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Growth performance, immune response, and digestive enzyme activity in Pacific white shrimp, *Penaeus vannamei* Boone, 1931, fed dietary microbial lysozyme

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ABSTRACT

The present study investigates the effects of the dietary microbial lysozyme (ML) as an immunostimulant, on the growth performance, some immune parameters and digestive enzyme of Pacific white shrimp, *Penaeus vannamei.* Six hundred shrimps were obtained and randomly allocated into four groups as follows with three replicates. The shrimps were fed diets supplemented with 0 (control), 0.5, 1, and 2 g kg^{-1} ML for 4 months. The results indicated that dietary supplementation of ML significantly improved final weight, weight gain, average daily weight gain rate (ADG), feed conversion rate (FCR), and feed efficiency rate (FER) compared to the control ($P < 0.05$). However, weight gain specific growth rate (SGR) and survival rate were not significantly affected by dietary ML ($P > 0.05$). Dietary ML had a progressive effects on some immune parameters status including total antioxidant capacity (TAOC), superoxide dismutase (SOD), glutathione peroxidase (GPX), lysozyme (LYZ), alkaline phosphatase (ALP), phenoloxidase (PO) and acid phosphatase (ACP) activity as well as differential haemocyte count (DHC) and total haemocyte count (THC), in shrimps treated with the lysozyme than untreated shrimps ($P < 0.05$). However, feeding with ML had no significant effect on plasma malondialdehyde (MDA) level ($P > 0.05$). Furthermore, intestinal digestive enzymes (lipase, protease, and amylase) in shrimp fed with dietary ML were significantly ($P < 0.05$) higher than those fed with non-supplemented control basal diet. Thus, the results indicate that oral administration of ML can be recommended for shrimp feed to improve immune response as well digestive enzymes activity modulation.

1. Introduction

Pacific white shrimp, *Penaeus vannamei* is the most economically important species for aquaculture in an extensive, semi-intensive, and intensive systems in many parts of the world [1,2]. Shrimp culture industry has been threatened by a tension of diseases which considered as a primary concern and a limiting factor [3]. It is very difficult to keep a shrimp free of pathogens [4], infectious diseases [5,6] stress and pollution.

Crustaceans including shrimp must rely on innate defense mechanisms [7] and non-specific immunity to ensure efficient defense responses against bacterial, fungal and viral attack that continually threaten their survival [8]. In principles, antibiotics administration in diets for growth promotion and disease prevention [9] caused excessive use of antimicrobials which negatively affects the shrimp health status [5], and also led to the development of drug residues in aquatic animal [2], food safety problems, decrease their effectiveness [10] and environmental threats [11]. Therefore, it is necessary to use the healthy feed additives supplement to improve the immune response [12] and keep shrimps protected against pathogen outbreaks [13]. Immunostimulants are a group of biological and synthetic compounds [14] (e.g. probiotics, prebiotics and symbiotic) [15,16] that stimulate the nonspecific cellular and humoral defense in animal's immune responses [17]. Among all types of immunostimulants, one way for improving the feed safety and control diseases is to use antimicrobial enzymes in diet [11,18–21].

Lysozyme (EC 3.2.1.17) is recognized as a mucolytic enzyme [11] constructed of 130 amino acids with a molecular weight of 14.5 kD [22,23]. This enzyme found in animals, plants, and microorganisms [24] secretions and is considered as an important portion of the innate immune system [19]. Lysozymes specified by their ability to break down the β-(1, 4)-glycosidic bond between *N*-acetylmuramic acid and

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N-acetylglucosamine in peptidoglycan cell wall [11]. The lysozyme has antimicrobial activity against both gram-negative [25] and gram-positive bacteria [26]. Meanwhile, antibacterial, antiviral, antifungal and antiparasitic activities, as well as antiinflammatory and antioxidant action of lysozyme, is suitable for use in aquaculture [27,28]. However, because of the lacking operability of injections in shrimp culture, oral administration of lysozyme is performed.

There is limited available information regarding administration of microbial lysozyme as feed additive in aquaculture. Although few studies demonstrate that lysozyme is beneficial for the growth and immune status of fish [11,18,29,30], a few studies investigate its effects on the immune response of shrimp [24,25]. Considering the lack of knowledge about different aspects of ML administration as feed additive on shrimps, the present study was performed to investigate possible effects on growth performance, some immune parameters and digestive enzyme of Pacific white shrimp, *Penaeus vannamei.*

2. Material and methods

2.1. Experimental animals and conditions

Six hundred apparently healthy Pacific white shrimps were obtained from a valid private sector farm with an average body weight of 1.32 ± 0.02 g. The experiment was conducted at Kolahi Breeding Center (KBC) in southern Iran. Shrimps were randomly distributed into 12 indoor cylindrical fiberglass tanks and fed the commercial diet for 7 days to acclimatize to experimental conditions. After the acclimatization period, the experiment started for 4 months with 3 experimental groups and a control, each treatment repeated in triplicates. All the experimental tanks contained aerated filtered seawater (salinity 35 ± 0.4 g l^{-1}, pH 8.2 ± 0.3, dissolved oxygen level $^{>}$4.0 mg O l^{-1} and temperature 32 ± 0.4 °C) with constant aeration using air-stones connected to an air pump. Every three days once, the water was partially changed (about 50%). The light regime was set at 14 h light and 10 h dark. Shrimps were hand-fed three times (7:00, 12:00, and 17:00) daily at a rate of 3% of body weight. Based on every 10-days biometric survey, the feeding ratio was corrected. Utmost care was taken to avoid feed lost.

2.2. Key resource table

Resource	Source	Identifier
Chemical		
4.6-Ethylidene		
acetate		
ammonium bicarbonate		
cumene hydroperoxide		
diethanolamine		
DOPA-chrome		
ethylene diaminetetraacetic acid		
fatty acid		
glutathione		
H2O2		
L-DOPA		
malondialdehyde		
MDA		
MgCl		
MgCl2		
NADP		
NADPH		
NaOH		
n-butanol		
pyridine		
SOD		
sodium		
sodium chloride		
sulfate		
Tetramethoxy propane		
thiobarbituric acid		
trichloroacetic acid		
Tris		
trisodium citrate		
ρ-nitrophenol		
ρ-nitrophenyl		
ρ-nitrophenyl phosphate		
ProteinPeptide		
LYZ		
phosphatase		
trypsin		

2.3. Experimental diet

A commercial diet (Faradaneh Co., Iran) was used as basal diet in this experiment. The chemical composition of the basal diet is shown in Table 1. Microbial lysozyme 10% (Fermented lysozyme use as feed additives) supplemented by Zhejiang Aegis Biotech Co.,Ltd (China) and added to the basal diet at 0 (control), 0.5, 1, and 2 g kg^{-1} diet. The inclusion levels were selected based on the previous study [30]. To prepare each experimental diet, the proper amount of ML was dissolved in 50 ml of distilled water and sprayed on 1 kg diet. The control diet was sprayed with an equivalent volume of distilled water to preserve an equal level of moisture. The diets were kept at 4 °C until use.

2.4. Sample collection

2.4.1. Immunological assays

At the end of the 129-day feeding trial, the shrimps have fasted for 24 h before sampling. Haemolymph was collected from 6 individuals from each treatment by a 1-ml sterile syringe (equipped with a 25-gauge needle) containing 0.9 ml precooled anticoagulant (30 mM trisodium citrate, 388 mM sodium chloride, 115 mM glucose and10 mM ethylene diaminetetraacetic acid) from the ventral sinus cavity, located at the base of the first abdominal segment. The drawn haemolymph was divided into two parts. One part was used to measure the total haemocyte count (THC) and differential haemocyte count (DHC). The remaining was centrifuged at 4600 *g* for 10 min at 4 °C and the supernatant was collected in a fresh sterile tube and stored at −80 °C to determine the total antioxidant capacity (TAOC), superoxide dismutase (SOD), glutathione peroxidase (GPX), lysozyme (LYZ), alkaline phosphatase (ALP), malondialdehyde (MDA), phenoloxidase (PO) and acid phosphatase (ACP).

2.4.2. Digestive enzyme assays

To evaluate the effects of dietary lysozyme on digestive enzymes activity including amylase, protease, and lipase, the intestines from 6 shrimps from each treatment were dissected and removed. Then each dissected intestine homogenized by adding 0.9% ice-cold sterile saline solution to prepare 10% (W:V) homogenates. The obtained homogenates were centrifuged at 1000 *g* for 10 min at 4 °C. These samples were stored at −20 °C until analysis which were done within 24 h.

Table 1
Analysis of the commercial feed for shrimp (Faradaneh, Iran).

Analyses	Composition
Protein	41–43%
Lipid	7–10%
Crude fiber	2–4%
Ash	8–13%
Moisture	5–10%
Total P	1.24–1.5%
Digestible energy	3.95–4 kcal g^{-1}

2.5. Analytical methods

2.5.1. Growth performance

The mean weight gain (WG; g), feed conversion ratio (FCR), feed efficiency (FER %), average daily weight gain rate (ADG %), and survival rate (SR %) for all groups were calculated with the following equations:

Weight gain (WG; g) = (final body weight – initial body weight) / initial body weight

Feed conversion rate (FCR) = feed intake (g) / weight gain (g)

Average daily weight gain rate (ADG (% day^{-1})) = 100 × [(final weight) – (initial weight)] / days

Feed efficiency rate (FER %) = (100 × fresh body weight gain) / dry feed intake

Survival rate (SR %) = 100 × (final number of shrimps / initial number of shrimps)

2.5.2. Immunological parameters analysis

Anticoagulant-haemolymph mixture was placed on a haemocytometer (Neubauer, Germany) to measure the total haemocyte count (THC), hyaline cells (HC), semi-granular cells (SGC), and granular cells (GC). The number of cells ml^{-1} was counted using a light microscope (Optika, Italy) at 100 × magnification.

The LYZ activity (U ml^{-1}) in the shrimp haemolymph was measured by the turbidimetric assay. According to Ellis [31], haemolymph with hen egg white lysozyme as standard was placed in a plate and suspension of *Micrococcus luteus* (Sigma, USA) was added. One unit of lysozyme activity was determined as a reduction in absorbance of 0.0001 per minute.

The SOD activity was measured by a commercial kit (ZellBio GmbH, Germany) according to the manufacture's instruction. In this assay, the SOD activity unit (U ml^{-1}) considered as the amount of the sample that will catalyze the decomposition of 1 μmole of O_2^- to H_2O_2 and O_2 in 1 min. In this procedure, the final product made a specific chromogen to a color which was measured calorimetrically at 420 nm [32].

The MDA was assayed according to Ohkawa et al. [33]. Briefly, the plasma supernatant was mixed with sodium dodecyle sulfate, acetate buffer and an aqueous solution of thiobarbituric acid. After heating, the red pigment produced was extracted with an *n*-butanol-pyridine mixture and estimated by the absorbance at 535 nm. Tetramethoxy propane was used and lipid peroxide level was expressed in μmol l^{-1}.

The PO activity was determined photometrically [34]. Obtained plasma samples were diluted in TBS (10 mM Tris, 336 mM NaCl, 5 mM $CaCl_2$, 10 $MgCl_2$, pH 7.6) and pre-incubated with an equal volume of the enzyme inducer trypsin (Sigma). Trypsin and plasma were replaced by TBS. After incubation, the red pigment L-DOPA was added and the formation of DOPA-chrome was monitored after 0, 5, 15 and 25 min. PO activity was expressed as the variation in absorbance (490 nm) per minute and the level expressed in μmol l^{-1}.

The GPX activity was assayed according to Paglia and Valentine [35]. The oxidized form of glutathione converted to the reduced form with oxidation of NADPH to $NADP^+$ with ELISA reader and calculate GPX activity with a commercial kit (ZellBio, Germany). Diluted plasma was added to the reaction mixture containing cumene hydroperoxide and buffer. The density of NADPH was measured at 412 nm, and the rate of reaction was estimated from the absorbance of cumene hydroperoxide. The level expressed as U ml^{-1}.

The phosphatase (ACP and ALP) activity was estimated photometrically based on the method described by Burtis et al. [36]. Crude enzyme solutions were incubated with substrate ρ-nitrophenyl phosphate in diethanolamine (Pars Azmoon, IRAN) -MgCl buffer. Afterward, nitrophenolphosphate was added and the absorbance measured photometrically at 405 nm and the activity expressed as mg ml^{-1}.

The value of the TAOC was determined using a commercial Kit (ZellBio, Germany). The supernatant sample was added to the reagents and incubated. The absorbance was measured at 490 nm with a microplate reader [37]. The level expressed as μmol l^{-1}.

2.5.3. Digestive enzymes analysis

Protease activity was measured based on Ross et al. method [38]. An equal volume of category enzyme extraction (CME) was incubated with ammonium bicarbonate buffer containing azocasein. The reaction was stopped by adding trichloroacetic acid and cooled on ice. Then it was centrifuged and the supernatant was added to a microplate containing an equal volume of NaOH (Sigma). Trypsin (Sigma) and buffer were used instead of the sample as positive and negative controls, respectively. The protease activity was measured by the absorbance at 450 nm and the level expressed as U mg protein $^{-1}$.

Amylase was determined according to the substrate 4.6-Ethylidene-(G7)-ρ-nitrophenyl-(G1)-α-D-maltoheptaoside to reduce oligosaccharide, producing glucose and ρ-nitrophenol. The increase of absorbance at 405 nm represents the total amylase activity [39] and the amount expressed as U mg protein $^{-1}$.

Lipase activity was determined by fatty acid release and hydrolysis of triglycerides in an emulsion of olive oil [40]. Enzyme level measured from the absorbance using a photometer by the absorbance at 580 nm. Lipase activity was expressed as U mg protein $^{-1}$.

The total protein activity was measured according to Bradford [41] method by using bovine plasma albumin as standard.

2.6. Statistical analysis

After checking the normality and homogeneity of variance through Shapiro-Wilk and Leven's test respectively, statistical analysis was performed using One way ANOVA followed by Duncan's multiple-range test if the prerequisites of the parametric test were met. Otherwise, equivalent non-parametric test, Kruskal-Wallis and Mann-Whitney *U* test, were applied. Mean values were considered significantly different at $P < 0.05$. Data are expressed as mean values ± S.E. All statistical analyses were conducted using the statistic software SPSS version 16.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Growth performance

The growth performance indices of the specimen during a 129-day feeding trial, are shown in Table 2. There were no significant differences ($P > 0.05$) for initial weight, weight gain, and SGR for shrimp fed with different dietary lysozyme levels compared to the control. On the other hand, the value of the final weight was significantly higher in shrimps of the both 1 and 2 g kg^{-1} ML ($P < 0.05$) than the shrimps fed 0.5 g kg^{-1} and the control. Shrimp fed 2 g kg^{-1} ML had the highest FER. Meanwhile, the FCR in shrimp treated with 2 g kg^{-1} dietary lysozyme was significantly the lowest ($P < 0.05$). Although the FER revealed no significant difference between the groups fed with 0.5 g kg^{-1} dietary lysozyme and the control ($P > 0.05$), the value increased with the level of the dietary lysozyme ($P < 0.05$) in the other treatments.

Moreover, no significant difference was observed in the survival rate ($P > 0.05$) among different treatments.

3.2. Immunological parameters of the plasma

Immune parameters of shrimp fed different levels of dietary ML are presented in Fig. 1. The results indicated that the value of the MDA activity is the same in all the treatments ($P > 0.05$).

The level of ALP, GPX and ACP activity in the shrimps fed ML were higher compared to those in shrimps fed control diet. The values

Table 2
Effect of different dietary lysozyme levels on growth performance of *P. vannamei*.

Parameter	Dietary ML level (g kg^{-1})			
	0	0.5	1	2
Initial weight (g)	1.27 ± 0.04^a	1.36 ± 0.04^a	1.30 ± 0.05^a	1.35 ± 0.03^a
Final weight (g)	28.03 ± 0.49^a	28.11 ± 0.59^a	30.36 ± 0.65^b	31.84 ± 0.39^c
Weight gain (g)	21.22 ± 0.90^a	19.78 ± 1.01^a	22.37 ± 1.11^b	22.50 ± 0.37^c
ADG (% day^{-1})	20.75 ± 0.10^a	20.76 ± 0.21^a	22.52 ± 0.36^b	23.53 ± 0.20^c
FCR	1.20 ± 0.01^c	1.20 ± 0.01^c	1.10 ± 0.02^b	1.06 ± 0.01^a
FER (%)	83.48 ± 0.39^a	83.53 ± 0.86^a	90.62 ± 1.44^b	94.67 ± 0.80^c
Survival rate (%)	66.67 ± 1.76^a	66.00 ± 4.62^a	62.67 ± 2.67^a	67.33 ± 4.67^a

Results are presented as Mean ± S.E. of triplicate observations.
Means in the same row with different superscripts are significantly different ($P < 0.05$). Data analyzed with ANOVA followed by Duncan's multiple-range test.
ADG, average daily weight gain rate; FCR, food conversion rate; FER, food efficiency rate.

corroborated no significant differences among the treatments with 0.5, 1 and 2 g kg^{-1} ML ($P > 0.05$). The plasma PO levels were significantly higher in the treatments fed 1 and 2 g kg^{-1} ML compared to the shrimps fed 0 and 0.5 g kg^{-1} ML ($P < 0.05$). The mean value of plasma TAOC was significantly higher for the treatments fed 1 and 2 g kg^{-1} ML compared to the control ($P < 0.05$); however, there was no significant difference demonstrated between 0.5 g kg^{-1} ML and the control ($P > 0.05$). Also the shrimp fed 2 g kg^{-1} ML had a higher level of the plasma LYZ than the shrimp fed 0.5 g kg^{-1} ($P < 0.05$) whereas, this value was statistically the same among other treatments ($P > 0.05$).

The SOD was significantly affected by dietary ML in a dose-dependence manner. This value was significantly higher in the shrimp fed 2 g kg^{-1} ML ($P < 0.05$). The shrimp in control had the lowest SOD value in the plasma among the treatments ($P < 0.05$).

3.2.1. Haemocyte analysis

The differential haemocyte counts of shrimps fed different levels of dietary ML are presented in Table 3, and the three main types of the shrimp haemocytes under light microscopy are shown in Fig. 2. Hyaline cells have rounded shape, with regular borders, and lack intracellular granules. The semi-granular cell (SGC), has an oval to spindle shape with round and dark small granules. The third type of cells has numerous large granules, was considered as the granular cell (GC). The THC of shrimp fed dietary ML was significantly increased when compared with those of untreated shrimp ($P < 0.05$). The number of GC and SGC were also higher in treated groups than control ($P < 0.05$) whereas there was no significant variation in the hyaline counts ($P > 0.05$).

3.3. Digestive enzyme activity

Fig. 3 represents the effects of feeding on different levels of dietary ML on digestive enzymes activities. The highest protease activity was recorded in the shrimp fed 0.5 g kg^{-1} ML ($P < 0.05$). There was no significant difference in lipase levels among the shrimp fed 0, 0.5, and 1 g kg^{-1} ML ($P > 0.05$). Furthermore, Shrimp fed 0.5 and 1 g kg^{-1} ML showed a notable increase in amylase activity when compared with other groups ($P < 0.05$).

4. Discussion

The results from the present study clearly demonstrated that oral administration of ML effectively improved the growth performance and immunological as well as biochemical parameters in Pacific white shrimp, *P. vannamei*. Although previous studies presented that the lysozyme were able to be an immune modulator, and disease resistance in fish, information on the function of the lysozyme in shrimp garnered less attention.

The obtained results revealed that dietary ML could significantly improve the final weight of *P. vannamei*, and affect the shrimp weight gain. These findings are in accordance with the results reported in case of rainbow trout (*Oncorhynchus mykiss*) fed 0.5–1.5 mg kg^{-1} ML [18,30]. On the other hand, several researchers imply that there is a link between growth and immunostimulants [42,43]. When a diet supplemented with immunostimulants, they showed better growth and feed conversion rates [44]. The growth parameters and feed intake were increased in rainbow trout (*Oncorhynchus mykiss*) feeding 150–600 mg kg^{-1} [18], gible carp (*Carassius auratus gibelio*) fed supplemented diet with 1000 mg kg^{-1} [11], broiler chicken administrated 100–300 mg kg-1 [19] and 70–120 mg kg-1 dietary lysozyme [45]. Despite the positive effects of lysozyme administration on growth and immune response in different animals, the mechanisms underlying its mode of action are poorly understood. This positive influence has been attributed to the bactericide effects of this compound. Inclusion of antibiotics in the diet of chicks resulted in increased growth rate and improved feed efficiency compared with groups fed antibiotic-free diets [45,46]. One of the prominent effects of antibiotics including lysozyme is the detrimental effects on the gram-positive intestinal bacteria which results in vitamin-sparing effects and decrease in the production of toxic substances by the gut microbiota. Also, it may be assumed that lysozyme has positive effects on length, width, and perimeter or surface area of intestinal microvilli [11] and epithelial cells [19,47], besides, facilitating enzyme secretion for better nutrients digestion and absorption [45]. The effects of dietary lysozyme on growth performance indices could vary and it may depend on some factors like stress, season, sex, species, and environmental conditions.

Among the immunostimulants, β1-3 glucan, β1-6 glucan [48,49], levamisole [50], saponin [51], lactoferrin [52], chitin [53], chitosan [54], and vitamin C [55] are practiced in aquaculture to prevent free radical damage [56], protect haemocyte, activate some antimicrobial compounds [57], and improving the immune status of shrimp [55]. The health of aquatic organisms is depended on the production of oxygen species and antioxidants like SOD and GPX which protect the animal cells against the free radicals [58]. Evaluation of the antioxidant and immune factor activity is a good health status indicator [59]. MDA is a direct evidence of the toxic processes caused by free radicals [5]. In the present study, dietary ML had no significant effect on plasma MDA activity in *P. vannamei*. Similar results have been reported for gibel carp (*Carassius auratus gibelio*) followed by treatment with lysozyme [11], and for Nile tilapia (*Oreochromis niloticus*) fed *Astragalus* polysaccharides as an immunostimulant substances [60]. As MDA is the main component of lipid peroxides and its concentration is widely used assay for lipid peroxidation, no increased levels in this indicator can either reflected the non-significant peroxidation levels of lipids in the shrimp after ML supplementation or increasing the activity of antioxidants like GPX and SOD after this usage. However, the results obtained about GPX and SOD in plasma, make the last assumption more feasible. Glutathione peroxidase protects the organism against oxidative damage, it can reduce lipid hydroperoxides and free hydrogen peroxide to water [61]. In the present study, the supplementation with

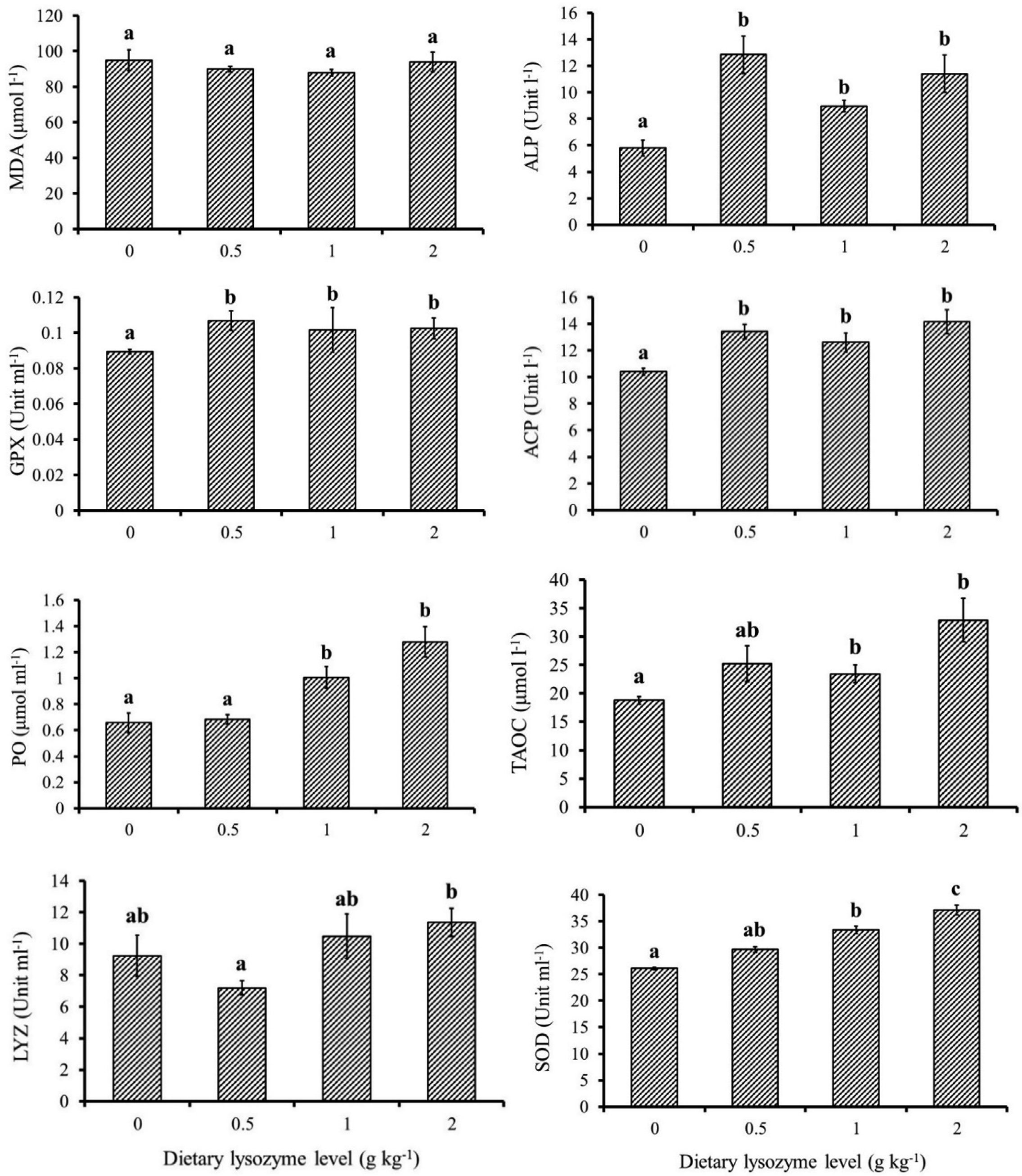

Fig. 1. The plasma immune parameters of *P. vannamei* fed different levels of dietary ML. Each bar represents the mean value from three replicate with the standard error. The bar assigned with different letters are significantly different ($P < 0.05$). Data analyzed with Kruskal-Wallis and Mann-Whitney *U* test except than SOD which analyzed with ANOVA followed by Duncan's multiple-range test. MDA, malondialdehyde; ALP, alkaline phosphatase; GPX, glutathione peroxidase; ACP, acid phosphatase; PO, phenoloxidase; TAOC, total antioxidant capacity; LYZ, lysozyme; SOD, superoxide dismutase.

Table 3
Mean (± SE) THC (total haemocyte count), GC (granular cells), SGC (semi-granular cells) and HC (hyaline cells) in *P. vannamei* which had been fed by different dietary ML levels.

Parameter	Dietary ML level (g kg^{-1})			
	0	0.5	1	2
THC (× $10^6 ml^{-1}$)	12.4 ± 0.17^{a}	12.9 ± 0.39^{ab}	13.4 ± 0.31^{b}	13.8 ± 0.23^{b}
GC (× $10^6 ml^{-1}$)	1.8 ± 0.08^{a}	1.9 ± 0.12^{ab}	2.3 ± 0.13^{b}	2.3 ± 0.13^{b}
SGC (× $10^6 ml^{-1}$)	4.2 ± 0.08^{a}	4.5 ± 0.13^{ab}	4.6 ± 0.19^{ab}	4.9 ± 0.19^{b}
HC (× $10^6 ml^{-1}$)	6.4 ± 0.07^{a}	6.5 ± 0.19^{a}	6.5 ± 0.18^{a}	6.6 ± 0.14^{a}

Data in the same row having different superscripts are significantly different ($p < 0.05$) among treatments. Data analyzed with ANOVA followed by Duncan's multiple-range test.

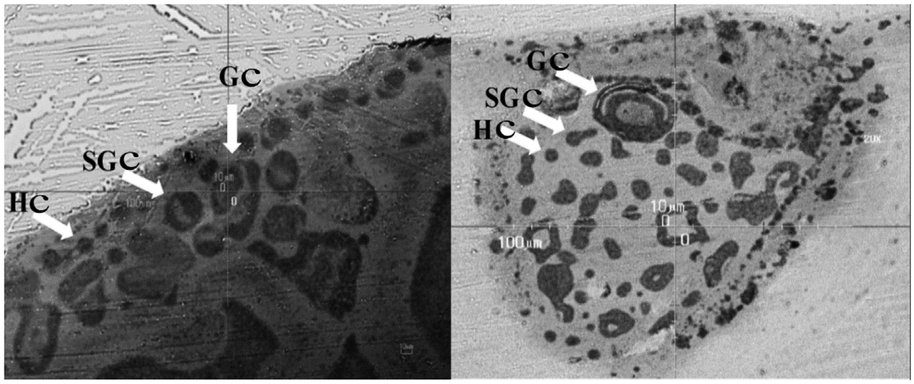

Fig. 2. Haemocyte in the *P. vannamei* stained with Giemsa observed under light microscopy. (HC) Hyaline cell; (GC) Granular cell (SGC); Semi-granular cell.

dietary ML resulted in an increase of the GPX level in shrimp plasma in all the treated groups. Likewise, in rainbow trout (*Oncorhynchus mykiss*), 150 mg kg^{-1} ML could increase the plasma GPX activity [18], whereas in juvenile gibel carp (*Carassius auratus gibelio*) there was no significant difference among treatments [11]. It is possible that oral lysozyme administration plays an important role in the activity of SOD. Superoxide dismutase catalyzes the dismutation of the superoxide (O_2^{-}) radical into either molecular oxygen (O_2) or hydrogen peroxide (H_2O_2) [62]. Increases in this antioxidant were in accordance with increased immune responses. These findings are in agreement with the increase of SOD level in rainbow trout (*Oncorhynchus mykiss*) utilized 150, 300, 450 and 600 mg kg^{-1} dietary lysozyme [18].The ALP and ACP levels increased in shrimp fed by ML supplemented diets. Phosphatase is a type of enzyme, used to free attached phosphoryl groups from pathogens and is stored in lysosomes [63]. Although information on the mechanism of action of the dietary ML on phosphatase has less attention, researchers analyzed the effects of other immunostimulants agents, like β-glucan on phosphatase activity. In a study on mitten crab (*Eriocheir sinensis*), ALP value was higher in the untreated group on day 14th but a significant difference was found on day 28th in β-glucan-0.5% group and ACP was not significantly affected [64].

The pro-phenoloxidase system present in invertebrates was involved in phagocytosis, melanization, cytotoxic reactions, particle encapsulation, and nodules and capsules formation [65,66]. In the present trial, administration of 1 and 2 g kg^{-1} ML improved plasma PO levels and it might promote shrimp immune system by enhancing phagocytic activity and increasing respiratory burst activity [65]. Likewise, the injection of recombinant lysozyme protein significantly increased PO and block White Spot Syndrome Virus (WSSV) infection in *Litopenaeus stylirostris* [24]. The total antioxidant is a biomarker for shrimp immune response, increasing trend indicated that the antioxidant capacity of *P. vannamei* was enhanced by adding a proper amount of lysozyme in the diet. The previous studies analyzes the TAOC levels, for example, TAOC activity in *L. vannamei* fed 3% poly-β-hydroxybutyrate (PHB) was significantly higher than 1% PHB and 5% PHB groups [2]. Also, 2 g kg^{-1} dietary ML had significant effect on the activity of plasma LYZ which is an effective antibacterial agent. This observation is similar to the results of rainbow trout (*Oncorhynchus mykiss*) serum LYZ that significantly increased after injection one to three times of 10 and 100 µg kg^{-1} dimerized lysozyme (KLP-602) [67].

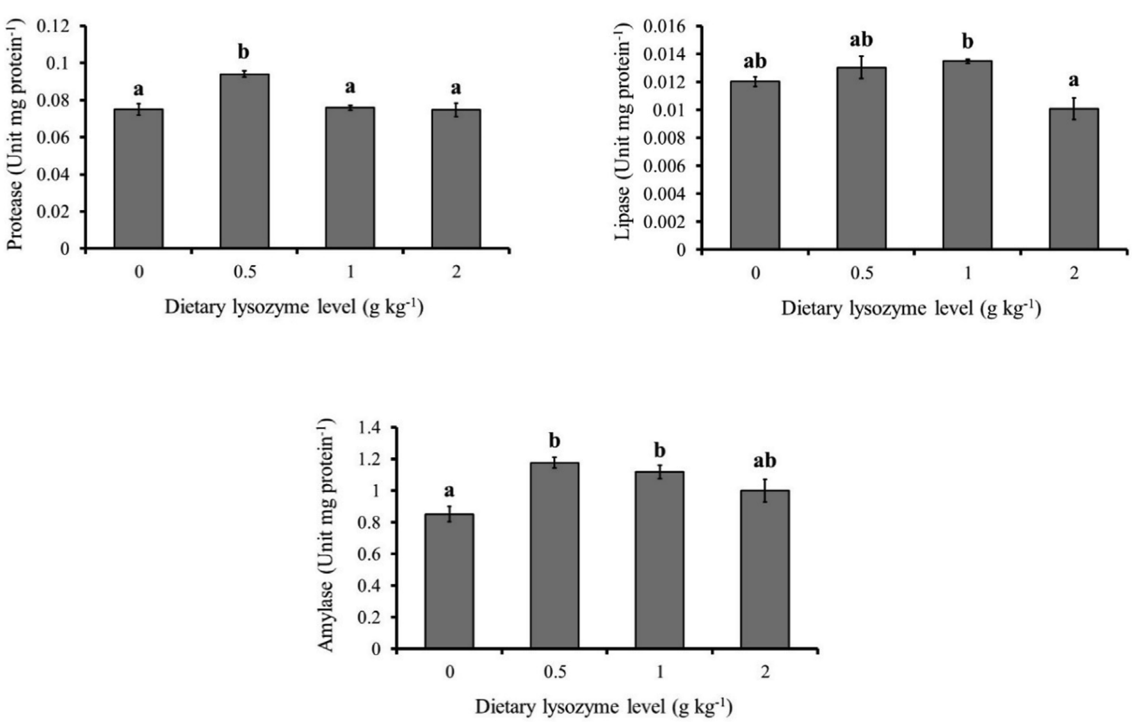

Fig. 3. The specific activity of intestinal enzymes of *P. vannamei* fed different levels of dietary ML. Each bar represents the mean value from three replicate with the standard error. The bars assigned with different letters are significantly different ($P < 0.05$). Data analyzed with ANOVA followed by Duncan's multiple-range test.

Both life cycle and nutrition, affect the quantity and quality of haemocyte count in crustaceans [68,69]. In the present study, ML treatment enhanced the THC, SGC, and GC levels, but not the hyaline cells. These results indicate that treatment with ML may induce haemocyte production in shrimp circulatory system. This is inconsistent with Mai and Wang [24] that the THC of *Litopenaeus stylirostris* treated with 8 mg of shrimp lysozyme gene, increased significantly after 4 h, then decreased but was still higher in the shrimp received the lysozyme gene.

Carbohydrate metabolism, nutrient transport, and immunological functions [24] such as encapsulation, phagocytosis, and nodule formation modulate by haemocytes [70]. They are a great source for many immune molecules [71]. Granular and semi-granular cells contain the proPO system [66,72] leading to both disease and stress resistance [15,24]. Although the immune-modulatory of the lysozyme is not yet fully understood, lysozyme has defensive mechanisms, it is able to help to overcome oxidative stress, facilitate the function of phagocytosis and improve the amount of immune enzyme and antioxidants by increasing the number of haemocytes and their bactericidal activities [73]. Another hypothesis currently assumes that lysozyme can affect the non-specific immune system of shrimp and their resistance to pathogens and environmental stresses after connecting to the haemocyte receptors. Moreover, the influence of immunostimulants in animal immunity may depend on animal health, species, developmental stage, dose, and co-operation with other immune substances [73].

Exogenous enzymes in diet might improve the total enzyme activity of the gut, and the presence of the immunostimulants like lysozyme might stimulate the qualitative and quantitative variations of endogenous enzymes by shrimp. The activities of digestive enzymes (e.g. amylase, lipase, and protease) are recognized as an important indicator of digestive function [2] (nutrients digestion and absorption) and may effect on the growth and the molt cycle [74]. According to the results, 0.5 and 1 g kg^{-1} ML in the diet might benefit for digestion and nutrient absorption in shrimp intestine. In line with other investigations, amylase activity showed highly significant differences on the day 14th among all groups of Nile tilapia (*Oreochromis niloticus*) treated with different concentration of *Astragalus* polysaccharides, whereas no significant difference was observed in lipase activity [75]. The optimum dose of immunostimulants have synergistic action along the intestinal tract and might be a growth promoter for intestinal tissue, and brush borders, subsequently, modulate intestinal microbial status [11] and stimulate enzyme expression. There are a few studies analyses the influence of lysozyme on digestive enzymes and extra research should be operated to clarify its function.

5. Conclusion

In summary, this preliminary experiment provides an insight into how a natural immunostimulant product (microbial lysozyme) can play a critical role in regulating the immune system of shrimp. This finding corroborated no detrimental impacts of lysozyme administration on nutrient digestibility or growth performance when added to shrimp basal diet. Furthermore, this study was based on a trial in the laboratory conditions, and practical shrimp farming may differ due to their environments. The related mechanism remains to be pursued in further investigation.

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