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Effects of different levels of commercial food supplement, Biotronic® Top3 on biochemical and immune parameters of *Litopenaeus vannamei*

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Abstract

The effect of different dietary levels of Biotronic® Top3 (Biomin, Austria) on hemolymph biochemical parameters, hemolymph enzymes and immune parameters of white leg shrimp (*Litopenaeus vannamei*) was investigated. 375 Shrimps (mean initial weight: 4.28±0.05g) collected from the commercial farm and stocked randomly in 300L polyethylene tanks in triplicates. The shrimps were fed with commercial diet (4005, Faradaneh Company) supplemented with different levels of Biotronic® Top3 (0: Control, 0.5%, 1%, 2%, and 4%) for 56 days (8 weeks). At the end of the experiment, the biochemical indices, levels of hemolymph enzymes (AST, ALT, ALP and LDH), hemolymph immune parameters (Lysozyme and Phenoloxidase), and cellular hemolymph parameters of experimental shrimps were compared. The dietary inclusion of 2% and 4% of Biotronic® Top3 led to an increase in hemolymph enzymes. Shrimp were fed with supplemented diets also had lower levels of glucose, cholesterol and triglyceride and higher levels of protein, calcium and creatinine. Addition of Biotronic® Top3 in the feed had a positive effect on the cellular and humoral immune responses of shrimps. The results of this study showed that the best performance of dietary supplementation of Biotronic® Top3 on *L. vanammei* was observed at level of 1%.

Key words: Organic acids, *Litopenaeus vannamei*, Immune parameters, Hemolymph enzymes, Biochemical parameters

Introduction

Aquaculture has become one of the most critical parts of providing protein and food production and creating food security for society requirements (Rahman et al. 2015). Aquaculture is successful and sustainable when it contributes to aquatic health and optimizes culture conditions, achieving maximum aquaculture growth as well as reducing the side costs of the production process (Mente et al. 2011).

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Today, the principal concern of the aquaculture industry is to increase the performance of the aquatic immune system, improve the quality of the formulated diet to optimize the growth rate and promote health. In this regard, one of the ways to improve the growth parameters, nutrition, and survival of aquatic animals is to use food supplements (Zakaria and Sodagar 2016). Probiotics, prebiotics, carotenoids, amino acids, vitamins, minerals, enzymes, and metabolic modifiers are various dietary supplements that have an impact on health, growth, and meat quality in aquatic animals (Ebrahimi and Birghadar 2006). In many countries, banning or restricting the use of antibiotics as a dietary supplement in the aquaculture industry as a result of bacterial resistance increases attention to the use of other nutritional supplements such as prebiotics and probiotics and symbiotics. Shrimp actively feed on the floor. This behavior increases the risk of proliferation and spread of unwanted and dangerous bacteria in the shrimp's body, reducing the efficiency of the digestive system and causing disease. Herbal extracts, also called phytobiotics, can help grow and multiply beneficial bacteria in the gut by modulating shrimp's immune system and making aquatic animals resistant to disease (Coutteau 2010). In recent years, more attention has been paid to medicinal plants to control and prevent diseases. The use of medicinal plants has attracted a lot of attention around the world due to its many benefits, including having fewer side effects on the health of living organisms and the environment, being cheap, not creating drug resistance in bacteria, and being stable and available (Punitha et al. 2008).

One of the dietary supplements is Biotronic® Top3. Biotronic® Top3 is an active formula including organic acids, a phytochemical compound, and a permeabilizing complex. There have been limited studies on the effects of Biotronic® Top3, including research by Jadidi et al. (2016) that showed that Tiger Oscar fish fed 8 g/kg food of Biotronic® Top3 had the highest growth and nutrition indices. Menanteau-Ledouble et al. (2017), showed that using Biotronic® Top3 increases the immunity of Rainbow trout and increases fish resistance to furunculosis. They confirmed the immunostimulatory effects of this dietary supplement. Riemensperger et al. (2012) reported that Biotronic® Top3 supplementation had antimicrobial activity against gram-negative bacteria (*Salmonella enteritidis*, and *Salmonella typhimurium*). Langfield et al. (2004) also reported by using the microdilution method, that the Biotronic® Top3 supplement can inhibit the growth of pathogenic bacteria. Given that no research performed on the immunogenic effects of this compound and supplement on *L. vannamei*, this study examines the immunogenic effects, changes in hemolymph biochemical indicators, and hepatic toxicity.

Materials and Methods

Experimental treatments and rearing stages

350 juveniles of *L. vannamei* (mean weight: 4.28±0.05 g), which were healthy, randomly collected from rearing ponds and transferred to 300-liter polyethylene tanks. The shrimp then divided between treatments at a density of 25 pieces per tank. The shrimps were adapted to experimental conditions for 8 days. The experimental period was 8 weeks (56 days). Shrimp fed with experimental diets (basic commercial food and Biotronic® Top3 supplements). The implementation environment of the project simulated similar to the cultural environment. The water quality parameters of the treatments controlled by 10% daily water exchange. Also, the waste and unused food removed from the tanks. To reduce the shrimp stress, water temperature and pH were measured twice a day (morning and evening), and the amount of dissolved oxygen and water salinity was measured once a day by the multiparameter device. Tanks were fully aerated. Feeding was done

according to the shrimp feeding table and on the satiety method four times a day.

Five treatments considered in triplicates (four supplemented treatments with levels of 0.5, 1, 2, and 4% of Biotronic® Top3 in the diet and one treatment as control treatment without any supplementation). The composition of commercial supplement (Biotronic® Top3) used in this experiment made by Biomin Company (Austria), which prepared by Etoukfarda Company (Tehran, Iran). Basic commercial food (4005 Feed for shrimp, Faradaneh company) with defined chemical analysis (protein: 43-41%, lipid: 7-10%, moisture 5-10%), prepared and stored in plastic bags at -20^{o}C until consumption.

Sampling

Sampling was performed randomly at the end of the experimental period. An anticoagulant solution used for sampling (10 ml Tris HCl, 250 ml Sucrose, 100 ml Citrate Sodium with 6.7 pH = 1: 1 ratio) (Samadi et al. 2016). Immediately after taking hemolymph from abdominal sinus, the contents of the syringe transferred to a sterile microtubule and kept in a liquid nitrogen tank at -196^{o}C for 48 hours before analysis.

Measurement of hemolymph biochemical parameters

For measuring of hemolymph biochemical parameters, hemolymph samples centrifuged at 6000 rpm at 4 ^{o}C, for 10 minutes (Hettich D-7200 Tuttlingen centrifuge, Germany). Then, the supernatant was separated and transferred to 1.5 ml microtubules for biochemical analysis of hemolymph plasma as well as the study of other indicators studied in this study.

The hemolymph biochemical parameters including Glucose, Protein, Triglyceride, Cholesterol, Creatinine, Calcium were determined using biochemical analysis kits (Pars Azmoun, Iran) as described by company.

Measurement of hemolymph enzymes

The hemolymph AST (Aspartate aminotransferase), ALT (Alanine transaminase) and ALP (Alkaline phosphatase) activity were measured using the quantitative diagnostic kits (Pars Azmoun Company, Iran) by photometric method (Moss and Henderson 1999). Also, the amount of lactate dehydrogenase (LDH) in hemolymph was measured using the quantitative diagnostic kits (Pars Azmoun Company, Iran) by a photometric method and by DGKC method (Standard of the Germany Biochemical Association) at a wavelength of 405 nm (Shahsavani et al. 2010).

Measurement of hemolymph immune parameters

Measurement of phenoloxidase activity

The phenoloxidase activity of hemolymph determined using a spectrophotometer based on production of chrome-DOPA pigment following oxidation of L-DOPA (Sigma) substrate. In this method, cacodylate buffer (CAC) and trypsin-stimulating enzyme (1%) (Sigma) used, and at the end, the absorption rate read at 490 nm. The chrome-DOPA complex measured after 0, 5, and 10 minutes (each unit of enzyme activity was equivalent to a change in 0.0001 absorptions/minute/mg of protein at 35 ^{o}C) (Hall and Soderhäll 1989).

Measurement of lysozyme activity

A turbidimetric method recommended by Ellis (1990) used to measure the hemolymph lysozyme activity. In this respect, 10 µL of hemolymph plasma sample with 200 µL of 0.2 mg/ml suspension of Micrococcus lysodeikticus (Sigma) in sodium phosphate buffer (0.05 mol, pH = 6.2) mixed in the ELISA plates and the absorbance measured at wavelength 530 nm (at 25 ^{o}C, 1 and 6 minutes after mixing). Lysosome activity causes bacterial lysis and reduces absorbance. A unit of lysozyme activity determined with a decrease in absorbance of 0.001 per minute per ml of hemolymph plasma.

Measurement of hemolymph cellular characteristics

Total hemocytes count

The hemolymph samples homogenized with the Vertex device for 20 seconds. To count the total number of hemocytes, 100 µL from each sample were mixed with an equal volume of formalin buffer (10%) for 30 minutes, and then calculated the total number of hemocytes (THC) by Neubauer slide (Germany) using an optical microscope (Song et al. 2003, Le Moullac et al. 1997).

Differential cell count

The hemolymph smear prepared by adding 50 µL of the hemolymph sample on a microscopic slide. Then the smear fixed by methanol, stained by the may-Grunwald Giemsa method. Then the stained smears surveyed under an optical microscope and different hemocytes counted and presented in percentage.

Statistical analyses

Data in the results reported based on mean±standard errors. Data analyzed by SPSS software version 20, using a One-way ANOVA analysis. Duncan's post-test used to test the significance of the mean differences. In all studies, the significance level of the tests was 95%. Excell software version 2013 used to draw the graphs.

Results

Hemolymph biochemical parameters

Table 1 presents the results of levels of glucose, total protein, cholesterol, triglycerides, calcium, and creatinine. The results showed that the total protein content was not significantly different between control treatment and treatments containing different levels of Biotronic® Top3 ($p>0.05$). There are significant differences between control and experimental treatments for levels of glucose, cholesterol, triglycerides, calcium, and creatinine ($p<0.05$). Based on the results, the decreasing trend of hemolymph glucose levels was observed by increasing Biotronic levels. The lowest level of glucose was observed in the treatment 4 (12.67±1.76 mg/dl) and treatment 5 (13.33±1.33 mg/dl), respectively and the highest level of hemolymph glucose was observed in the control treatment (22.67±0.66 mg/dl) ($p<0.05$). The decreasing trend of cholesterol levels was seen from control treatment to the highest level of Biotronic supplemented diet. So, the highest cholesterol level in hemolymph observed in control treatment (62.00±1.15 mg/dl) and the lowest cholesterol level recorded in treatment 5 (27.33±5.33 mg/dl) ($p<0.05$). The highest level of hemolymph triglyceride was observed in control treatment (130.00±5.29 mg/dl) and the lowest level of triglycerides was recorded in treatment 5 (33.33± 2.40 mg/dl) with a decreasing trend from control treatment to treatment 5 ($p<0.05$). The highest level of calcium was observed in treatment 5 (18.66±1.87 mg/dl) and the lowest calcium level measured in the control treatment (10.06±1.16 mg/dl) ($p<0.05$) and there was a fluctuation trend for calcium levels between treatments. The lowest level of creatinine was observed in the control treatment (0.52±0.01 mg/dl) and the highest level was observed in treatment 5 (0.90±0.05 mg/dl); ($p<0.05$) with an increasing trend from control treatment to treatment 5.

Table 1: The Mean±SE of hemolymph biochemical parameters of *L. vannamei* fed on diets supplemented by different levels of Biotronic® Top3

Hemolymph biochemical parameters	Experimental treatments				
	Treatment 1 (control)	Treatment 2 (0.5%)	Treatment 3 (1%)	Treatment 4(2%)	Treatment 5 (4%)
Glucose (mg/dl)	22.67±0.66[a]	20.00±1.15[ab]	18.00±1.15[b]	12.67±1.76[c]	13.33±1.33[c]
Total protein (g/dl)	3.16±0.67[a]	4.41±0.99[a]	4.08±0.56[a]	4.28±0.85[a]	5.95±0.91[a]
Cholesterol (mg/dl)	62.00±1.15[a]	47.33±1.76[bc]	48.67±6.36[b]	35.33±2.66[cd]	27.33±5.33[d]
Triglyceride (mg/dl)	130.00±5.29[a]	95.33±7.42[b]	90.67±5.33[b]	62.00±7.21[c]	33.33±2.40[d]
Calcium (mg/dl)	10.06±1.16[c]	15.20±1.31[ab]	15.06±1.36[ab]	13.20±0.20[bc]	18.66±1.87[a]
Creatinine (mg/dl)	0.52±0.01[c]	0.54±0.02[bc]	0.64±0.04[b]	0.66±0.01[b]	0.90±0.05[a]

* All data are expressed as mean±standard error. The significance level is 95% (p<0.05).
** The presence of different letters in each row indicates a significant difference between the experimental treatments.

Hemolymph enzymes assay

Table 2 shows the levels of hemolymph enzymes included AST, ALT, ALP, and LDH. The results showed an increasing trend in all the above-mentioned parameters from control treatment to treatment 5 (p<0.05). The highest level of AST was recorded in treatment 4 (300.00±34.48 U/L) and 5 (289.33±42.62 U/L), respectively and the lowest AST level was recorded in the control treatment (85.33±2.90 U/L) (p<0.05). The highest ALT level measured in treatment 5 (128.67±7.68 U/L) and the lowest ALT level was measured in treatment 2 (38.00±3.05 U/L) and control treatment (49.33±11.39 U/L) (p<0.05). The highest level of LDH was measured in treatment 5 (706.67±60.66 U/L) and the lowest level of LDH was measured in the control treatment (159.33±28.15 U/L) (p<0.05). The highest level of ALP recorded in treatment 5 (1430.00±95.68 U/L), and the lowest ALP level was recorded in the control treatment (449.33±11.62 U/L) (p<0.05).

Table 2: The Mean±SE of hemolymph enzymes activity of *L. vannamei* fed on diets supplemented by different levels of Biotronic® Top3

Hemolymph biochemical parameters	Experimental treatments				
	Treatment 1 (control)	Treatment 2 (0.5%)	Treatment 3 (1%)	Treatment 4(2%)	Treatment 5 (4%)
SGOT (U/L)	85.33±2.90[b]	87.33±3.71[b]	86.00±8.08[b]	300.00±34.48[a]	289.33±42.62[a]
SGPT (U/L)	49.33±11.39[c]	38.00±3.05[c]	107.33±12.34[ab]	88.67±12.71[b]	128.67±7.68[a]
LDH (U/L)	449.33±11.62[d]	600.67±82.56[cd]	688.67±42.11[bc]	876.67±39.90[b]	1430.00±95.68[a]
ALP (U/L)	159.33±28.15[c]	192.67±22.04[c]	244.67±4.80[c]	433.33±8.74[b]	706.67±60.66[a]

* All data are expressed as mean±standard error. The significance level is 95% (p<0.05).
** The presence of different letters in each row indicates a significant difference between the experimental treatments.

Hemolymph immune parameters

Table 3 shows the results of hemolymph lysozyme and phenoloxidase activity. The results showed that there are significant differences in levels of lysozyme and phenol oxidase activities between treatment 5 and other treatments (p<0.05). Based on the results, the lowest level of lysozyme activity (0.39±0.05 U/min/mg Protein) was recorded in control treatment and the highest level of lysozyme activity was recorded in treatment 5 (0.81±0.04 U/min/mg protein) ($p<0.05$). The lowest activity of phenoloxidase was recorded in control treatment (0.10±.00 Absor/min/mg protein), and the highest level of phenoloxidase activity was recorded in treatment 5 (0.14±0.01 Absor/min/mg Protein) ($p<0.05$).

Table 3: The Mean±SE of humoral immune response of *L. vannamei* fed on diets supplemented by different levels of Biotronic® Top3

Humoral immune parameters	Experimental treatments				
	Treatment 1 (control)	Treatment 2 (0.5%)	Treatment 3 (1%)	Treatment 4(2%)	Treatment 5 (4%)
Lysozyme (U/min/mg Protein)	0.39±0.05^{b}	0.41±0.00^{b}	0.42±0.06^{b}	0.42±0.02^{b}	0.81±0.04^{a}
Phenoloxidase (Absor/min/mg protein)	0.10±0.00^{b}	0.11±0.00^{b}	0.12±0.01^{b}	0.12±0.00^{ab}	0.14±0.01^{a}

* All data are expressed as mean±standard error. The significance level is 95% ($p<0.05$).
** The presence of different letters in each row indicates a significant difference between the experimental treatments.

Hemolymph cellular parameters

Table 4 shows the results of total hemocytes count (THC), percentage of hyalinocyte (HC), small granulocytes (SGC), and granulocytes (GC). The results showed that there was a significant increasing trend in THC from control treatment to treatment 5. So, the highest THC was measured in treatment 5 (151.67±3.38 ×10^5/ml) and the lowest THC (118.33±0.88 ×10^5/ml) was measured in control treatment ($p<0.05$). Although, no significant differences were observed between control treatment with treatment 2 and between treatment 3 and treatment 4, respectively ($p<0.05$). The highest rate of hyalinocyte was measured in treatment 2 (81.50±2.29%) and the lowest rate of hyalinocyte was measured in the control treatment (68.4±3.13%) ($p<0.05$). The highest rate of small granulocytes (24.16±1.90%) was measured in control treatment and the lowest rate of small granulocytes was measured in treatment 2 (11.02±0.95%), and there was a significant difference between the control treatment and all Biotronic® Top3 treatments ($p<0.05$). The highest and the lowest rates of granulocytes were recorded in treatment 5 (16.54±0.57%) and treatment 2 (6.68±0.02%), respectively ($p>0.05$).

Table 4: The Mean±SE of hemolymph cellular parameters of *L. vannamei* fed on diets supplemented by different levels of Biotronic® Top3

Hemolymph cellular parameters	Experimental treatments				
	Treatment 1 (control)	Treatment 2 (0.5%)	Treatment 3 (1%)	Treatment 4(2%)	Treatment 5 (4%)
THC (×10^5 cell/ml)	118.33±0.88^{c}	122.00±2.08^{c}	138.33±2.40^{b}	139.33±0.88^{b}	151.67±3.38^{a}
Hyolinocyte (%)	68.04±3.12^{b}	81.50±2.29^{a}	73.96±3.53^{ab}	78.13±3.09^{a}	72.40±0.65^{a}
SGC (%)	24.16±1.90^{a}	11.79±2.27^{b}	15.57±0.46^{b}	14.30±1.66^{b}	11.02±0.95^{b}
GC (%)	7.48±1.33^{b}	6.68±0.02^{b}	13.45±4.54^{ab}	7.55±1.76^{b}	16.54±0.57^{a}

* All data are expressed as mean±standard error. The significance level is 95% ($p<0.05$).
** The presence of different letters in each row indicates a significant difference between the experimental treatments.

Discussion

Based on the results of this study, the supplementing Biotronic® Top3 to the diet of *L. vannamei* changed the levels of hemolymph biochemical parameters. Hemolymph biochemical parameters are valued indicators for health monitoring and physiological responses to nutritional status. Because serum and blood parameters show adverse conditions much faster than other diagnostic factors, they are widely used to describe the animal's health condition. So, biochemical parameters used to assess existing stress and physiological responses (Arshadi et al. 2018).

According to the results of the present study, at the end of the experimental period, the level of glucose decreased with supplementing increased levels of Biotronic® Top3. Various studies on the use of organic acids in diets showed no significant effect on the levels of serum glucose in different aquatic animal species (Shah et al. 2015, Saei et al. 2016). On the other hand, Banaee et al. (2011) studied the effects of oral administration of cinnamon supplementation in rainbow trout. They observed a decrease in serum glucose levels in the studied fish. Based on the results provided by Banaee et al. (2011), the addition of cinnamon supplement to the diet of rainbow trout probably led to an increase in insulin biosynthesis and an increase in its release, resulting in a decrease in serum glucose levels. Cinnamon is abundant in polyphenols and cinnamaldehyde which are essential for the improvement in blood glucose metabolism and breakdown of fatty acids (Khan et al. 2016). High density lipoproteins level was also enhanced by the bioactive compounds present in cinnamon leading to lowering of cholesterol and triglycerides level. Prominently, polyphenols are a source of mimetic insulin which activate and stimulate glucose metabolism led to decrease in hemolymph glucose.

Measuring blood protein concentrations in simple groups of crustaceans can provide valuable information to determine their physical condition (Ozbay and Riley 2002). Based on the results of the present study, an increasing trend in hemolymph protein levels has observed in experimental treatments. This incremental trend has shown in various studies on organic acids and plant extracts (Banaee et al. 2011 (*Oncorhynchus mykiss*), He et al. 2017 (*Litopenaus vannamei*), Samadi et al. 2016 (*Litopenaeus vannamei*), Su et al. 2014 (*Litopenaus vannamei*)). Flora et al. (1998) and El-Kamary et al. (2009) reported that cinnamon consumption caused a significant increase in ribosome formation and DNA synthesis, as well as an increase in protein synthesis and facilitated the hepatic production. Since there is a close relationship between protein synthesis in liver tissue and total plasma protein concentration, an increase in total hemolymph protein levels in shrimp treated with Biotronic® Top3 may be due to increased protein synthesis in hepatopancreatic tissue. Based on the results, decreasing trends in hemolymph cholesterol and triglyceride levels observed in experimental treatments. The effects of depressing serum cholesterol and triglycerides have shown in different studies on organic acids and herbal supplements. The cinnamon in Biotronic® Top3 supplement can affect the quantity and quality of membrane lipids included cholesterol and phospholipids (Basiglio et al. 2009). Sobolova et al. (2006) observed that due to the inhibition of cholesterol absorption by silymarin, it plays a critical role in regulating plasma cholesterol profile. On the other hand, inhibiting the activity of cholesterol-acetyl transferase may be the main way to reduce cholesterol absorption and biosynthesis of lipoproteins (Sobolova et al. 2006).

The use of organic acids appears to increase the concentration of lipoproteins in hepatocytes by increasing protein synthesis (Sheikh et al. 2010). On the other hand, some studies have shown that organic acids significantly increase the height of intestinal villi and increase the depth of crypts. Therefore, it seems that these effects of organic acids lead to improved digestion and absorption of energy and protein and all digestive substances in the intestine and improve the process of lipoprotein synthesis (Fouladi et al. 2014). Wilder et al. (2003) reported that the mean levels of hemolymph calcium in *L. vannamei* and *Macrobrachium resenbergii* were 70 mg/dl and 64 mg/dl, respectively, which is higher than the levels recorded in the present study. This difference can fluctuate under the influence of different factors and conditions, such as the stage of molting and growth, skeletal calcium transfers to hemolymph and stomach, the type of diet, methods of measuring biochemical parameters, and the shrimp biological stages (Chen et al. 1995). Several studies have shown that the use of organic acids increases the digestibility and absorption of minerals (He et al. 2017, Gislason et al. 1994). Acidic anions improve their absorption from the intestine by combining them with mineral elements such as calcium, phosphorus, zinc, and magnesium (da Silva et al. 2013). In general, organic acids increase the solubility of minerals by reducing the pH of digestive contents, possibly increasing their ability to be used (da Silva et al. 2013).

Abdel-Fattah et al. (2008) stated that adding different levels of citric acid (1.5 and 3%) increases affects the blood concentration of calcium and phosphorus. However, the components of herbal extracts may also be effective in increasing calcium absorption from the intestine and increasing serum and hemolymph calcium (Samadi et al. 2016). The results of this study indicate the increasing trend in the hemolymph creatinine level, focused on treatment 5. High creatinine levels in shrimp hemolymph in supplemented treatments with higher levels of Biotronic® Top3 may be due to damage to skeletal muscle or dysfunction of the shrimp excretory system in the excretion of excess creatinine from hemolymph (Samadi et al. 2016).

Comparison of the results of the present study at the end of the experiment, the increasing trend of hemolymph enzymes levels (included AST, ALT, LDH, and ALP) were observed ($p<0/05$), among the Biotronic® Top3 treatments. In the meantime, treatment 5 (4% Biotronic® Top3) showed higher levels of hemolymph enzymes. These hemolymph enzymes are indicators of hepatopancrease health. So, their increase indicates an increase in hepatopancreatic activity or possible damage to the hepatopancreas. Silymarin (*Silybum marianum*) is a medicinal plant that has significant medicinal and nutritional applications. It is mainly used in hepatic diseases because medicinal plants have specifically repaired the activity of hepatic enzymes due to their antioxidant properties. It removes free radicals and chelates metal ions, inhibiting lipid peroxidation, protecting cell membrane permeability, and preventing hepatic glutathione depletion (Borsari et al. 2001, Ramadan et al. 2002). However, high levels of silymarin can lead to cell damage and hepatotoxicity. Eventually lead to increased levels of hepatic enzymes (Stickel et al. 2005, Banaee et al. 2011). Different studies have also shown that the administration of organic acids increases hepatic enzymes by increasing anabolic and catabolic activities and totally, by increasing cellular metabolism in the liver (Fouladi et al. 2014, He et al. 2017). Increases in hepatic enzymes have observed in some similar studies on organic acids in aquatic diets (Maktabi 2019).

Hemocytes play a critical role in host immune responses, including the detection of pathogens, phagocytosis, melanization reaction, and cytotoxicity (Johansson et al. 2000). Based on the results of the present study, with increasing levels of Biotronic® Top3 in experimental treatments, an increasing trend in the total homocytes was observed. Hemocytes play very important roles in the shrimp's immune system and are divided into three cell types based on the presence of cytoplasmic granules: hyalinocytes (HCs), small granulocytes (SGCs), and granulocytes. (GCs). Hyalinocytes are involved in the process of phagocytosis and small granulocytes and granulocytes are involved in the processes of encapsulation and secretion and release of prophenoloxidase (proPO) (Jiravanichpaisal et al. 2006). Based on the results, the addition of Biotronic® Top3 supplements led to an increase in the total hemocytes, a relative increase in hyalinoctyes and granulocytes. Also, the use of Biotronic® Top3 supplementation led to an increase in the levels of prophenoloxidase and lysozyme in the treated experiments. These results indicate the effects of Biotronic® Top3 immunostimulant and immune regulatory effects on the experimental shrimp. The antioxidant activity of organic acids and sylimarine in Biotronic® Top3 supplements plays a role in the regulatory effects of the immune system, which protects hemocytes from oxidative damage (Bendich 1989, da Silva et al. 2013, He et al. 2017, Arshadi et al. 2018). In addition, the effects of plant compounds in Biotronic® Top3 on enhancing and improving cellular and humoral immune responses in vertebrates have been demonstrated in previous studies (Bendich 1989, Chew and Park 2004, Samadi et al. 2016). Samadi et al. (2016) stated that *L. vannamei*, which has been exposed to plant extracts, has been associated with an increase in the total hemocytes, which has been shown to proliferate hemocytes in hematopoietic tissues. An overall increase in the homocytes has also been shown in some other studies on plant extracts and organic acids in shrimp (Chuchird et al. 2015, Su et al. 2014). In aquaculture, immune system stimulants can stimulate the innate immune system in shrimp by activating and increasing the activity of granulocytes and hyalimocytes and increasing the process of phagocytosis (Sakai 1999, da Silva et al. 2013, He et al. 2017, Arshadi et al. 2018). They can increase protein synthesis to increase the production of protein molecules involved in the innate immune system, such as lysozyme (Ng et al. 2015, Romano et al. 2015). Menanteau-Ledouble et al. (2017) observed an increase in resistance to infection with *Aeromonas salmonocida*, by adding Biotronic® Top3 supplement to the diet of rainbow trout. ProPO and lysozyme are important immune response factors in shrimp. ProPo activation system is an important defense system in detecting external factors in shrimp (Cardenas and Dankert 1997), which indicates the health status and defense sensitivity of shrimp body. Prophenoloxidase is an enzyme that accelerates the oxidation of phenolic compounds such as tyrosine and L-DOPA and is the source of a complex molecular sequence that peaks with the formation of melanin. During this sequence, several intermediate compounds (such as quinone) have antimicrobial effects (Sritunyalucksana and Soderhall 2000). Phenoloxidase is directly dependent on disease changes and environmental conditions (Li et al. 2006, Wang and Chen 2005). Lysozyme can also hydrolyze acetylsalicylic acid in the bacterial wall and kill bacteria. Due to the relative and significant increase in granulocytes compared to other hemocysts in hemolymph and the increase hemolymph phenoloxidase levels, it can be concluded that the effects of Biotronic® Top3 on the immune system of *L. vannamei*, mainly conducted by increasing the conversion of small granulocytes to granulocytes and increase the granule release and melanization reaction. However, the phagocytosis process is not out of the question due to the relative increase in hyalinocytes. The similar results were reported by He et al. (2017), Su et al. (2014), Romano et al. (2015) and Ng et al.

(2015). He et al. (2017) Reported that AviPlus® (organic acids and essential oils blend) application significantly enhanced relative expression of immune related genes including lysozyme, penaeidin and catalase at 48 h post challenge. They stated that the tested organic acids and essential oils mixture beneficially affects intestinal microflora and improves immune response and disease resistance of *L. vannamei*.

According to the present study, supplementing Biotronic® Top3 to the diet of *L. vannamei* in eight weeks feeding period decreases glucose, cholesterol and triglyceride levels and increases the levels of protein, calcium, and creatinine of hemolymph, especially in treatment 4 (2% Biotronic® Top3) and 5 (4% Biotronic® Top3). Supplementing Biotronic® Top3 also increased hemplymph enzymes, especially in treatments 4 (2% Biotronic® Top3) and 5 (4% Biotronic® Top3). Also, the positive trend of the effects of Biotronic® Top3 on the cellular and humoral immune system of *L. vannamei* fed with different nutritional levels of Biotronic® Top3 was evident. Due to the positive immunological effects and the negative effects of increasing hemolymph enzymes and hemolymph creatinine level in treatments 4 and 5, and the positive effects of different levels of dietary Biotronic Top3 on growth performance, feed utilization and body biochemical composition of the white leg shrimp (*Litopenaeus vannamei*) which were reported by Zahizadeh et al. (2020), the best performance of Biotronic® Top3 was observed in treatment 3 (1% Biotronic® Top3).

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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References

Abdel-Fattah, S.A., El-Sanhoury, M.H., El-Mednay, N.M. & Abdel-Azeem, F. (2008). Thyroid activity, some blood constituents, organs morphology and performance of broiler chicks fed supplemental organic acids. *International Journal of Poultry Science*, 7, 215-222.

Arshadi, A., Yavari, V., Oujifard, A., Mousavi, S.M., Gisbert, E. & Mozanzadeh, M.T. (2018). Dietary nucleotide mixture effects on reproductive and performance, ovary fatty acid profile and biochemical parameters of female Pacific shrimp *Litopenaeus vannamei*. *Aquaculture Nutrition*, 24, 515– 523.

Artiss, J.D. & Zak, B. (1997). Measurement of cholesterol concentration. Handbook of Lipoprotein Testing. AACC Press, Washington: 99- 114.

Banaee, M., Sureda, A., Mirvaghefi, A.R. & Rafei, G.R. (2011). Effects of long-term silymarin oral supplementation on the blood biochemical profile of rainbow trout (*Oncorhynchus mykiss*). *Fish Physiology and Biochemistry*, 37, 885–896.

Basiglio, C.L., Sa´nchez Pozzi, E.J., Mottino, A.D. & Roma, M.G. (2009). Differential effects of *silymarin* and its active component *silibinin* on plasma membrane stability and hepatocellular lysis. *Chemico-Biological Interact*, 179, 297–303.

Bauer, P.J. (1981). Affinity and stoichiometry of calcium binding by arsenazo III. *Analytical Biochemistry*, 110(1), 61- 72.

Bendich, A. (1989). Cartenoids and the Immune response. *Nutrition*, 119(1), 112-115.

Borsari, M., Gabbi, C., Ghelfi, F., Grandi, R., Saladini, M., Severi, S. & Borella, F. (2001). Silybin, a new iron-chelating agent. *Journal of Inorganic Biochemistry*, 85, 123–129.

Cárdenas, W. & Dankert, J.R. (1997). Phenoloxidase specific activity in the red swamp crayfish *Procambarus clarkia*. *Fish & Shellfish Immunology*, 7, 283–295.

Chen, J.C., Lin, M.N., Ting, Y.Y. & Lin, J.N. (1995). Survival, haemolymph osmolality and tissue water of *Penaeus chinensis* juveniles acclimated to different salinity and temperature levels. *Comparative Biochemistry and Physiology*, 110 A, 253-258.

Chew, B.P. & Park, J.S. (2004). Carotenoid action on the immune response. *Journal of Nutrition*, 134, 257S–261S.

Chuchird, N., Rorkwiree, Ph. & Rairat, T. (2015). Effect of dietary formic acid and astaxanthin on the survival and growth of Pacific white shrimp (*Litopenaeus vannamei*) and their resistance to *Vibrio parahaemolyticus*. *Springer Plus*, 4:440, 1-12.

da Silva, B.C., do Nascimento Vieira, F., Mouriño, J.L.P., Ferreira, G.S. & Seiffert, W.Q. (2013). Salts of organic acids selection by multiple characteristics for marine shrimp nutrition, *Aquaculture*, 384–387, 104-110.

Ebrahimi, A. & Beiraghdar, A. (2006). Nutrition and fish requirements in aquaculture (with emphasis on cultural species in Iran). Isfahan University of Technology, Jihad Daneshgahi Publications. 1st edition, 304 pages (in Persian Language).

El-Kamary, S.S., Shardell, M.D., Abdel-Hamid, M., Ismail, S., El-Ateek, M., Metwally, M., Mikhail, N., Hashem, M., Mousa, A., Aboul-Fotouh, A., El-Kassas, M., Esmat, G. & Stricklanda, T. (2009). A randomized controlled trial to assess the safety and efficacy of silymarin on symptoms, signs and biomarkers of acute hepatitis. *Phytomedicine*, 16, 391–400.

Ellis, A.E. (1990). Lysozyme Assays. Techniques in fish immunology, 101-103.

Foster, L.B. & Hocholzer, J.M. (1971). A single reagent manual method for directly determining urea nitrogen in serum. *Clinical Chemistry*, 17, 921-925.

Fouladi, P., Ebrahimnezhad, Y., Shahryar, H.A., Maheri, S.N. & Ahmadzadeh, A.R. (2014). Effects of organic acids supplement on performance and gut parameters in male Japanese quail (*Coturnix coturnix*). *Journal Biological International*, 6(2), 102-109.

Gislason, G., Olsenm R.E. & Ringø, E. (1994). Lack of growth-stimulating effect of lactate on Atlantic salmon, *Salmo sal*ar L. *Aquaculture and Fisheries Management*, 25, 861- 862.

He, W., Rahimnejad, S., Wang, L., Song, K., Lu, K. & Zhang, Ch. (2017). Effects of organic acids and essential oils blend on growth, gut microbiota, immune response and disease resistance of Pacific white shrimp (*Litopenaeus vannamei*) against *Vibrio parahaemolyticus*, *Fish & Shellfish Immunology*, 70, 164-173.

Jiravanichpaisal, P., Lee, B.L. & Söderhäll, K. (2006). Cellmediated immunity in arthropods. hematopoiesis, coagulation, melanization and opsonization. *Immunobiology*, 211, 213-36.

Johansson, M.W., Keyser, P., Sritunyalucksana, K. & Söderhäll, K. (2000). Crustacean haemocytes and haematopoiesis. *Aquaculture*, 191 (1–3), 45–52.

Khan, M.U., Qurashi, N.A., Khan, M.S., Jabeen, F., Umar, A., Yaqoob, J. & Wajid. M. (2016). Generation of reactive oxygen species and their impact on the health-related parameters: A critical review. *International Journal of Biosciences*, 9(1), 303-323.

Le Moullac, G., Le Groumelle, M., Ansquer, D., Froissard, S. & Levy, P. (1997). Haematological and phenloxidase activity change in the shrimp *Penaeus stylirostris* in relation with the moult cycle: protection against vibriosis. *Fish & Shellfish Immunology*, 7, 227-234.

Li, J.T., Lee, P.P., Chen, O.C. & Chen, W. (2005). Dopamine depresses the immune ability and increases susceptibility to *Lactococcus garvieae* in the freshwater giant prawn, *Macrobrachium rosenbergii*. *Fish & Shellfish Immunology*, 19, 269-280.

Maktabi, P., Mohammadi Azarm, H., Peighan, R., Mousavi, S.M. & Zarei, M. (2019). Effect of Formic Acid, Potassium D-Format Salt and Formic Acid Nanochitosan Solution on Survival Rate, Growth Parameters and Carcass Composition in Common Carp (*Cyprinus carpio*). *Journal of Wetland Ecological Research*, Islamic Azad University, 10 (38), 41-54. (in Persian Language).

Menanteau-Ledouble, S., Krauss, I., Goncalves, R.A., Weber, B., Santos, G.A. & El-Matbouli, M. (2017). Antimicrobial effect of the Biotronic® Top3 supplement and efficacy in protecting rainbow trout (*Oncorhynchus mykiss*) from infection by *Aeromonas salmonicida subsp. Salmonicida*. *Research in Veterinary Science*, 114, 95–100.

Mente, E., Karalazos, V., Karapanagiotidis, I. & Pita, C. (2011). Nutrition in organic aquaculture: an inquiry and a discourse. *Aquaculture Nutrition*, 17, 798-817.

Ng, W.K., Koh, Ch.B., Teoh, Ch.Y. & Romano, N. (2015). Farm-raised tiger shrimp, *Penaeus monodon*, fed commercial feeds with added organic acids showed enhanced nutrient utilization, immune response and resistance to *Vibrio harveyi* challenge, *Aquaculture*, 449, 69-77.

Ozbay, G. & Riley, J.G. (2002). An analysis of refractometry as a method of determining blood total protein concentration in the American lobster *Homarus americanus* (Milne Edwards). *Aquaculture Research*, 33, 557-562.

Punitha, S.M.J., Babu, M.M., Sivaram, V., Shankar, V.S., Dhas, S.A., Mahesh, T.C., Immanual, G. & Citarasu, T. (2008). Immunostimulating influence of herbal biomedicines on nonspecific immunity in Grouper *Epinephelus tauvina* juvenile against *Vibrio harveyi* infection. *Aquaculture International Journal*, 16, 511-523.

Rahman, M.M., Normawaty, M.N., Shahbudin, S. & Kamaruzzaman, Y. (2015). Effects of human activity and the distance from shoreline. Desalin Water treat. Coastal water quality of Tioman Island. Doi: 10.108./ 19443994. 2. 15. 100682.

Ramadan, L.A., Roushdy, H.M., Abu-Senna, G.M. & Amin, N.E. (2002). Radio-protective effect of silymarin against radiation induced hepatotoxicity. *Pharmacological Research*, 45(6), 447–454.

Rifai, N., Bachorik, P.S. & Albers, J.J. (1999). Lipids, lipoproteins, and apolipoproteins.In: Burtis, C.A., Ashwood, E.R. (Eds.), Tietz Textbook of Clinical Chemistry. WBSaunders, Philadelphia, Pennsylvania, pp. 809–861.

Romano, N., Koh, Ch.B. & Ng, W.K. (2015). Dietary microencapsulated organic acids blend enhances growth, phosphorus utilization, immune response, hepatopancreatic integrity and resistance against *Vibrio harveyi* in white shrimp, *Litopenaeus vannamei*, *Aquaculture*, 435, 228-236.

Sacks, D.B. (2011). A1C Versus glucose testing: a comparison, *Diabetes Care*, 34, 518-523.

Saei, M.M., Beiranvand, K., Taee, H.M. & Nekoubin, H. (2016). Effects of Different Levels of BioAcid Ultra on Growth Performance, Survival, Hematological and Biochemical Parameters of Fingerlings Rainbow Trout (*Oncorhynchus mykiss*). *Journal of Aquaculture Research and Development*, 7(455), 1-5.

Sakai, M. (1999). Current research status of fish immunostimulants. *Aquaculture*, 172(1–2), 63-92.

Samadi, L., Zanguee, N., Mousavi, S.M. & Zakeri, M. (2016). Effect of dietary Garlic extract on growth, feeding parameters, hematological indices and body composition *of Litopenaeus vannamei*. *Journal of the Persian Gulf (Marine Science)*, 7(24), 29-41.

Shah, S.Z.H., Afzal, M., Khan, S.Y., Hussain, S.M. & Habib, R.Z. (2015). Prospects of using citric acid as fish feed supplement. *International Journal of Agriculture and Biology*, 17, 1−8.

Shahsavani, D., Kazerani, H.R., Kaveh, S. & Gholipour-Kanani, H. (2010). Determination of some normal serum parameters in starry sturgeon (*Acipenser stellatus* Pallas, 1771) during spring season. *Comparative Clinical Pathology*, 19, 57–61.

Sheikh, A., Tufail, B., Gulam, A.B., Masood, S.M. & Manzoor, R. (2010). Effect of dietary supplementation of organic acids on performance, intestinal, histomorphology and serum biochemistry of broiler chickens. *Veterinary Medicine International*, 479485-479487.

Sobolova, L., Skottova, N., Vecera, R. & Urbanek, K. (2006). Effect of silymarin and its polyphenolic fraction on cholesterol absorption in rats. *Pharmacological Research*, 53, 104–112.

Söderhäll, K. & Hall, L. (1984). Lipopolysaccharide-induced activation of the prophenoloxidase activating system in crayfish haemocytelysate. *Biochimica et Biophysica Acta*, 797, 99-104.

Song, L., Yu, I., Lien, W. & Huang, C. (2003). Haemolymph parameters of *Litopenaeus vannamei* infected with Taura syndrome virus. *Fish & Shellfish Immunology*, 14, 317-331.

Stickel, F., Patsenker, E. & Schuppan, D. (2005). Herbal hepatotoxicity. *Journal of Hepatology*, 43, 901–910.

Su, X., Li, X., Leng, X., Tan, C., Liu, B., Chai, X. & Guo, T. (2014). The improvement of growth, digestive enzyme activity and disease resistance of white shrimp by the dietary citric acid. *Aquaculture International*, 22, 1823–1835.

Wang. F.I. & Chen. J.C. (2006). The immune response of tiger shrimp *Penaeus monodon* and its susceptibility to *Photobacterium damselae* subsp. Damselae under temperature stress. *Aqaculture*, 258, 34-41.

Zahizadeh, A., Zakeri, M., Mousavi, S.M., Kochanian, P. & Souri, M. (2020). Effect of different levels of dietary Biotronic Top3 on growth performance, feed utilization and body biochemical composition of the white leg shrimp (*Litopenaeus vannamei*). *Journal of Fisheries*, 73(4), 515-527.

Zakaria, H. & Sodagar, M. (2016). Aquatic plants and their role in the aquarium. *Ornamental Aquatic Extension Scientific Journal*. 3(2), 25-30. (in Persian Language).

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اثرات سطوح مختلف مکمل تغذیه‌ای تجاری Biotronic® Top3 بر پارامترهای بیوشیمیایی و ایمنی میگوی سفید غربی (*Litopenaeus vannamei*)

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چکیده

اثر سطوح مختلف مکمل غذایی Biotronic® Top3 (Biomin، اتریش) بر پارامترهای بیوشیمیایی همولنف، آنزیم‌های همولنف و پارامترهای ایمنی میگوی سفید غربی (*Litopenaeus vannamei*) بررسی شد. ۳۷۵ میگو (با میانگین وزن اولیه: ۰/۰۵±۴/۲۸ گرم) از یک مزرعه تجاری جمع‌آوری شده و به طور تصادفی در مخازن پلی‌اتیلن ۳۰۰ لیتری و با تراکم ۲۵ عدد در هر مخزن و با ۳ تکرار، ذخیره‌سازی شدند. میگوها با رژیم‌های غذایی آزمایشی با افزودن سطوح مختلف (۰: شاهد ، ۰/۵٪ ، ۱٪ ، ۲٪ و ۴٪ از مکمل Biotronic® Top3) به خوراک میگوی تجاری (۴۰۰۵ ، شرکت فرادانه) تغذیه شدند. میگوها به مدت ۵۶ روز و با توجه به نرخ استاندارد تغذیه چهار بار در روز تا حد سیری، تغذیه شدند. در پایان آزمایش، شاخص‌های بیوشیمیایی، سطح آنزیم‌های همولنف (AST, ALT, ALP, LDH)، پارامترهای ایمنی (لیزوزیم و فنول اکسیداز) و سلولی همولنف میگوها در تیمارهای آزمایشی و شاهد مقایسه شدند. در رژیم غذایی ۲ درصد و ۴ درصد مکمل Biotronic® Top3 منجر به افزایش آنزیم‌های همولنف همولنف شد. میگوهایی که از این رژیم‌ها تغذیه نمودند، همچنین دارای مقادیر کمتری از گلوکز، کلسترول و تری گلیسرید همولنف بودند و سطوح پروتئین، کلسیم و کراتینین بالاتری داشتند. افزودن Biotronic® Top3 در خوراک تأثیر مثبتی بر پاسخ ایمنی سلولی و هومورال میگوها داشت. نتایج این مطالعه نشان داد که بهترین عملکرد Biotronic® Top3 در سطح ۱ درصد این مکمل، مشاهده شد.

کلمات کلیدی: اسیدهای آلی، میگوی سفید غربی، پارامترهای ایمنی، آنزیم‌های همولنف، پارامترهای بیوشیمیایی همولنف

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