



ENHANCEMENT OF GROWTH PERFORMANCE, MEAT QUALITY, DIGESTIVE SYSTEM, AND IMMUNITY OF *LITOPENAEUS VANNAMEI* FED WITH ADVANCED CHELATE COMPOUNDS-BASED MINERALS: A NEW PERSPECTIVE ON CHELATION TECHNOLOGY

Moslem Sharifinia^{1*}, Mohammad Behzadi Tayemeh², Aghil Dashtiannasab¹, Ali Kaviani¹, Shahin Faghih³, Mohammad Khali Pazir¹, Mehrzad Keshavarzifard¹, Khosrow Aeinjamshid¹, Somayeh Kalanaky², Saide Fakharzade², Yahya Jand², Mohammad Hassan Nazaran^{2*}

¹Shrimp Research Center, Iranian Fisheries Sciences Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Bushehr 75169-89177, Iran

²Department of Research and Development, Sodour Ahrar Shargh Company, Tehran, Iran

³Department of Medicinal and Aromatic Plants, Estahban Branch, Islamic Azad University, Fars, Iran

*Corresponding authors: moslem.sharifinia@yahoo.com; m.sharifinia@areeo.ac.ir (M. Sharifinia); mnazaran@nanochelatingtechnology.com (M.H. Nazaran)

Abstract

This study aimed to explore the effects of essential nutrients on various aspects of Pacific white shrimp, *Litopenaeus vannamei*, including growth performance, survival rates, tissue mineralization, digestive enzyme activity, and redox and immune status. Under the brand name Bonza® shrimp, the chelated supplement, containing Zn, Cu, Mn, Mg, Ca, P, Fe, and Se, was manufactured based on the advanced chelate compound technology, registered with the United States Patent (No. US8288587B2). Then the shrimp (weight of 8.55 ± 0.24 g; $n = 420$) were randomly assigned into six experimental groups (Bonza® shrimp mixed in diet treatments: MT1: 250; MT2: 500; MT3: 1000 mg kg⁻¹, and sprayed on diet treatments: ST1: 250; ST2: 500; ST3: 1000 mg kg⁻¹) and one control treatment (CT). At the end of the feeding trial (60 days), the shrimp fed with diets containing Bonza® shrimp (especially MT3 treatment) showed a significant enhancement in growth performance, i.e., weight gain, survival, and feed conversion ratio, compared to the CT treatment ($P < 0.05$). Moreover, the supplementation of Bonza® shrimp at the highest dose markedly increased the levels of the applied minerals and ash in the muscle tissue and whole body. Likewise, the administration of the organic supplement markedly raised the levels of total protein, glucose, cholesterol, and triglyceride, particularly at the highest dosage, i.e., the MT3 treatment. Besides, the inclusion of this supplement considerably increased the activities of amylase, lipase, phenoloxidase, lysozyme, superoxide dismutase, alkaline phosphatase, and acid phosphatase enzymes ($P < 0.05$). Also, the total hemocyte count in shrimp fed with the supplement (MT2 and MT3) was significantly higher than the other treatments. Therefore, the supplementation of Bonza® shrimp could not only improve the growth performance, macro- and microelement levels in the body, and digestive enzyme activities but also enhance the redox and immune status. The outcomes recommend that the supplement could adjust the energy allocation among various traits probably through optimizing the metabolism. According to the fitted linear regressions, furthermore, the method in which this organic supplement (at the level of 1000 mg kg⁻¹) was premixed with the diet can be more effective than the top-dressing method. Together, this study introduces the mineral supplement, synthesized using a patented procedure, as a metabolism optimizer to enhance growth performance, meat quality, digestive system, and immunity of *L. vannamei*.

Key words: minerals, growth performance, antioxidant capacity, innate immunity, chelation technology

Aquaculture is one of the fastest growing food production industries in the world and is considered as the greatest potential for meeting the growing demand for seafood (Khanjani et al., 2021, 2022; Khanjani and Sharifinia, 2021; Sharifinia, 2024). Shrimp, as one of the most delicious seafood, is an important traditional food with high quality and nutrients that are beneficial for human health (Sharifinia et al., 2023 a, b; Yao et al., 2022; Yeganeh et al., 2020). The protein in shrimp (up to 20%) is of high quality and contains all the necessary amino acids for growth (Akonor et al., 2016; Dong et al., 2020). Due to the lack of connective tissues, shrimp protein easily digests (Pan et al., 2019), and in turn the foods containing shrimp can be a good option for groups of people, especially elderly ones who have difficulty in chewing and digesting food (Liu

and Ralston, 2021). Furthermore, omega-3 fatty acids are found in abundance in shrimp. These unsaturated fatty acids can boost the immune system and be effective in reducing the risks of heart disease, diabetes, and stroke (Elagizi et al., 2021; Smith and Guentzel, 2010). Therefore, Pacific white shrimp (*Litopenaeus vannamei*) with an annual production of 4.9 million tons in 2018 is the most consumed and cultivated shrimp species in the world (FAO, 2020). Due to the importance of this crucial crustacean in the food industry, nutritionists have extensively investigated the effects of food additives on its growth performance, meat quality, and immunity. However, further research needs to be done to assess nutritional requirements of *L. vannamei* with focus on macro- and microelements (Truong et al., 2022).

Marine species actively absorb the minerals they need through the water they ingest. However, shrimp cannot supply the essential ions for their physiological activities just through ingested seawater, so they need a dietary supplement for healthy growth and metabolism (Bharadwaj et al., 2014; Huang et al., 2017; Truong et al., 2022). According to the results of earlier studies, dietary values of macro- and microelement requirements for Pacific white shrimp include copper (Cu; 32 mg kg⁻¹), zinc (Zn; 33 mg kg⁻¹), manganese (Mn; 70 mg kg⁻¹), magnesium (Mg; 2.6–3.5 g kg⁻¹), calcium (Ca; 5 g kg⁻¹), phosphorus (P; 5–10 g kg⁻¹) and potassium (K; 10–15 g kg⁻¹) (Cheng et al., 2006; Davis et al., 1993; Huang et al., 2017; Liu et al., 2014; Roy et al., 2007). These biologically important nutrients regulate metabolism and have a great impact on the performance and health of shrimp (Katya et al., 2016; Yuan et al., 2019 a).

During recent decades, macro- and microminerals have attracted considerable attention chiefly because of their biologically important roles, although the mineral requirements of shrimp had not been a research focus area before (Truong et al., 2022). Generally, minerals in diets are more bioavailable when they are in certain forms, but inorganic salts often are not readily absorbed by farmed aquatic animals (Apines-Amar et al., 2004; Truong et al., 2022). Nutritionists argue that the limited bioavailability of such inorganic salts can be related to their antagonistic effects (i.e., chemical incompatibility) and interactions with anti-nutrients such as phytate. In plant-based diets, in other words, phytic acid binds to divalent cationic minerals and in turn reduces the absorption of these ionic nutrients through formation of insoluble complexes, thereby disposing of them as waste products in the environment (Katya et al., 2016). Moreover, competitive antagonism among inorganic salts can lead to reduced absorption chiefly because of the formation of insoluble precipitates. Moreover, competitive antagonism among inorganic salts can lead to reduced absorption, primarily due to the formation of insoluble precipitates. Excessive supply of these salts can penetrate environments, potentially threatening aquatic fauna and flora (Ghasemi et al., 2022; Gheisari et al., 2011). To overcome such challenges, hence, vigorous efforts have been made to identify an alternative approach. As a result, chelated macro- and microelements have attracted considerable attention thanks to their improved bioavailability compared to inorganic salts (Truong et al., 2022).

The technology of advanced chelate compound, a self-assembly technique, is capable of designing and synthesizing regular structures in several industries, e.g., agri- and aquaculture, medicine, and animal nutrition (Ghasemi et al., 2022; Hoseini-Alfatemi et al., 2020). Generally, chelated elements can be synthesized using the different types of ligands, including amino acids, peptides, polysaccharides, and organic acids (Byrne et al., 2021). In the context of advanced chelate compound technology, the elements are bonded with polymerized organic acids based on their preference for specific co-

ordination sites of chelating agents, thus providing more stable and bioavailable crystalline complexes (Banadaky et al., 2021). Accordingly, our previous research has confirmed the impressive capacity of organic acid-chelated supplements to considerably enhance production/growth performance, immunity, and metabolism in fish (Rashidian et al., 2023), ostriches (Seyfori et al., 2019), chickens (Ghasemi et al., 2020), and dairy cows (Banadaky et al., 2021). Upon reviewing the literature, however, no information is available about the nutritional impacts of chelated macro- and/or microelements on Pacific white shrimp, even though a few studies have published the nutritional advantages of amino acid-chelated minerals for both fish and shrimp (El-Sayed et al., 2023; Yang et al., 2023). However, the bioavailability of these minerals in conventional inorganic salt forms remains a challenge, leading to exploration of alternatives. Chelated minerals offer a promising solution, thanks to their superior bioavailability compared to inorganic salts. This study aims to explore the effects of an organic acid-chelated supplement, enriched with essential macro- and microelements (Zn, Cu, Mn, Mg, Ca, P, Fe, and Se), on the growth performance, survival rate, tissue mineralization, digestive enzyme activity, and immune status of Pacific white shrimp. By addressing this knowledge gap, our research seeks to contribute valuable insights to the field of aquaculture nutrition.

Material and methods

Preparation of chelated supplement

Utilizing the self-assembly method, the organic acid-chelated supplement, under the brand name Bonza[®] shrimp, was manufactured by Sodour Ahrar Shargh Company on the basis of the advanced chelate compound technology (Nazaran, 2012). Under No. US8288587B2, this technology is patented in the United States Patent and trademark office, and Table S1 shows the analysis of the Bonza[®] shrimp supplement containing organic acid-chelated macro- and microelements. Moreover, dynamic light scattering (DLS; Malvern-M3-PALS) was employed to measure the hydrodynamic size, polydispersity index (PDI), and zeta potential of the organic acid-chelated complex, and Fourier transform infrared spectrometer (FTIR; IR-Prestige-21, Shimadzu, Kyoto, Japan) was utilized to assess infrared spectra of the chelated supplement. The crystalline phase was determined using a Phillips X-ray diffractometer (XRD) employing a scanning rate of 0.033° s⁻¹ in a 2θ range from 5° to 80° with Cu Kα radiation (λ = 1.5418 Å). Moreover, scanning electron microscope (SEM) was applied to depict the morphology of the supplement.

Experimental design

This research was performed at the Persian Gulf SPF Shrimp National Research Center in Bushehr, Bushehr Province, Iran. To acclimate to experimental conditions,

juvenile healthy white shrimp, *L. vannamei*, were fed with a commercial diet for 7 days. Afterward, a total of 420 juveniles with an average initial weight and length of 8.55 ± 0.24 g and 9.54 ± 0.19 cm, respectively, were randomly distributed into 21 cylindrical fiberglass tanks (300 L tanks filled with 200 L of seawater) at a stocking density of 20 shrimp per tank. The experimental design included six experimental treatments (mixed in pellet treatments: MT1: 250; MT2: 500; MT3: 1000 mg kg⁻¹, and sprayed on pellet treatments: ST1: 250; ST2: 500; ST3: 1000 mg kg⁻¹) and one control treatment (CT). All treatments were performed in three replicates, and the shrimp were fed with the experimental diets four times a day at regular intervals (6:00, 12:00, 18:00, and 24:00) for 60 days. During the study, the animals were precisely weighed biweekly to fine-tune their daily feed ration, and dead shrimp were immediately removed from the tanks, weighed, and recorded. To maintain a healthy environment, daily cleaning of the tanks was implemented by siphoning out accumulated waste materials, and more than 60% of the tank seawater was exchanged before the first feeding of the day. The photoperiod was maintained at 14L: 10D during the study, and tank aeration was ensured via air stones to maintain dissolved oxygen levels near saturation. Furthermore, the experimental conditions used to experiment were: temperature, 26–28°C; salinity, 27–29 g L⁻¹; pH, 8.1–8.9; and dissolved oxygen, 6.2–6.8 mg L⁻¹.

Experimental diets

To prepare the experimental diets, a commercial feed, containing 34% crude protein, 8% crude fat, 4% crude fiber, 14% ash, and 10% moisture, was purchased from Faradaneh Company (Shahr-e Kord, Chaharmahal and Bakhtiari Province, Iran). Then the commercial diet was sprayed or mixed with aforementioned concentrations of the chelated supplement (i.e., Bonza® shrimp) and corn starch as binder (2%). That is, the chelated supplement was employed in two ways: mixed into the diet, or sprayed onto the diet for the easier application on farms. In the mixing method, the commercial diet was thoroughly powdered and then the suspended supplement and corn starch were added. Finally, the resulting paste was pelleted by a grinder machine and kept in an oven at 60°C for 6 h to eliminate moisture. In the top-dressing method, the suspended supplement was sprayed onto the commercial feed surface. Without adding Bonza® shrimp, moreover, the commercial diet was employed as the control treatment.

Growth performance, feed utilization, and morphometric parameters

At the end of the feeding experiment, all shrimp per tank were weighed and counted to calculate the weight gain (WG), body weight index (BWI), specific growth rate (SGR), feed conversion ratio (FCR), and survival rate (SR). In addition, the length and hepatopancreas weight of the shrimp were measured to compute the

length gain (LG), condition factor (CF), and hepatosomatic index (HSI) as follows:

$$CF = (\text{weight (g)} / \text{length (cm)}^3) \times 100$$

$$HSI = (\text{hepatopancreas weight (g)} / \text{total weight (g)}) \times 100$$

$$WG \text{ (g)} = \text{Final weight} - \text{Initial weight}$$

$$LG \text{ (cm)} = \text{Final length} - \text{Initial length}$$

$$FCR = (\text{Feed given (g)}) / (\text{Live weight gain (g)})$$

$$SR (\%) = \left[\frac{(\text{Final number of shrimp})}{(\text{Initial number of shrimp})} \right] \times 100$$

$$HSI (\%) = \left[\frac{(\text{Hepatopancreas wet weight (g)})}{(\text{Wet body weight (g)})} \right] \times 100$$

$$CF \text{ (g cm}^{-3}\text{)} = \left[\frac{(\text{Body weight (g)})}{(\text{Body length}^3 \text{ (cm}^3\text{)})} \right] \times 100$$

Whole-body proximate composition and mineral concentrations

Standard methods were applied to assess the crude protein, crude lipid, moisture, and ash content of the whole body. The moisture content was assessed by drying the samples in an oven at 105°C until a constant weight was achieved. The Kjeldahl method after acid digestion using an Auto Kjeldahl System and the ether-extraction method with a Soxtec System were employed to evaluate crude protein and crude lipid, respectively. The methods are widely recognized and commonly used in the analysis of shrimp and other aquatic species (AOAC, 2006). The mineral content of tissue samples was calculated using a multi-step method. First, uniform tissue samples were obtained and subjected to wet digestion using a nitric-perchloric acid solution. After digestion, the resulting solution was diluted with deionized-distilled water (Hill et al., 1983). Finally, the concentrations of macro- and microelements, i.e., Mg, Cu, Ca, Mn, Se, P, Fe, and Zn, were assessed using an inductively coupled plasma-mass spectrometer (ICP-MS).

Hemolymph biochemical parameters

Hemolymph biochemical factors including total protein (TP), glucose (Glu), cholesterol (Chol), triglyceride (TG), and albumin (Alb) were evaluated spectrophotometrically (ERMA Inc., Tokyo, Japan) through applying

commercial diagnostic kits (Pars Azmoon Co., Tehran, Iran).

Digestive enzyme activities and tissue protein of intestine

The protein content of the samples was evaluated using the Bradford method (Bradford, 1976), with bovine serum albumin as the standard. Moreover, amylase activity was assessed using the Rick and Stegbauer method, which involved the use of iodine solution to detect non-hydrolyzed starch (Rick and Stegbauer, 1974). Lipase activity was determined by measuring the release of fatty acids resulting from the enzymatic hydrolysis of triglycerides in a stabilized olive oil emulsion, as previously described by Borlongan (1990). The activities of the digestive enzymes were reported in units per milligram of protein.

Enzymes and immunological parameters

For the cell counts, a drop of the hemolymph-anti-coagulant mixture (diluted hemolymph) was placed on a hemocytometer (Neubauer, Germany) to measure the total hemocyte count (THC) using a light microscope (Ceti, Triton II, England) at 100 $\times$ magnification.

To determine the phenoloxidase (PO) activity, the formation of the red pigment DOPA-chrome was measured spectrophotometrically at 490 nm after the oxidation of L-dihydroxyphenylalanine (L-DOPA), which is the enzyme substrate. This method was described by Perazzolo and Barracco (1997) and involved diluting samples (four pools of three shrimp per treatment) in tris-buffered saline solution (TBS-1) at a ratio of 1:8. Then, 50 μ L of this solution was pre-incubated with trypsin (SIGMA, 1 mg mL⁻¹) at 20°C for 15 minutes in 96-microwell plates with flat bottoms. Control samples were prepared using TBS-1 instead of trypsin and serum. Following incubation, 50 μ L of L-DOPA (3 mg mL⁻¹) was added to the wells, and the formation of DOPA-chrome was monitored at 0, 5, 15, and 25 minutes. PO activity was calculated as the variation in absorbance (490 nm) per minute and per milligram of total protein in the samples. Enzymatic activity was expressed in units, with one unit being equivalent to a change of 0.001 in the absorbance/min/milligram of protein at 20°C (Leadprathom et al., 2012).

The lysozyme (LZM) activity (U mL⁻¹) was measured by turbidimetric assay according to Ellis (1990). Hemolymph with hen egg white lysozyme as standard was placed in a plate and the suspension of *Micrococcus lysodeikticus* (0.2 mg mL⁻¹ 0.05 M sodium phosphate buffer pH 6.2; SIGMA, USA) was added. One unit of lysozyme activity was determined as a reduction in absorbance of 0.0001 per minute.

The superoxide dismutase (SOD) activity was measured according to the manufacturer's instruction of antioxidant Enzyme ZellBio test kits (ZellBio GmbH, Germany). For analysis of SOD, the hepatopancreas samples were homogenized in the phosphate buffer (0.025 M KH₂PO₄, 0.025 M Na₂PO₄·12H₂O, pH 6.4). Total SOD

activity (U mg⁻¹) in supernatant samples was measured following the method described by Bolann and Ulvik (1991). The absorbance was measured at 420 nm. One unit of SOD activity was defined as the amount of SOD required to inhibit the rate of xanthine reduction by 50% in a 1 ml reaction system. Special SOD activity was expressed as a unit per mg of protein.

The acid phosphatase (ACP) and alkaline phosphatase (ALP) activities were determined photometrically according to the method described by Burtis and Bruns (2014). Crude enzyme solutions were incubated with substrate *p*-nitrophenyl phosphate in diethanolamine-MgCl buffer (Pars Azmoon Co., Tehran, Iran). Afterward, nitrophenolphosphate was added and the absorbance measured photometrically at 405 nm as well as the activity expressed as units L⁻¹.

Statistical analysis

The Shapiro-Wilk test and normal Q-Q plot were applied to check the normality of data, and Levene's test was employed to assess the equality of variances. To determine whether there were significant differences among the groups, a one-way analysis of variance (ANOVA) was conducted. Also, to perform multiple comparisons of means, post hoc Duncan's test was employed. All statistical analyses were carried out using SPSS ver. 22.0 software (Chicago, Illinois, USA). For all statistical tests, mean values were considered to be significantly different at a significance level of $P < 0.05$. Results of the assays were presented as mean $\pm$ standard deviation. Using R software, a simple linear regression was fitted to investigate the relationships between weight gain and the levels of the chelated supplement.

Results

Characterization of chelated supplement

Based on DLS data, the hydrodynamic size, zeta potential, and PDI displayed values of 449.2 nm, -35.7 mV, and 0.257, respectively (Figure S1). Moreover, the infrared spectra of the chelated supplement are depicted in Figure S2, indicating the chelation of the macro- and microelements. In other words, the FTIR spectrum related to the carbonyl group at around 1700 cm⁻¹ translocated to 1599 cm⁻¹. The XRD patterns measured for the chelated supplement are represented in Figure S3, and the diffraction peaks, related to diffraction plane of 111 and 200, demonstrated high crystallinity of the chelated supplement at the 2 θ values of 43.25° and 78.69°, respectively. In addition, the SEM image (Figure S4) illustrates the morphology of the supplement.

Growth performance and morphometric parameters

IBW, WG, LG, FCR, SR, HSI, and CF are shown in Table 1. Generally, diets containing the organic macro- and microelements positively affected WG, LG, SR, and

FCR of Pacific white shrimp. That is, shrimp that received the organic macro- and microelements exhibited enhanced growth performance and a higher survival rate as compared to the CT treatment. The lowest and highest values of WG, LG, and SR were obtained in the CT and MT3 treatments, respectively. Moreover, shrimp fed with the basal diet displayed the highest FCR (1.64 ± 0.10), while the MT3 treatment demonstrated the lowest FCR (1.33 ± 0.04). Although the highest values of HSI and CF were detected in the MT3 treatment, there were no significant differences in their values among the treatments ($P > 0.05$). Based on the fitted linear regressions (Figure 1), furthermore, the current study illustrated that the mixed method (adjusted R-squared: 0.88; p-value: 0.04078) was more efficient than the top-dressing method (adjusted R-squared: 0.95; p-value: 0.0155). In other words, to reach the best growth observed in the mixed method at the dosage of 1000 mg kg⁻¹, the supplement should be applied at the dosage of about 1500 mg kg⁻¹ in the top-dressing method.

Whole-body proximate composition and mineral concentrations

Table 2 shows the wet-weight based whole-body composition of shrimp. No significant differences were observed in the protein and lipid levels of the whole body among all treatments ($P > 0.05$), although their levels were higher in the mineral-supplemented treatments compared to the control treatment. On the other hand, the ash and moisture contents displayed significant differences among the treatments and their highest levels were found in ST3 treatment ($P < 0.05$).

Table 3 represents the concentrations of macro- and microelements (Zn, Cu, Mn, Mg, Ca, P, Fe, and Se) in the muscle tissue of shrimp that received various doses of the organic elements for 60 days. The concentrations of these elements in the muscle tissue of shrimp fed the control diet were significantly lower ($P < 0.05$) than those that were fed with the diets containing the organic elements. Generally, the highest levels of all elements were found in the shrimp fed the MT3 treatment.

Hemolymph biochemical parameters

The effects of different dosages of the organic elements on hemolymph biochemical parameters including TP, Glu, Chol, TG, and Alb are presented in Figure 2. When compared to the control group, generally, the administration of the organic supplement considerably elevated the levels of TP, Glu, Chol, and TG particularly at the highest dosage, i.e., the MT3 treatment. However, no significant differences were statistically found for Alb between the organic element-supplemented groups and the control one ($P < 0.05$).

Digestive enzyme activity and protein of intestine tissue

Figure 3 depicts the results of intestinal digestive enzyme activities and protein of intestine. Overall, there was a significant increase in the level of intestinal protein, especially at the highest concentration (i.e., both MT3 and ST3 treatments). Likewise, the inclusion of the organic acid-chelated elements considerably increased amylase and lipase activities ($P < 0.05$).

Table 1. Growth performance and morphological indices of juvenile *Litopenaeus vannamei* fed different dosage (250, 500, and 1000 mg kg⁻¹) of Bonza® shrimp (mixed treatments: MT1, MT2, and MT3; and sprayed treatments: ST1, ST2, and ST3) for 60 days

Treatments	Parameters							
	IBW (g)	WG (g)	IBL (cm)	LG (cm)	SR (%)	FCR	HSI (%)	CF (%)
CT	8.57±0.21 a	9.61±0.12 d	9.70±0.05 a	2.42±0.03 b	85.00±1.00 c	1.64±0.10 a	3.92±0.19 a	1.02±0.01 a
MT1	8.57±0.19 a	9.78±0.06 cd	9.60±0.11 a	2.55±0.10 b	86.00±0.57 bc	1.61±0.05 a	4.06±0.16 a	1.02±0.03 a
MT2	8.56±0.25 a	10.12±0.05 ab	9.50±0.09 a	2.67±0.08 a	95.00±1.00 ab	1.40±0.07 bc	4.13±0.30 a	1.04±0.02 a
MT3	8.55±0.24 a	10.28±0.12 a	9.55±0.10 a	2.69±0.06 a	98.33±0.57 a	1.33±0.04 c	4.20±0.16 a	1.04±0.01 a
ST1	8.58±0.33 a	9.64±0.03 d	9.60±0.08 a	2.57±0.04 ab	86.67±0.58 bc	1.61±0.05 a	3.97±0.08 a	1.01±0.01 a
ST2	8.56±0.31 a	9.79±0.04 cd	9.60±0.11 a	2.57±0.06 a	90.00±1.00 bc	1.52±0.08 ab	4.01±0.26 a	1.02±0.01 a
ST3	8.55±0.29 a	10.02±0.06 bc	9.50±0.09 a	2.69±0.11 a	93.33±1.16 abc	1.44±0.04 bc	4.02±0.31 a	1.03±0.03 a

Data are presented as means ± standard deviation. If values in the same column have different letters, they are considered to be significantly different at a significance level of $P < 0.05$. The abbreviations used in the table correspond to the following parameters: IBW (initial body weight), WG (weight gain), IBL (initial body length), LG (length gain), SR (survival rate), FCR (feed conversion ratio), HSI (hepatosomatic index), and CF (condition factor).

Table 2. The proximate composition of the whole body of Pacific white shrimp (*L. vannamei*) that were fed diets with varying levels of the chelated complex for a duration of 60 days

Treatments	Crude protein (% DW)	Ash (% DW)	Crude fat (% DW)	Moisture (%)
CT	23.86±0.18	1.38±0.03 bc	0.88±0.04	53.78±0.20 b
MT1	23.99±0.22	1.37±0.03 bc	0.98±0.09	59.98±0.14 ab
MT2	24.08±0.19	1.43±0.04 b	0.97±0.05	63.54±0.29 c
MT3	24.13±0.17	1.43±0.06 b	0.94±0.03	58.48±0.22 d
ST1	23.83±0.25	1.43±0.04 b	0.92±0.05	57.92±0.10 e
ST2	23.84±0.14	1.32±0.04 c	0.91±0.08	64.56±0.31 d
ST3	23.90±0.26	1.59±0.03 a	0.92±0.04	64.12±0.22 a

Data are presented as means ± standard deviation. If values in the same column have different letters, they are considered to be significantly different at a significance level of $P < 0.05$.

Table 3. Concentrations of macro- and microelements (Zn, Cu, Mn, Mg, Ca, P, Fe, and Se) in the muscle tissue of *L. vannamei* that received various doses of the organic elements for 60 days

Treatments	Mg (ppm)	Zn (ppm)	Ca (%)	P (%)	Fe (ppm)	Cu (ppm)	Se (ppm)	Mn (ppm)
CT	222.14±6.15 e	10.05±0.07 g	0.61±0.010 e	0.246±0.006 c	2.59±0.03 g	2.72±0.05 f	0.123±0.006 d	0.083±0.006 c
MT1	265.11±3.46 c	10.90±0.02 e	0.71±0.005 b	0.273±0.006 ab	2.91±0.04 f	3.37±0.02 d	0.163±0.006 bc	0.156±0.006 b
MT2	289.28±1.92 a	11.81±0.03 c	0.76±0.010 a	0.277±0.006 a	3.75±0.05 c	3.52±0.02 c	0.174±0.005 abc	0.187±0.015 b
MT3	296.18±0.89 a	12.63±0.02 a	0.75±0.005 a	0.287±0.005 a	4.95±0.03 a	4.48±0.03 a	0.190±0.010 a	0.250±0.020 a
ST1	243.58±1.81 d	10.64±0.08 f	0.64±0.005 cd	0.263±0.006 b	3.20±0.01 e	3.07±0.05 e	0.120±0.010 d	0.100±0.020 c
ST2	258.29±2.12 c	11.63±0.04 d	0.64±0.006 de	0.267±0.005 b	3.52±0.05 d	3.36±0.03 d	0.156±0.006 c	0.166±0.006 b
ST3	280.19±1.14 b	12.11±0.10 b	0.67±0.020 c	0.283±0.005 a	4.09±0.03 b	3.95±0.02 b	0.183±0.005 ab	0.230±0.010 a

Data are presented as means ± standard deviation. If values in the same column have different letters, they are considered to be significantly different at a significance level of $P < 0.05$.

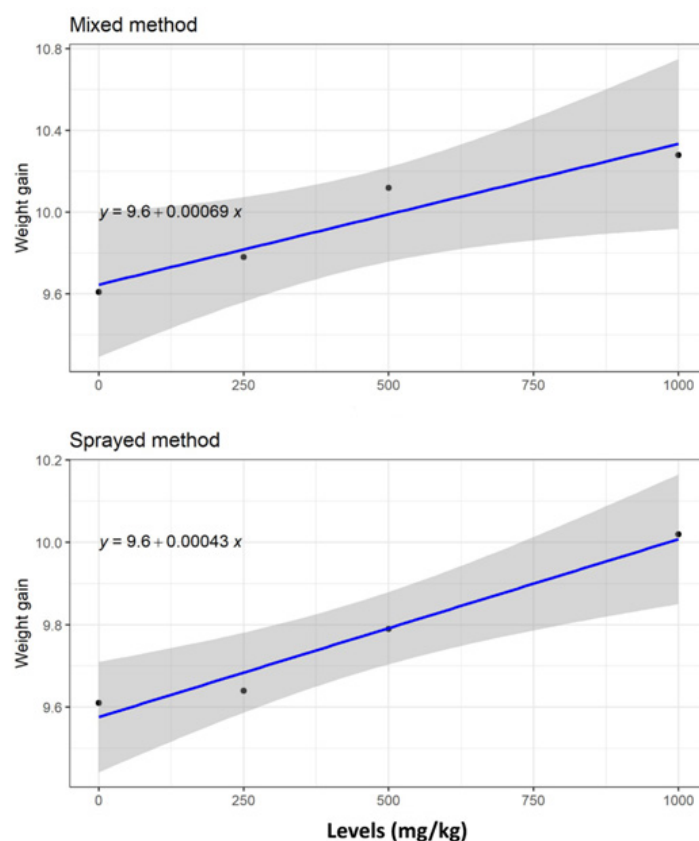


Figure 1. Simple linear regressions fitted to investigate the relationships between weight gain and the levels of the chelated supplement

Enzymes and immune response

Figure 4 illustrates the activities of PO, SOD, LZM, ALP, and ACP enzymes, along with THC, in both the hemolymph and hepatopancreas of *L. vannamei*. Generally, the PO activity (Figure 4a) in the hepatopancreas of shrimp fed with the organic elements (MT1, MT2, and MT3) was significantly higher than in those fed with other diets ($P < 0.05$). However, no statistically significant differences ($P > 0.05$) were observed among the control (CT), ST1, ST2, and ST3 treatments. Regarding PO activity, the lowest levels were observed in the CT treatment, whereas the highest levels were found in the MT3 treatment. Additionally, SOD activity significantly increased in response to the organic elements premixed in the diet (MT2 and MT3) compared to other treatments ($P < 0.05$). Similarly, the inclusion of the organic elements (especially MT2 and MT3) markedly elevated LZM ac-

tivity in the hemolymph ($P < 0.05$). Additionally, it was found that the LZM activity in the hemolymph of shrimp fed with the CT was significantly lower than the other treatments ($P < 0.05$). According to the data presented in Figure 4, shrimp fed with the organic elements mixed in the diet treatments exhibited significantly higher ALP and ACP activities in the hemolymph compared to the other experimental groups ($P < 0.05$). The lowest ALP and ACP activities were observed in the CT treatment, while the highest were seen in the MT3 treatment. Moreover, the supplementation of organic macro- and microelements notably increased THC levels; specifically, the THC in shrimp fed with supplements mixed in diet treatments (MT2 and MT3) was significantly higher compared to other treatments. The lowest THC levels were observed in the CT treatment, while the highest were found in the MT3 treatment.

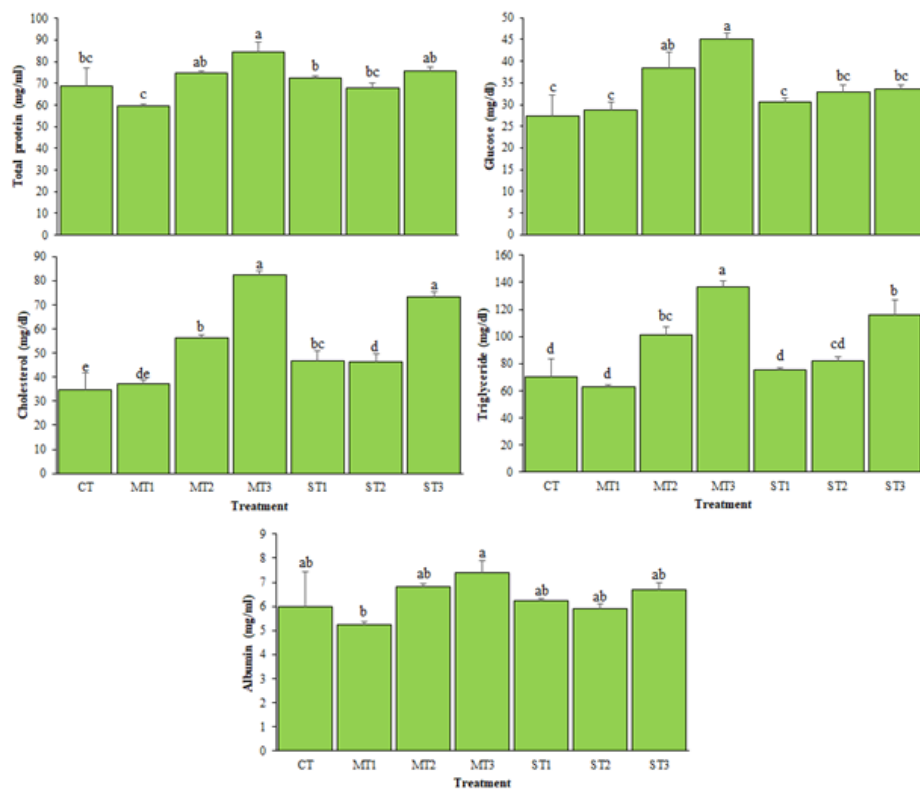


Figure 2. Hematological and biochemical parameters of juvenile *L. vannamei* after feeding with different dosages (250, 500, and 1000 mg kg⁻¹) and forms (mixed in and sprayed on diet) of Bonza® shrimp for 60 days. The data are expressed as means with standard deviations. The use of distinct letters signifies that there are significant statistical differences among the experimental groups at a significance threshold of P<0.05

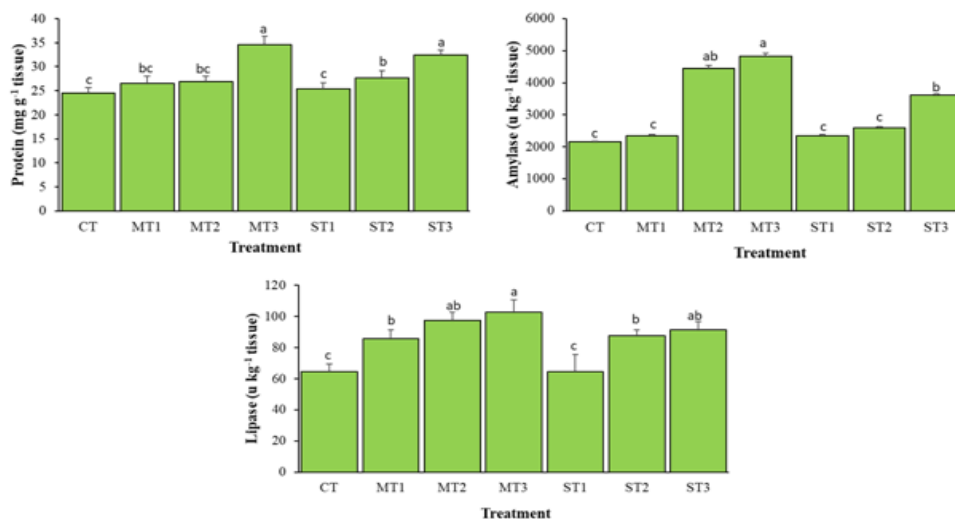


Figure 3. Impacts of Bonza® shrimp administered at different dosages (250, 500, and 1000 mg kg⁻¹) and forms (mixed in and sprayed on diet) on the total protein content and digestive enzyme activities, including amylase and lipase, of *L. vannamei* during a 60-day feeding trial. The data are presented as means ± standard deviations, and significant differences (P<0.05) among means are indicated by different letters

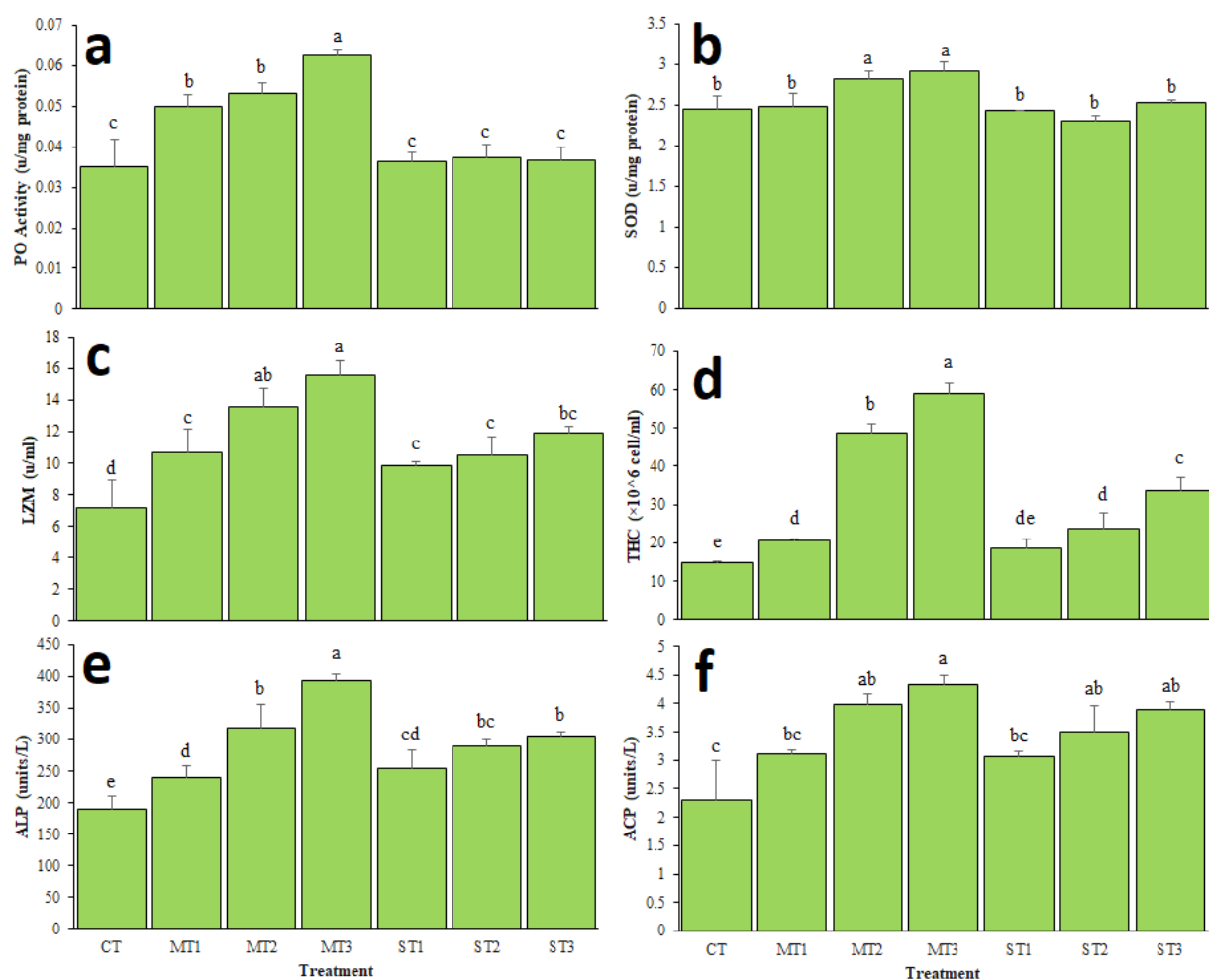


Figure 4. Effects of different dosages (250, 500, and 1000 mg kg⁻¹) of Bonza® shrimp (mixed treatments: MT1, MT2, and MT3; and sprayed treatments: ST1, ST2, and ST3) on hepatopancreas phenoloxidase (PO; a), superoxide dismutase (SOD; b), hemolymph lysozyme (LZM; c), total hemocyte count (THC; d), alkaline phosphatase (ALP; e), and acid phosphatase (ACP; f) of juvenile *Litopenaeus vannamei*. Values are presented as the means with standard deviations represented by vertical bars. Values with different letters are significantly different ($P < 0.05$)

Discussion

To the best of our knowledge, this is the first study to investigate the effects of dietary organic acid-chelated macro- and microelements on growth performance, survival, tissue mineralization, digestive enzymes, and redox and immune status of *L. vannamei*. In the present study, shrimp fed with the diets containing the organic supplement indicated more effective growth promotion due in part to the high biological availability of chelated minerals. Existing literature has provided clues that the bioavailability of minerals can be influenced by various factors such as supplement levels, chemical sources, mineral forms, and nutrient interactions (Lin et al., 2013; Yuan et al., 2019 b). Chelating an element with an organic compound can significantly improve its absorption, either by releasing it as an ion at the absorption site or by absorbing it as an intact chelate. Through chelation, elements are prevented from converting into insoluble compounds in the intestine, thus enhancing their absorption (Katya et al., 2016). More precisely, the current study illustrated

that the administration of the organic mineral supplement could greatly decrease FCR and also increase WG and SR of *L. vannamei*. These results are consistent with the fact that the addition of chelated minerals could improve growth performance of shrimp. Despite extensive research, little is known about the nutritional effects of chelated supplements containing both macro- and microelements on Pacific white shrimp. However, valuable but limited studies have investigated the effects of chelated minerals, comprising just one element, on Pacific white shrimp (Bharadwaj et al., 2014; Lin et al., 2013; Roy et al., 2007; Shi et al., 2021 b; Yuan et al., 2019 b, 2020). For example, Katya et al. (2016) examined the impacts of various levels of traditional inorganic and commercial chelated sources of dietary trace minerals (copper, zinc, and manganese premixes), on the growth performance of Pacific white shrimp fed plant protein-based diets. Their results indicated that the inclusion of the chelated elements could significantly enhance the FW and WG of the shrimp. Moreover, Bharadwaj et al. (2014) showed that different levels of chelated copper could significantly in-

duce the positive effect on the growth performance of Pacific white shrimp. According to Yuan et al. (2019 b) and Shi et al. (2021 b), similarly, both amino acid-chelated zinc and copper could enhance the growth performance of shrimp. The experimental results of the present study are similar to the results of previous studies (Shi et al., 2021 a, b), which showed that chelated minerals can improve FCR in Pacific white shrimp. Concerning dietary effects of macro-elements, Roy et al. (2007) observed significant differences in growth performance of Pacific white shrimp supplemented with chelated potassium (10 g kg⁻¹ diet). Moreover, it has been shown that both inorganic and organic magnesium can boost the growth performance of Pacific white shrimp, with optimal dosages being approximately 4.25 g kg⁻¹ for inorganic magnesium and 2.38 g kg⁻¹ for organic magnesium (Panmei et al., 2023). Despite the positive findings reported by such studies regarding the growth performance of shrimp fed with chelated mineral sources, it has been emphasized that growth performance alone cannot be deemed sufficient to conclusively establish the potential benefits of chelated minerals. Indeed, further supporting evidence is required to draw concrete conclusions.

Tissue mineralization has been taken into consideration as an important parameter for assessing the bioavailability of mineral supplements (Katya et al., 2016). Overall, the results of this study indicated that the supplementation of the organic elements especially at 1000 mg kg⁻¹ could increase the ash content; more precisely, the concentrations of the macro- and trace-elements of Mg, Zn, Ca, P, Fe, Cu, Se, and Mn in the muscle tissue were significantly elevated following administration of the chelated minerals. This suggests that the chelation technology is able to level up the absorption and utilization of minerals. In this context, few studies have investigated the effect of chelation process on the bioavailability of minerals in shrimp. For instance, Katya et al. (2016) evaluated the effects of inorganic and chelated trace mineral premixes on mineral content in Pacific white shrimp that received diets containing mineral inhibitor phytic acid. Their results showed that the shrimp fed diets supplemented with chelated trace mineral premixes had higher whole-body Cu and Zn concentrations compared to those fed inorganic premixes. Also, it has been confirmed that chelation technology could improve the tissue mineralization of macro-elements, i.e., magnesium content in hepatopancreas of *P. vannamei* juveniles (Panmei et al., 2023). On the contrary, a study by Yuan et al. (2020) reported that both inorganic and organic zinc had higher bioavailability than control group in the muscle tissue of shrimp.

Hematological parameters such as triglyceride, total protein, and cholesterol have been considered to be well-known indicators of animal health (Zhou et al., 2006). As far as we know, there is a limited number of published reports that have investigated the impact of chelated minerals on biochemical characteristics in shrimp (Katya et al., 2016; Yuan et al., 2019 b). The findings of the current

study demonstrated a significant increase in the levels of TP, Glu, Chol, and TG in the hemolymph of the shrimp fed with the diets containing the organic elements, as compared to those fed with the control diet. The highest values of these parameters were obtained in the MT3 treatment. Similarly, Katya et al. (2016) and Yuan et al. (2019 b) noted that such hematological characteristics were affected by levels and sources of trace minerals. The content of TP in the hemolymph partially reflects the level of protein in feeds and the amount of digestion and absorption of protein in animals. When protein synthesis and nitrogen deposition increase in animals, the total protein content in the serum increases (Li et al., 2022). According to the current study, the findings indicate that the organic acid-chelated macro- and microelements could promote the absorption and synthesis of protein. While the mechanism underlying the effect of chelated minerals on biochemical parameters in shrimp is not fully understood, there might be possible explanations. In general, this could be related to the reason that the chelation process increased the bioavailability and absorption of minerals considered as cofactors for enzymes involved in metabolism and nutrient utilization. Zinc, for example, is an essential cofactor for several enzymes and, in turn, is involved in carbohydrate and protein metabolism (Katya et al., 2016; Lin et al., 2013; Suttle, 2022). Additionally, minerals may promote the growth of beneficial gut bacteria, potentially leading to improved gut health and nutrient utilization (Suttle, 2022). However, while these mechanisms may contribute to the effects of chelated minerals on biochemical parameters in shrimp, further research is needed to fully understand the precise mechanisms.

Digestive enzymes, synthesized and secreted by the digestive gland, can be influenced by the type, source, and content of nutrients in penaeid shrimp (Xu and Pan, 2012), and are able to regulate growth and molting cycle through playing a leading role in nutritional physiology; for example, literature has documented that trace elements such as Cu and Zn could positively affect the activities of digestive enzymes which, in turn, could enhance digestion and nutrient absorption (Muralisankar et al., 2016, 2015). In this regard, the present study displayed that the inclusion of chelated minerals in the diet could improve the activities of intestinal digestive enzymes. Generally, chelation process could increase the bioavailability and stability of the minerals. As a result, the activities of digestive enzymes (such as amylase and lipase) can be enhanced, leading to improved digestion and nutrient utilization. For example, it has been documented that another advanced chelate compound-based supplement containing several microelements could increase the activity of protease in rainbow trout (Rashidian et al., 2023).

Investigation of the immune system status of shrimp is very important for prevention and control of diseases, and the total number of hemocytes has been used as an indicator of aquatic organisms' health (Perazzolo et al.,

2002). Like other invertebrates, shrimp lack an adaptive immune system and rely on innate immunity. Hemocytes are essential components of the immune system in invertebrate aquatic animals due to their ability to carry out various immune activities, e.g., encapsulation, phagocytosis, the activation of the prophenoloxidase system, and the production of antimicrobial lysozyme and peptides (Licona-Jain et al., 2020). Therefore, loss and damage of hemocytes can increase susceptibility to pathogens and even endanger the survival of farmed shrimp via depressing the immune ability (Xian et al., 2014). In this study, the current findings demonstrated that the supplementation of the organic elements, especially at the highest dosage (1000 mg kg⁻¹) and in the mixed method, could increase the number of hemocytes as well as the activities of PO and LZM enzymes, indicating the strengthening of the non-specific defense system of shrimp. In this regard, literature has proved that minerals can improve the immunity of aquatic crustacean; For example, it has been shown that the administration of zinc improved the immune response and disease resistance in Chinese mitten crab (Wang et al., 2022). In line with the current results, studies have reported that the non-specific defense system of shrimp could be influenced by chelated minerals more than inorganic ones. For instance, a fairly similar study has demonstrated that the supplementation of organic zinc could considerably increase THC and the activity of PO compared to *L. vannamei* that received zinc sulfate (Lin et al., 2013). Similarly, Yuan et al. (2019 b) have published that the administration of chelated Cu could improve the innate immunity of *L. vannamei* through increasing the enzyme activities of PO in hemolymph and LZM in hepatopancreas. The authors also believe that the organic copper can be more effective than the inorganic one. Even though a few studies have assessed the effects of chelated complexes containing only one element on the immune system of shrimp, no information is available about the impact of chelated complexes consisting of both macro- and microelements. Hence, further research is necessary to fully clarify the potential role of chelated minerals in enhancing the immune system of shrimp.

Both ACP and ALP enzymes exhibit significant hydrolytic activity towards phosphate-containing compounds, engage in the degradation of external carbohydrates, proteins, and lipids, as well as act as transphosphorylase (Yuan et al., 2019 b). Moreover, ALP is considered as a lysosomal enzyme and a critical indicator of macrophage activation (Gobi et al., 2016). Also, Tan and Mai (2001) have documented that this zinc-containing enzyme (i.e., ALP) can be a useful indicator for determining the bioavailability of zinc. In the present study, interestingly, significantly higher ALP and ACP activities were observed in the hemolymph of shrimp fed the organic elements premixed in the diet treatments, as compared to the other diets. This finding suggests that the organic macro- and microelements may level up the health status and immune system of shrimp. Similarly, Yuan et al.

(2019 b) detected that the activities of both ALP and ACP enzymes in the hemolymph of Pacific white shrimp could increase in response to chelated copper. Furthermore, another comparable study has reported that the supplementation of chelated zinc could raise the ALP activity in the hemolymph of *L. vannamei* (Lin et al., 2013).

In addition to the immune functions, Mohseni et al. (2014) have highlighted that the antioxidant status could be affected by dietary components, particularly essential nutrients, and antioxidant-related enzymes such as SOD have been widely accepted as an important marker for assessing oxidative stress. Moreover, SOD has been identified as a reliable indicator for assessing the mineral nutrition status of aquatic animals (Lin et al., 2013, 2010; Yuan et al., 2019 b). In this context, shrimp fed with the organic elements had significantly higher SOD activity in comparison with the control group, suggesting an improvement in the antioxidant capacity. This finding could be due to the higher effective concentrations of chelated minerals in the hepatopancreas and hemolymph. Similarly, Yuan et al. (2019 b) assessed the effect of amino acid-chelated Cu on SOD activity and observed that this organic element can elevate the activity of this enzyme. Also, the authors believe that this may be associated with higher effective Cu concentration in hepatopancreas of *L. vannamei*. Another fairly similar study has exhibited that the supplementation of organic zinc could considerably increase the activity of SOD, compared to shrimp that received zinc sulfate, thanks to its superior bioavailability (Lin et al., 2013). In addition, it has been claimed that dietary chelated magnesium could alleviate oxidative stress in white-leg shrimp through affecting antioxidant-related enzymes such as CAT and SOD (Panmei et al., 2023).

Energy trade-offs are considered to be a crucial strategy for all creatures to grow, reproduce, and survive. In other words, limited resources such as essential nutrients and energy might result in the increasing energy allocation to some traits rather than other ones (Garland Jr et al., 2022; Ng'oma et al., 2017; Zerjal et al., 2021). For instance, it has been claimed that increasing energy allocation to one trait such as immunity requires reduction in another one (e.g., growth) (van der Most et al., 2011). However, the ongoing study demonstrated that the administration of organic-acid chelated macro- and micro-elements could simultaneously improve growth performance, immunity, and meat quality of the shrimp. The findings suggest that the supplement could adjust the energy allocation among various traits probably through optimizing the metabolism. Even though considerable studies have assessed such phenomena in wild animals, further studies need to be performed to assess this in farmed species (Zerjal et al., 2021).

Conclusion

This study broadens our perspective on the effects of the advanced chelate compound-based macro- and microelements on growth performance, survival, tissue miner-

alization, digestive enzymes, and redox and immune status of *L. vannamei*. The most obvious finding emerging from this study is that the administration of the organic elements could not only improve the growth performance, macro- and microelement levels in the body, and digestive enzyme activities but also enhance the redox and immune status through increasing the SOD, PO, and LZM activities as well as THC. Based on the results, moreover, supplementing the diet with this organic supplement at a level of 1000 mg kg⁻¹ can be more effective than spraying it on diets. Taken together, this study introduces an efficient supplement to enhance growth performance, meat quality, digestive system, and immunity of *L. vannamei*.

Declaration of competing interest

Bonza® shrimp is synthesized based on Advanced Chelate Compound technology which was invented by Mohammad Hassan Nazaran. The authors declare that they have no conflicts of interest.

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