
Survival (%)	25.00 ± 5.20^{a}	37.50 ± 1.44^{b}	23.33 ± 4.64^{a}	24.17 ± 0.83^{a}	29.17 ± 0.83^{ab}

Means in the same row with different superscripts (a,b) are significantly different ($P < 0.05$).
IBW: initial body weight (g); FBW: final body weight (g).
WG (%): weight gain = 100 × (FBW − IBW)/IBW.
SGR (%/d): specific growth rate = 100 × (ln FBW − ln IBW)/70 days.
Survival rate (%) = 100 × final shrimp number/initial shrimp number.

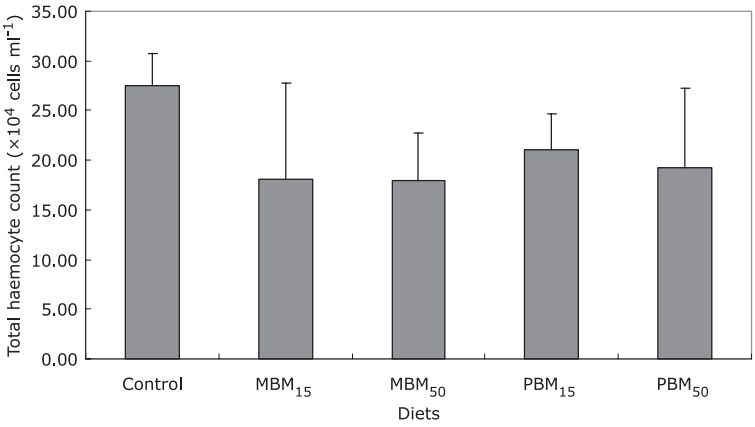

Fig. 1. Mean (±SE) total haemocyte count of *M. nipponense* fed different diets.

4. Discussion

MBM and PBM have been widely studied as two important alternative protein sources for fish meal in fish diets. Generally, MBM is rarely included in commercial aquafeeds at levels higher than 20% [21]. However, more positive results have been reported with sea bream *Sparus aurata* [22], rainbow trout *Salmo gairdneri* [23] and tilapia *Oreochromis mossambicus* Peters [24]. PBM seemed to be a more promising protein and generally could efficiently substitute up to 50% fish meal protein in fish diets [25–27]. When high quality PBM was used, many species could tolerate up to 100% replacement of fish meal [27–30].

In the present study, shrimps fed diets containing MBM or PBM all showed good growth performance. The highest final body weight in PBM_{15} group was 20% higher than that of the control, which might be due to the higher digestibility of PBM [31], thereby enhancing growth. However, the fact that 50% replacement of PBM caused low SGR as compared to the replacement of PBM at 15% might be due to the poorer essential amino acid balance than PMB_{15}. The utilization of PBM depends on the quality of the products, which vary among producers [32]. Our results are in agreement with those of Davis and Arnold [33], who observed that co-extruded soybean poultry by-product meal and flash dried poultry by-product meal could

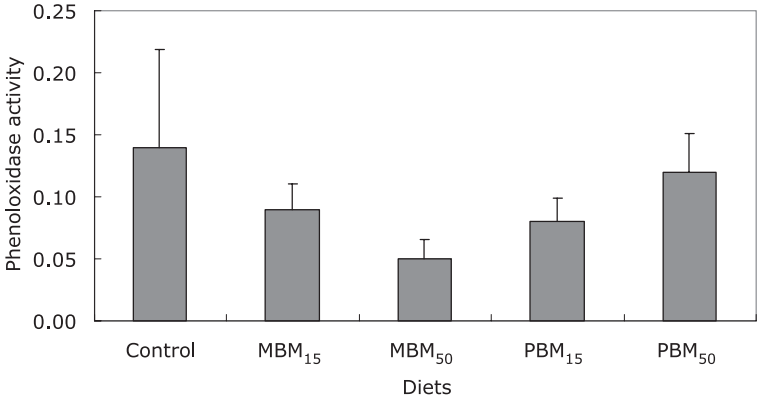

Fig. 2. Mean (±SE) phenoloxidase activity in the haemocytes of *M. nipponense* fed different diets.

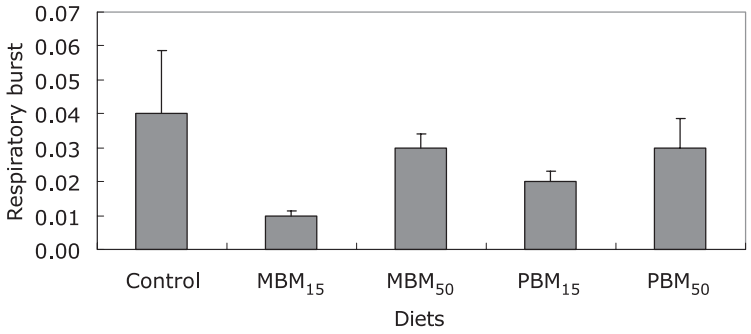

Fig. 3. Mean (±SE) respiratory burst in the haemocytes of *M. nipponense* fed different diets.

efficiently substitute a certain percentage of fish meal in practical diets for juvenile *L. vannamei* (mean initial wt. ± S.D., 0.37 ± 0.015 g) without an adverse effect on growth performance. Similar results were also observed in chinook salmon *Oncorhynchus tschawytscha* W. [26] and gilthead seabream [29].

In several marine species, replacement of fish meal with MBM generally negatively affected the growth performance of fish [34,35]. However, Stone et al. [36] reported that MBM products were successfully used to replace 50% fish meal protein in silver perch *Bidyanus bidyanus* diets. Similarly, gilthead bream can tolerate up to 40% substitution of MBM (providing about 40% protein) for fish meal in their diets [22]. More positive results have been reported by Davies et al. [24], who found that an optimum ratio of MBM could effectively replace up to 75% of fish meal in practical diets for tilapia, and diets containing MBM were superior as complete alternative protein to fish meal. Watanabe and Pongmaneerat [23] reported that the growth rate of rainbow trout fed five diets with MBM as the sole protein at different dietary protein levels (5–40%) was higher than those fed fish meal diets. Our results supported these reports. High replacement of MBM (50%) showed better growth, but experienced higher mortality as compared to the replacement of MBM at 15%. The lowest survival rate in the MBM_{50} group, which was not significantly different to the control group, suggests that MBM could replace fish meal protein up to 50%. However, Teskeredzic et al. [37] suspected that diets containing MBM at 60–65% caused high mortality in rainbow trout fry.

In decapod crustaceans, circulating haemocytes are associated with cellular defence [38]. It was known that THC is a useful indicator of shrimp health [39]. In our present study, the total haemocyte count of *M. nipponense* varied from $18.00 \pm 4.79 \times 10^4$ to $27.50 \pm 3.18 \times 10^4$ cells ml^{-1} (means ± SE). It was lower than those reported in *M. rosenbergii* [16] and *P. monodon* [40]. This could be partly attributed to the lower rearing temperature in our experiment. It is well known that several extrinsic factors like temperature, pH, salinity and dissolved oxygen affect the circulating haemocyte count of crustaceans [7]. A certain increase in water temperature has been reported normally to increase THC in several crustaceans [7]. *M. rosenbergii* reared at temperatures of 27–28 °C and 30–31 °C had significantly higher THC than those reared at 20–21 °C and 33–34 °C [41]. In *P. stylirostris* exposed to low temperature (18 °C), a significant drop in THC (40%) was observed compared to that for prawns at 27 °C [7]. Increase of temperature from 10 to 20 °C, and from 18 to 32 °C has been reported to increase THC of *Carcinus maenas* and *Penaeus californiensis*, respectively [42,43]. On the other hand, differences in THC are also found between species [44]. No available figure of THC in *M. nipponense* could be compared before our present study.

The important role of the prophenoloxidase system in invertebrate defense system has been largely documented [11,45]. The proPO system can be activated and converted to active PO. The PO activity is activated by several microbial polysaccharides, and is dependent on high temperature and divalent calcium and magnesium cations (Ca^{2+} and Mg^{2+}) in *P. monodon* [46]. Similar to that in other crustaceans, PO activity in *Penaeus paulensis* and *M. rosenbergii* was enhanced by suitable content of Ca^{2+} and Mg^{2+} and

was inhibited by high concentrations of Ca^{2+} and Mg^{2+} [46,47]. MBM generally contains higher ash content and Ca^{2+} concentration than fish meal. In the present study, dietary Ca^{2+} concentration increased with increased MBM inclusion while it decreased with increased PBM inclusion. This might account for a similar tendency in the PO activity though they were not significant. Further research to study this correlation of Ca^{2+} concentration and PO activity is warranted. However, the highest PO activity was observed in the control group. Lee and Shiau [40] reported the depressing effect of the high level supplementation of C2MP-Mg on the PO activity in *P. monodon*. In the present study, Mg^{2+} contents in different diets were similar. No obvious correlation was observed between dietary Mg^{2+} content and PO activity.

Phagocytosis is an important reaction of cell defense to eliminate microorganisms or foreign particles in invertebrate animals since they lack true antibodies and have to rely solely on the innate immune mechanism [15]. During phagocytosis, reactive oxygen species are produced. This phenomenon, known as respiratory burst, plays an important role in microbicidal activity [13]. Because O_2^- is the first product released from a respiratory burst, O_2^- measurement has been accepted as an accurate method for estimating the cell's capability to generate a respiratory burst [13,15]. It is well known that O_2^- production can be affected by environmental factors [7] and can be enhanced by treatment with immunostimulants [46], but the effect of nutritional factors on O_2^- production has received little attention. Lee and Shiau [40] first reported that intracellular superoxide anion production of the haemocytes in *P. monodon* fed diets containing ascorbate supplements was approximately 1.27–2.24 times higher than that of shrimp fed unsupplemented diets. It has been demonstrated that O_2^- generation by shrimp *P. stylirostris* [14] or oyster *Crassostrea gigas* [48] haemocytes depended on the presence of NADPH oxidase enzyme, a cell membrane-bound enzyme. In the present study, superoxide anion production by the haemocytes in groups fed with MBM or PBM diets was not significantly different from that of the control. This fact indicated that the NADPH oxidase was not affected by current nutritional treatment. The present results showed that neither O_2^- production nor THC of *M. nipponense* was significantly affected by MBM or PBM inclusion. The superoxide anion decrease in hypoxic *P. stylirostris* related to the decrease in THC has been reported by Le Moullac et al. [14].

In the authors' opinion, either MBM or PBM could replace up to 50% fish meal protein in the diets for *M. nipponense* without significant negative effect on the growth and survival performance and immunological parameters.

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