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The interaction effects of dietary lipid, vitamin E and vitamin C on growth performance, feed utilization, muscle proximate composition and antioxidant enzyme activity of white leg shrimp (*Litopenaeus vannamei*)

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Abstract

This study aimed at evaluating the interactive effects of dietary lipid, vitamin E and vitamin C on growth performance, feed efficiency, muscle composition and antioxidant enzyme activity of *Litopenaeus vannamei* for a period of 56 days. A total of 384 shrimp with an initial weight of 5.3 ± 1.1 g were stocked in 24 tanks. Eight experimental treatments were designed with two different levels of lipid (70 and 140 g kg^{-1}), VitC (0.5 and 1.0 g kg^{-1}) and VitE (0.1 and 0.3 g kg^{-1}). The results showed that high levels of dietary VitE and VitC improved the survival rate and the growth, feed and antioxidant indices in shrimp. The best shrimp growth performance was observed at 70 g kg^{-1} of lipid, 1.0 g kg^{-1} of VitC and 0.3 g kg^{-1} of VitE. Dietary lipid levels had a significant effect on muscle lipid, moisture and VitE contents. Furthermore, the interaction between dietary lipid, VitC and VitE had a significant effect on VitC and VitE contents of muscle. The lowest T-AOC, SOD, CAT and GPX activity was observed at 70, 1.0 and 0.3 g kg^{-1} of dietary lipid, VitC and VitE respectively. The results also showed that the interaction of dietary VitE and VitC had a significant effect on growth and antioxidant enzyme activity of shrimp and improved their growth indices by reducing dietary lipid levels. Dietary lipid level of 70 g kg^{-1}, in interaction with 1.0 g kg^{-1} VitC and 0.3 g kg^{-1} VitE, resulted in better growth and antioxidant status in *L. vannamei*.

KEYWORDS
antioxidant enzyme activity, growth and feed indices, lipid, *Litopenaeus vannamei*, vitamin C, vitamin E

1 | INTRODUCTION

Appropriate feeding based on the nutrient requirements has significant effects on the production and health status of aquatic organisms. In general, the evaluation of nutrient components includes nutrient digestibility, nutrient intake and nutrient efficiency. A well-regulated diet is an important factor in aquaculture because it can provide the species with the nutrients required for proper growth and health (Glencross et al., 2007). A balanced diet should provide all the nutritional components needed. The ratio and the interaction between nutrients can be effective in determining the optimal ration (Alam et al., 2009). Therefore, it is important to study the changes in the nutrient interaction in formulated diets for aquatic animals.

As an available energy source and supplier of essential fatty acids, lipids are of great importance in aquafeeds. Lipids serve as the carriers of lipid-soluble vitamins and precursors of eicosanoids, hormones and enzymatic co-factors for crustaceans. The imbalance in dietary lipid levels can affect oxidative reactions (Chaiyapechara et al., 2003). Due to the ability of double bonds to be combined with oxygen, their presence in unsaturated fatty acids can lead to the production of peroxides (Sargent et al., 1999). The oxidative stress arising from the production of peroxide compounds can increase the production of free radicals in cellular cytoplasm, resulting in damage to the cell membrane and ultimately irreversible changes in cells and tissues (Kohen & Nyska, 2002). All types of free radicals have a natural tendency to destroy cellular constituents, such as lipids, carbohydrates and proteins; thus, they affect the growth and survival indices (Finkel & Holbrook, 2000).

Most cells have a protective substance, called antioxidants, to avoid oxidative stress. Antioxidant defence system includes a variety of non-enzymatic molecules, such as ascorbic acid and α-tocopherol, and enzymes. Antioxidant defence system counteracts and eliminates reactive oxygen species (ROS), including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) (Parrilla-Taylor et al., 2013). Vitamin C is also a powerful antioxidant that protects low-density lipoproteins from oxidation (Chen et al., 2015). Therefore, it can reduce lipid oxidative stress and, consequently, affect the growth performance and survival rate of organisms. Vitamin E is known to be effective in protecting membranes from polyunsaturated fatty acid (PUFA) peroxidation (Ruff et al., 2001). Vitamin E is particularly abundant in immune cell membranes and protects macrophage membranes from peroxidative damage induced by free radicals produced during the respiratory burst; therefore, this vitamin plays an important role in species survival (Waagbo, 1994). Vitamins C and E provide cellular defence against both the uncontrolled generation of ROS from normal aerobic metabolism and oxidative challenges, such as pollution, infection, tissue damage and oxidative stress, which together can damage biological membranes and DNA (Halliwell & Gutteridge, 1990). Furthermore, it has been confirmed that the lipid oxidation of the leucocyte membrane can change the synthesis of some complement proteins and consequently their functions (Obach et al., 1993). Hence, any changes which occur in the leucocyte membrane, in response to different intakes of vitamins E and C and the changes in the function of these vitamins through the ROS pathway, can modulate the complement system in crustaceans. Vitamins are supplied to aquatic animals mainly through dietary intake due to the lack of key enzymes in the gastrointestinal tract of aquatic species needed for the production of vitamins (Drouin et al., 2011). Inadequate supply of vitamins, especially vitamins C and E, in the diet can cause nutritional deficiency and have negative effects on growth. Vitamin E content is strongly correlated with lipid stability (Pazos et al., 2005).

A few studies have evaluated the relationship between the nutrient components in the shrimp diet. Some of the issues investigated in this regard include the interaction between dietary lipid and vitamin E in *Eriocheir sinensis* (Wang et al., 2015) and *Fenneropenaeus indicus* (Ouraji et al., 2011), and the interaction between dietary vitamins E and C in *Marsupenaeus japonicus* (Nguyen et al., 2012) and *L. vannamei* (Ruff et al., 2001). Due to the importance of the interaction between nutrients and the inadequate information available in this regard, this study was designed to evaluate the interactive effects of different levels of dietary vitamin E, vitamin C and lipid on growth indices, feed utilization, muscle biochemical composition and antioxidant enzyme activity of *L. vannamei*.

2 | MATERIALS AND METHODS

2.1 | Experimental treatments

Eight experimental treatments (2 × 2 × 2) were designed to evaluate the interactive effects of two dietary levels of vitamin C (0.5 and 1 g kg^{-1} of diet; Pitaksong et al., 2013), two dietary levels of vitamin E (0.1 and 0.3 g kg^{-1} of diet; Ouraji et al., 2011) and two dietary levels of lipid (70 and 140 g kg^{-1} of diet) on the growth performance, feed efficiency, muscle proximate composition and antioxidant enzyme activity of *L. vannamei*. Accordingly, the following 8 treatments were formulated: treatment 1 (L1C1E1), containing 0.1 g kg^{-1} vitamin E and 0.5 g kg^{-1} vitamin C in 70 g kg^{-1} of dietary lipid; treatment 2 (L1C1E2), containing 0.3 g kg^{-1} vitamin E and 0.5 g kg^{-1} vitamin C in 70 g kg^{-1} of dietary lipid; treatment 3 (L1C2E1), containing 0.1 g kg^{-1} vitamin E and 1.0 g kg^{-1} vitamin C in 70 g kg^{-1} of dietary lipid; treatment 4 (L1C2E2), containing 0.3 g kg^{-1} vitamin E and 1.0 g kg^{-1} vitamin C in 70 g kg^{-1} of dietary lipid; treatment 5 (L2C1E1), containing 0.1 g kg^{-1} vitamin E and 0.5 g kg^{-1} vitamin C in 140 g kg^{-1} of dietary lipid; treatment 6 (L2C1E2), containing 0.3 g kg^{-1} vitamin E and 0.5 g kg^{-1} vitamin C in 140 g kg^{-1} of dietary lipid; treatment 7 (L2C2E1), containing 0.1 g kg^{-1} vitamin E and 1.0 g kg^{-1} vitamin C in 140 g kg^{-1} of dietary lipid; and treatment 8 (L2C2E2), containing 0.3 g kg^{-1} vitamin E and 1.0 g kg^{-1} vitamin C in 140 g kg^{-1} of dietary lipid. Raw materials, including fish meal, soybean meal, shrimp by-product meal, squid powder, fish oil, wheat flour, mineral supplements, cholesterol, lecithin and gelatin (as a binder), for shrimp feed were purchased from a local market. Vitamin E (DL-α-Tocopherol acetate) supplement (SIGMA, T3376-25G) and vitamin C (L-Ascorbic acid) supplement (EMSURE ACS Merck, 1.00468.0100) were also purchased. The experimental diets were prepared after formulations (Table 1) by thoroughly mixing the dry ingredients with oil and vitamins in a food mixer and then adding water until a dough was formed. This was then passed through a mincer with a 2-mm mesh diameter, and resulting spaghetti-like strings were air-dried until the moisture content was reduced to less than 10% and finally broken into 2–4 mm pellets. For limiting the effect of heat on vitamin C efficiency, care was taken during the preparation of diets to prevent the temperature from exceeding 30–35°C. The dry pellets were placed in plastic bags and stored at −20°C until the beginning of the experimental culture.

TABLE 1 Ingredients, formulation and proximate composition of the experimental diets (g kg^{-1} dry diet)

Ingredients (g kg^{-1} dry diet)	Diets							
	L1C1E1	L1C1E2	L1C2E1	L1C2E2	L2C1E1	L2C1E2	L2C2E1	L2C2E2
Fish meal[a]	225	225	225	225	225	225	225	225
Soybean meal[a]	150	150	150	150	150	150	150	150
Shrimp by-product meal[a]	100	100	100	100	100	100	100	100
Squid meal[a]	50	50	50	50	50	50	50	50
Fish oil	50	50	50	50	120	120	120	120
Vitamin C[b]	0.5	0.5	1.0	1.0	0.5	0.5	1.0	1.0
Vitamin E[c]	0.1	0.3	0.1	0.3	0.1	0.3	0.1	0.3
Mineral premix[d]	10	10	10	10	10	10	10	10
Vitamin premix[e] (C and E free)	10	10	10	10	10	10	10	10
Cholesterol	5	5	5	5	5	5	5	5
Lecithin	5	5	5	5	5	5	5	5
Wheat flour[a]	354.4	354.2	353.9	353.7	284.4	284.2	283.9	283.7
Binder (Gelatin)	40	40	40	40	40	40	40	40
Proximate composition (n = 3)								
Crude protein	351 ± 31	351 ± 55	342 ± 80	341 ± 79	351 ± 12	352 ± 12	341 ± 30	352 ± 13
Crude lipid	70 ± 12	70 ± 35	67 ± 22	68 ± 17	140 ± 19	135 ± 11	136 ± 17	141 ± 20
Ash	110 ± 19	111 ± 15	111 ± 20	111 ± 14	101 ± 14	101 ± 19	100 ± 17	101 ± 12
Moisture	101 ± 11	101 ± 12	110 ± 18	101 ± 19	90 ± 15	91 ± 09	91 ± 13	90 ± 19
Vitamin C[f]	0.48 ± 0.1	0.49 ± 0.1	0.98 ± 0.7	0.99 ± 1.1	0.47 ± 0.2	0.48 ± 0.5	0.99 ± 1.0	0.98 ± 0.9
Vitamin E[f]	0.10 ± 0.1	0.29 ± 0.2	0.09 ± 0.1	0.28 ± 0.1	0.09 ± 0.1	0.30 ± 0.2	0.10 ± 0.1	0.29 ± 0.2
Carbohydrate[g]	368	367	370	379	318	321	332	316
GE (MJ kg^{-1})[h]	17.3	17.3	17.1	17.2	19.3	19.2	19.2	19.3

[a]Proximate composition as g kg^{-1} dry weight basis (Fish meal [655 g kg^{-1} crude protein, 67 g kg^{-1} crude lipid]; Soybean meal [440 g kg^{-1} crude protein, 13 g kg^{-1} crude lipid]; Shrimp by-product meal [428 g kg^{-1} crude protein, 20 g kg^{-1} crude lipid]; Squid meal [715 g kg^{-1} crude protein, 23 g kg^{-1} crude lipid]; Wheat flour [126 g kg^{-1} crude protein, 11 g kg^{-1} crude lipid]).

[b]L-Ascorbic Acid, EMSURE ACS Merck, 100468.0100.

[c]DL-α-Tocopherol acetate, SIGMA, T3376-25G.

[d]Per kilogram: Manganese, 5000 mg; Copper, 6000 mg; Ferrous, 6000 mg; Zinc, 10,000 mg; Selenium, 20 mg; Iodine, 600 mg; Cobalt, 100 mg; Choline chloride, 6000 mg.

[e]Per kilogram (Vitamin C and E free): vit. A, 600,000 IU; vit. D3, 400,000 IU; vit. K3, 1000 mg; vit. B1 (Thiamine mononitrate), 3000 mg; vit. B2 (Riboflavin), 5000 mg; vit. B6 (Pyridoxine hydrochloride), 3000 mg; vit. B12 (Cyano-cobalamin), 8000 mg; Nicotinic acid, 30,000 mg; D-calcium pantothenate, 9000 mg; Folic acid, 1600 mg; D-biotin, 160 mg; Inositol, 24,000 mg.

[f]The analyses of vitamin E and vitamin C were carried out following the method described by Huo et al. (1996) and Trenzado et al. (2007) respectively.

[g]Carbohydrates content obtained by subtracting the sum of protein, lipid, ash and moisture content from 1000.

[h]Gross energy, calculated based on 0.017, 0.0398 and 0.0237 MJ g^{-1} for carbohydrate, lipid and protein respectively.

2.2 | Culture system design and feeding trials

The shrimp were caught from a pond by seine nets from the Chouebdeh-Abadan shrimp culture site and transferred to the experiment site under continuous aeration. The shrimp were transferred to polyethylene tanks, which had a capacity of 1000 L and were equipped with an aeration system, and were kept there for 10 days for adaptation. During the adaptation period, the shrimp were fed four times daily to near satiation with a commercial diet, which contained 360 g kg^{-1} of crude protein, 80 g kg^{-1} of lipid, 30 g kg^{-1} of fibre, 140 g kg^{-1} of ash and 100 g kg^{-1} of moisture. After the end of the adaptation period, 384 shrimp, with an average initial weight of 5.3 ± 1.1 g, were stocked in 24 (300 L) polyethylene tanks (16 shrimp per replicate). To maintain water quality during the

experimental period, about 10% of water exchange was performed daily by siphoning to remove faecal and uneaten pellets. Each tank was equipped with an aeration system to provide dissolved oxygen. Feeding was done by hand to visual satiation four times daily (at 06:00, 12:00, 18:00 and 24:00 hours) for 56 days. Due to the total darkness at 24:00, lights were turned on with less light intensity than the day 10 minutes before feeding and were turned off 30 minutes after feeding. The uneaten diets were dried and weighed after each feeding. Temperature, pH and dissolved oxygen parameters were recorded twice a day, once in the morning and once in the evening. Salinity was measured daily by a portable multi-parameter (WTW model) with an accuracy of 0.01. The mean water temperature was kept at 26.1 ± 0.1°C. The pH and salinity levels were kept within the range of 8.2–8.5 and 17–20 ppt, respectively, during the experimental period. A photoperiod of 12-hour light/dark cycle was used throughout the experiment.

2.3 | Sample collection and analytical procedures

At the beginning and the end of the experimental period, the biometry of all shrimp in each tank was individually carried out. The following standard formulas were used to evaluate the effects of the interaction between different levels of dietary vitamins and lipids on the growth and feeding performance of *L. vannamei*.

$$\text{Weight gain (WG)} = \text{Final wet weight (g)} - \text{initial wet weight (g)}$$

$$\text{Specific growth rate (SGR)} = \left[\ln \text{final weight (g)} - \ln \text{initial weight (g)}\right] \times 100 / \text{total duration of the experiment}$$

$$\text{Feed conversion ratio (FCR)} = \text{Feed given (dry weight, g)} / \text{total wet weight gain (g)}$$

$$\text{Protein efficiency ratio (PER)} = \text{Total wet weight gain (g)} / \text{protein given (g)}$$

$$\text{Daily feed intake (DFI)} = \left[\text{Diet consumed (dry weight, g)} / \text{duration in days} / \text{shrimp number per tank}\right] \times 100$$

$$\text{Daily protein intake (DPI)} = \left[\text{Protein consumed (dry weight, g)} / \text{duration in days} / \text{shrimp number per tank}\right] \times 100$$

$$\text{Condition factor (CF)} = \left(\text{Shrimp mass (g)} / \text{shrimp total length}^3 \text{ (cm)}\right) \times 100$$

$$\text{Length gain} = \text{Final total length (cm)} - \text{initial total Length (cm)}$$

$$\text{Survival rate (\%)} = (\text{Final shrimp number} / \text{initial shrimp number}) \times 100$$

Samples of shrimp diets, as well as the homogenized initial and final shrimp muscle tissues, were analysed following the official method of AOAC (2000). Moisture was determined by drying the samples at 105°C to a constant weight, and ash was determined by combustion at 600°C for 2 hours. Total nitrogen was measured by a micro-Kjeldahl technique, and crude protein was calculated by multiplying total nitrogen by 6.25. Total lipid was determined gravimetrically following chloroform–methanol (2:1) extraction using the method suggested by Folch et al. (1957). The analyses of vitamin E and vitamin C were carried out by HPLC (Agilent-1100), following the methods described by Huo et al. (1996) and Trenzado et al. (2007) respectively.

Hepatopancreas samples were homogenized (1–9 W/V) in 20 mM phosphate-buffered (KCl 0.0027 M, NaCl 0.14 M, pH 7.4). The homogenates were centrifuged at 12879 *g* for 10 minutes at 4°C to remove debris, and the resultant supernatants were used directly for the measurement of antioxidant activity (Li et al., 2008). Total antioxidant capacity (T-AOC), superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities were assayed using the BXC0553A, BXC0531A and BXC0551A kits (Biorex®) respectively. Catalase (CAT) activity was measured using the ZB-CAT96 kit (ZellBio® GmbH). T-AOC, SOD, CAT and GPX activities were determined by an enzymatic-colorimetric method and using a spectrophotometer (Specord 250) on wavelength 660 nm (Erel, 2004), 505 nm (Imai et al., 2000), 405 nm (Aebi, 1984) and 660 nm (Flohe & Gunzler, 1984) respectively.

Data are expressed as mean ± standard error. The effects of different levels of dietary lipid, vitamin E, vitamin C and the interaction between them were determined by multivariate analysis of variance (ANOVA), using SPSS 23 statistical software. Differences were considered significant at an alpha level of 0.05 ($p < 0.05$).

2.4 | Ethical approval

All applicable international, national and/or institutional guidelines for the care and use of animals were followed by the authors.

3 | RESULTS

3.1 | Growth performances and survival rate

The results obtained at the end of the experiment on the effects of different concentrations of dietary lipid, vitamin C and vitamin E on the growth performance and survival rate of white leg shrimp are presented in Table 2. At the end of the trial period, the highest mean of the main effect concerning weight gain, specific growth rate, total length and survival rate was observed at 70 g kg^{-1} of lipid, 1.0 g kg^{-1} of vitamin C and 0.3 g kg^{-1} of vitamin E. Based on the results of multivariate ANOVA, weight gain and survival rate were significantly affected by different dietary levels of lipid, vitamin C and vitamin E ($p < 0.05$). Specific growth rate and total length were also found to be significantly affected by different dietary lipid and vitamin C levels ($p < 0.05$); however, the results showed that dietary vitamin E had no

TABLE 2 Growth performance and survival rate of *Litopenaeus vannamei* fed the different levels of dietary lipid, vitamin C and vitamin E (mean ± SE values [*n* = 3])

Diets (g)			Weight gain (g)	Specific growth rate (%)	Length gain (cm)	CF (%)	Survival rate (%)
Lipid	Vitamin C	Vitamin E					
Pooled treatment means							
70	0.5	0.1	5.29 ± 0.81	0.31 ± 0.04	2.64 ± 0.14	0.88 ± 0.08	87.51 ± 8.01
		0.3	4.72 ± 0.58	0.30 ± 0.04	3.24 ± 0.31	0.76 ± 0.09	85.42 ± 4.04
	1.0	0.1	8.85 ± 0.88	0.47 ± 0.03	5.10 ± 0.40	0.64 ± 0.05	89.56 ± 8.33
		0.3	10.07 ± 0.29	0.51 ± 0.02	4.12 ± 0.13	0.77 ± 0.02	97.93 ± 3.51
140	0.5	0.1	2.19 ± 0.40	0.15 ± 0.03	1.81 ± 0.53	0.77 ± 0.03	77.06 ± 3.35
		0.3	3.53 ± 0.33	0.24 ± 0.01	2.39 ± 0.21	0.78 ± 0.02	91.63 ± 4.81
	1.0	0.1	6.09 ± 0.82	0.36 ± 0.05	3.12 ± 0.26	0.78 ± 0.01	81.23 ± 8.33
		0.3	6.75 ± 0.66	0.36 ± 0.02	3.03 ± 0.49	0.84 ± 0.01	95.81 ± 3.33
Means of main effect							
70			7.23	0.40	3.78	0.76	90.07
140			4.64	0.28	2.58	0.79	86.43
	0.5		3.93	0.25	2.52	0.80	85.40
	1.0		7.93	0.43	3.84	0.76	91.11
		0.1	5.61	0.33	3.17	0.77	83.82
		0.3	6.27	0.35	3.19	0.79	92.68
ANOVA (*p*-values)*							
L			0.009	0.007	0.022	ns	0.040
C			0.005	0.006	0.028	ns	0.020
E			0.033	ns	ns	ns	0.011
L × C			ns	ns	0.027	ns	ns
L × E			ns	ns	ns	ns	ns
C × E			ns	ns	ns	ns	0.037
L × C × E			0.031	0.045	ns	ns	ns

*L, Lipid level; C, Vitamin C level; E, Vitamin E level; L × C, lipid × vitamin C interaction; L × E, lipid × vitamin E interaction; C × E, vitamin C × vitamin E interaction; L × C × E, lipid × vitamin C × vitamin E interaction and ns = $p > 0.05$.

significant effect on specific growth rate and total length. Although the interaction between lipid and vitamin C (L × C) and lipid and vitamin E (L × E) had no significant effect on the weight gain, specific growth rate and survival rate of the shrimp, the interaction between lipid and vitamin C (L × C) had a significant effect on the shrimp total length. The interaction between vitamin E and vitamin C (E × C) had no significant effect on the weight gain, specific growth rate and total length of the shrimp ($p > 0.05$). However, the interaction between these two vitamins had a significant effect on survival rate ($p < 0.05$). Weight gain and specific growth rate were also significantly affected by the interaction between dietary lipid, vitamin C and vitamin E (L × C × E, $p < 0.05$), but the interaction between these parameters did not have a significant effect on the total length and survival rate of the shrimp ($p > 0.05$).

3.2 | Feed utilization

At the end of the 56-day experimental period, the best feed conversion ratio was observed in shrimp fed with 140 g kg^{-1} of dietary lipid, 1.0 g kg^{-1} of vitamin C and 0.3 g kg^{-1} of vitamin E (Table 3). The highest protein efficiency ratio was found in shrimp fed with 140 g kg^{-1} of dietary lipid, 1.0 g kg^{-1} of vitamin C and 0.3 g kg^{-1} of vitamin E. The highest percentages of daily feed intake and daily protein intake were observed at 70 g kg^{-1} of dietary lipid, 1.0 g kg^{-1} of vitamin C and 0.1 g kg^{-1} of vitamin E. Based on the results of multivariate ANOVA, different levels of dietary lipid and vitamin E levels had a significant effect on all feed utilization indicators ($p < 0.05$). The dietary vitamin C had no significant effect on the feed indices. The interaction between dietary lipid and vitamin C (L × C) had no significant effect on feed utilization parameters; however, these parameters were significantly affected by the interaction between dietary lipid and vitamin E (L × E, $p < 0.05$). Feed conversion ratio and protein efficiency ratio were not affected by the interaction between dietary vitamin E and vitamin C (E × C), whereas daily feed intake and protein intake were significantly affected by the interaction between the dietary supplementation of vitamin E and vitamin C. The interaction between dietary lipid, vitamin C and vitamin E also had a significant effect on feed conversion ratio and protein efficiency ratio (L × C × E, $p < 0.05$).

TABLE 3 Feed utilization of *Litopenaeus vannamei* fed the different levels of dietary lipid, vitamin C and vitamin E (mean ± SE values [*n* = 3])

Diets (g)			Feed conversion ratio	Protein efficiency ratio (%)	Daily feed intake (%)	Daily protein intake (%)
Lipid	Vitamin C	Vitamin E				
Pooled treatment means						
70	0.5	0.1	3.07 ± 0.13	0.93 ± 0.01	4.70 ± 0.63	2.35 ± 0.31
		0.3	1.61 ± 0.18	1.68 ± 0.10	2.60 ± 0.51	1.30 ± 0.25
	1.0	0.1	2.87 ± 0.06	1.01 ± 0.01	7.79 ± 0.90	3.89 ± 0.45
		0.3	1.95 ± 0.03	1.51 ± 0.01	5.17 ± 0.35	2.5 ± 0.17
140	0.5	0.1	2.77 ± 0.28	1.04 ± 0.09	2.48 ± 0.58	1.24 ± 0.28
		0.3	1.82 ± 0.28	1.57 ± 0.08	2.90 ± 0.31	1.45 ± 0.15
	1.0	0.1	2.75 ± 0.12	1.07 ± 0.01	6.05 ± 0.25	3.02 ± 0.62
		0.3	1.54 ± 0.13	1.83 ± 0.02	4.50 ± 0.54	2.25 ± 0.27
Means of main effect						
70			2.37	1.28	5.06	2.53
140			2.22	1.37	3.98	1.99
	0.5		2.31	1.30	3.17	1.58
	1.0		2.27	1.35	5.88	2.94
		0.1	2.86	1.01	5.26	2.63
		0.3	1.73	1.64	3.79	1.89
ANOVA (*p*-values)*						
L			0.045	0.013	0.011	0.031
C			ns	ns	ns	ns
E			0.011	0.010	0.011	0.033
L × C			ns	ns	ns	ns
L × E			0.008	0.010	0.014	0.037
C × E			ns	ns	0.044	0.044
L × C × E			0.030	0.009	ns	ns

*L, Lipid level; C, Vitamin C level; E, Vitamin E level; L × C, lipid × vitamin C interaction; L × E, lipid × vitamin E interaction; C × E, vitamin C × vitamin E interaction; L × C × E, lipid × vitamin C × vitamin E interaction and ns = $p > 0.05$.

3.3 | Muscle proximate composition

The results on the interactive effects of different levels of dietary lipid, vitamin C and vitamin E on the muscle composition indices of *L. vannamei* at the end of the experimental period are presented in Table 4. The highest percentage of muscle protein, lipid, ash and vitamin C content was observed at 140 g kg^{-1} of dietary lipid, while the highest percentage of muscle moisture and vitamin E content was observed at 70 g kg^{-1} of dietary lipid. Except for the muscle protein and moisture content, the highest percentages of lipid, ash, vitamin C and vitamin E contents were recorded at 1.0 g kg^{-1} of dietary vitamin C. The highest percentage of protein content was observed at 0.1 g kg^{-1} of dietary vitamin E, whereas the highest levels of the other proximate composition indices were observed at 0.3 g kg^{-1} of dietary vitamin E. According to the results of multivariate ANOVA, different levels of dietary lipid had a significant effect on muscle lipid, moisture and vitamin E content ($p < 0.05$), but they had no significant effect on protein, ash and vitamin C content ($p < 0.05$). Different dietary vitamin C and vitamin E levels had significant effects on muscle ash, vitamin C and vitamin E contents but had no significant effect on other muscle composition indices. The interaction between dietary lipid and vitamin C (L × C) had no significant effect on muscle composition indices, except for moisture content. The interaction effect of dietary lipid and vitamin E (L × E) had a significant effect on muscle protein and vitamin E content but did not have any significant effect on other composition indices. The interaction between dietary vitamin E and vitamin C (E × C) and dietary lipid, vitamin C, and vitamin E (L × C × E) was found to have a significant effect on the ash, vitamin C and vitamin E contents of *L. vannamei* ($p < 0.05$).

3.4 | Antioxidant enzyme activity

The changes in the antioxidant enzyme activity of the hepatopancreas of *L. vannamei* fed with different dietary lipid, vitamin C and

TABLE 4 Muscle proximate composition of *Litopenaeus vannamei* fed the different levels of dietary lipid, vitamin C and vitamin E (mean ± SE values [*n* = 3])

Diets (g)								
Lipid	Vitamin C	Vitamin E	Moisture (g kg^{-1})	Crude protein (g kg wet $weight^{-1}$)	Crude lipid (g kg wet $weight^{-1}$)	Ash (g kg wet $weight^{-1}$)	Vitamin C (μg g dry $weight^{-1}$)	Vitamin E (μg g dry $weight^{-1}$)
Pooled treatment means								
70	0.5	0.1	762.1 ± 12.0	143.1 ± 7.6	14.8 ± 1.1	27.6 ± 1.7	61.20 ± 2.10	20.24 ± 0.97
		0.3	736.8 ± 9.7	168.8 ± 7.2	19.3 ± 1.2	31.6 ± 1.3	72.41 ± 2.31	23.55 ± 0.99
	1.0	0.1	737.9 ± 8.1	155.3 ± 9.6	18.6 ± 1.4	29.9 ± 1.0	67.20 ± 3.05	21.92 ± 1.09
		0.3	762.4 ± 11.0	141.1 ± 8.0	15.9 ± 1.2	32.5 ± 1.6	88.95 ± 2.21	24.85 ± 0.85
140	0.5	0.1	706.6 ± 9.0	173.5 ± 9.4	26.0 ± 2.2	26.4 ± 1.9	64.20 ± 3.10	19.68 ± 1.02
		0.3	735.7 ± 7.8	154.7 ± 5.2	19.9 ± 1.9	31.4 ± 1.0	78.81 ± 2.11	22.76 ± 0.95
	1.0	0.1	728.9 ± 10.3	165.2 ± 5.5	21.5 ± 2.8	30.8 ± 1.7	70.23 ± 3.15	20.82 ± 0.91
		0.3	705.5 ± 8.9	169.5 ± 7.5	29.2 ± 1.1	37.5 ± 1.1	8.84 ± 2.08	23.10 ± 1.00
Means of main effect								
70			749.8	152.1	17.2	30.4	72.44	22.64
140			719.2	165.7	24.1	31.5	75.52	21.59
	0.5		735.3	160.0	20.0	29.3	69.15	21.55
	1.0		733.7	157.8	21.3	32.7	78.81	22.67
		0.1	733.9	159.3	20.2	28.7	65.70	20.66
		0.3	735.1	158.5	21.1	33.2	82.25	23.56
ANOVA (*p*-values)*								
L			0.0012	ns	0.021	ns	ns	0.047
C			ns	ns	ns	0.039	0.011	0.041
E			ns	ns	ns	0.022	0.009	0.033
L × C			0.046	ns	ns	ns	ns	ns
L × E			ns	0.035	ns	ns	ns	0.042
C × E			ns	ns	ns	0.030	0.014	0.035
L × C × E			ns	ns	ns	0.041	0.041	0.048

*L, Lipid level; C, Vitamin C level; E, Vitamin E level; L × C, lipid × vitamin C interaction; L × E, lipid × vitamin E interaction; C × E, vitamin C × vitamin E interaction; L × C × E, lipid × vitamin C × vitamin E interaction and ns = $p > 0.05$.

vitamin E levels are presented in Table 5. At the end of the trial period, the lowest T-AOC, SOD, CAT and GPX activity was observed at 70 g kg^{-1} of dietary lipid, 1.0 g kg^{-1} of dietary vitamin C and 0.3 g kg^{-1} of dietary vitamin E. Based on the results of multivariate ANOVA, the antioxidant enzyme activity was significantly affected by different dietary levels of lipid, vitamin C and vitamin E ($p < 0.05$). The interaction between dietary lipid and vitamin C (L × C), lipid and vitamin E (L × E), and vitamin E and vitamin C (E × C) had a significant effect on the antioxidant enzyme activity. T-AOC, SOD, CAT and GPX activities were also significantly affected by the interaction between dietary lipid, vitamin C and vitamin E (L × C × E, $p < 0.05$).

4 | DISCUSSION

While the biochemical, biological and physiological effects of vitamins in mammalian nutrition have been extensively studied (Bender, 2003; Combs, 2012), studies on shrimp are limited. Most previous nutritional studies of dietary vitamins in aquatic species have focused on vitamins required for growth and tissue storage, the interactive effects of vitamins (NRC, 2011; Ogino et al., 1970), and the interaction between vitamins and other nutrients (Lim et al., 2000). Very few studies have investigated the interactive effects of dietary vitamins and lipid in shrimp.

In the present study, the best shrimp growth performance was observed at the lowest level of dietary lipid and the highest level of dietary vitamin E and vitamin C. The interaction between dietary lipid, vitamin C and vitamin E (L × C × E) also improved growth and survival rate of shrimp. Lipid oxidation and the release of free oxygen radicals in the shrimp body appeared to have been reduced by the antioxidant effects of vitamins. However, the interaction between dietary lipid and vitamin E (L × E) and dietary lipid and vitamin C (L × C) did not have a significant effect on growth indices and survival rate. Wang et al. (2015) observed that the interaction between vitamin E and lipid did not cause any significant difference in the weight gain and survival rate of *Eriocheir sinensis*. Ouraji et al. (2011)

TABLE 5 Antioxidant enzyme activity of *Litopenaeus vannamei* fed the different levels of dietary lipid, vitamin C and vitamin E (mean ± SE values [*n* = 3])

Diets (g)			T-AOC (mmol Trolox equivalent/mg protein)	SOD (U ml^{-1})	CAT (U ml^{-1})	GPX (U L^{-1})
Lipid	Vitamin C	Vitamin E				
Pooled treatment means						
70	0.5	0.1	158.74 ± 7.11	2.88 ± 0.09	21.18 ± 1.21	164.3 ± 6.44
		0.3	133.21 ± 8.45	1.87 ± 0.11	14.86 ± 2.45	117.84 ± 7.12
	1.0	0.1	138.54 ± 6.87	2.02 ± 0.14	16.2 ± 2.22	147.54 ± 6.54
		0.3	101.04 ± 6.92	1.50 ± 0.12	11.14 ± 1.59	92.11 ± 8.23
140	0.5	0.1	184.01 ± 7.09	3.61 ± 0.11	25.67 ± 1.87	175.94 ± 8.20
		0.3	152.77 ± 8.21	2.19 ± 0.017	20.21 ± 1.61	154.93 ± 6.55
	1.0	0.1	165.42 ± 6.65	2.44 ± 0.15	23.01 ± 2.18	162.27 ± 6.98
		0.3	122.10 ± 7.81	2.10 ± 0.08	15.88 ± 1.01	112.89 ± 8.25
Means of main effect						
70			132.88	2.07	15.85	130.45
140			156.07	2.59	21.19	151.51
	0.5		157.18	2.64	20.48	153.25
	1.0		131.77	2.02	16.56	128.70
		0.1	161.67	2.74	21.52	162.51
		0.3	127.28	1.92	15.52	119.44
ANOVA (*p*-values)*						
L			0.015	0.033	0.037	0.032
C			0.012	0.022	0.021	0.030
E			0.013	0.018	0.022	0.011
L × C			0.014	0.043	0.030	0.041
L × E			0.012	0.032	0.031	0.018
C × E			0.007	0.027	0.027	0.022
L × C × E			0.035	0.044	0.038	0.041

*L, Lipid level; C, Vitamin C level; E, Vitamin E level; L × C, lipid × vitamin C interaction; L × E, lipid × vitamin E interaction; C × E, vitamin C × vitamin E interaction; L × C × E, lipid × vitamin C × vitamin E interaction and ns = $p > 0.05$.

also reported similar results in their study on *Fenneropenaeus indicus*. These results indicated that the interaction between either dietary vitamin E or vitamin C and dietary lipid cannot affect growth indices. However, the interaction between both the vitamins and dietary lipid can reduce the damaging effects of free oxygen radicals and prevent lipid peroxidation, thereby improving growth performance, probably due to the synergistic effect of these vitamins (Wahil et al., 1998). As a result, increasing the amount of dietary vitamins E and C and their interaction with dietary lipid can lead to improved growth performance in shrimp.

The results of the present study showed that the amount of vitamin E required in *L. vannamei* varied with the changes in the dietary lipid level. Previous studies have also shown that vitamin requirements of aquatic species are affected by dietary lipid levels ((Kiron et al., 2004; Roem et al., 1990; Shiau, 2001). In the present study, the role of dietary vitamins and their effect on the growth performance and survival rate of *L. vannamei* depended on the level of dietary lipid. At 70 g kg^{-1} of dietary lipid, lipid oxidation was lower; this lower lipid oxidation and the antioxidant activity of vitamins E and C limited the harmful effects of lipid peroxidation and improved the shrimp growth performance (Tables 2 and 5). Dietary vitamins E and C could play this essential role in the growth performance of the experimental shrimp only at 70 g kg^{-1} of dietary lipid. However, compared to the lower levels of dietary lipid, higher levels of dietary lipid increased lipid oxidation and, consequently, resulting in reduced growth and survival of shrimp. Therefore, it could be stated that the increased dietary lipid levels led to an increase in the requirement of dietary vitamins E and C, which have important antioxidant roles in physiological responses (Conklin, 1989).

The role of dietary C in improving the growth performance has been reported by Lee and Shiau (2002) and Marchie et al. (1998) in *Penaeus monodon*, Shiau and Hsu (1994), Kontara et al. (1997), and Gomez-Jimenez et al. (2005) in *L. vannamei*, and Manush et al. (2013) in *Macrobranchium rosenbergii*. The function of dietary vitamin C and its effect on the growth and survival rate of *L. vannamei* was found to be further improved by its interaction with dietary vitamin E. Therefore, it could be suggested that a combination of dietary vitamin C and vitamin E appears to have better effects on preventing

lipid peroxide formation than does each vitamin individually. This suggestion could be further supported by the findings of the present study that the anti-stress effects of vitamins C and E improved the growth, survival rate and the antioxidant enzyme activity of the experimental shrimp. Dietary vitamin E had a significant effect on weight gain and survival rate, but it had no significant effect on the specific growth rate. Similar observations have also been reported by He and Lawrence (1993) and Liu et al. (2007) on *L. vannamei* and Reddy and Ganapathi (1999) and Lee and Shiau (2004) on *Penaeus monodon*.

Feed conversion ratio is one of the economic factors considered in aquaculture. In the present study, the best feed conversion ratio for *L. vannamei* was observed at high levels of dietary lipid and vitamin E ($p < 0.05$) but low levels of vitamin C ($p > 0.05$). In a study on *Macrobrachium malcolmsonii*, Asaikkutti et al. (2016) found that there was no significant change in the feed conversion ratio when different levels of dietary vitamin C were used. Reddy and Ganapathi (1999) also reported that vitamin C and vitamin E deficiency increased the feed conversion ratio in *P. monodon*.

According to the results of multivariate analysis of variance in the present study (Table 3), the amount of dietary vitamin E had a more significant role than vitamin C in improving feed conversion ratio. It, therefore, can be argued that the levels of dietary vitamin C could not affect feed conversion ratio; however, increased levels of dietary vitamin E can affect the nutritional status of aquatic animals. Since vitamin C is a water-soluble vitamin, it easily leaches in water and is excreted thereby having low stability in the diets of aquatic species. Thus, it can be stated that high levels of this vitamin are unstable in aquatic animal's body. This could explain the effects of vitamin E, compared to vitamin C, on feed conversion ratio. Zhang et al. (2013) reported that dietary lipid levels between 100 and 120 g kg^{-1} increased the growth performance of *L. vannamei*, but when dietary lipid level was increased to 140 g kg^{-1}, a corresponding increase in growth performance was not observed, probably due to the inability of shrimp to use the amount of available lipid. In the present study, 140 g kg^{-1} of dietary lipid reduced growth indices in *L. vannamei*. This could be due to the decrease in feed efficiency at high dietary lipid levels (Bogevik et al., 2010), early satiety following a high lipid diet, or the unpalatability of such diets (Ebrahimi et al., 2013).

Lipid oxidation at a high level of dietary lipid is more likely to interfere with the antioxidant activity of dietary vitamins and prevent these vitamins from improving growth performance. The beneficial effect of dietary vitamins on feed utilization and growth performance was less observable at 140 g kg^{-1} of dietary lipid than at 70 g kg^{-1} of dietary lipid. The interaction between dietary lipid and high levels of vitamin E (L × E) had a significant effect on feed indices and improved nutritional indicators. Dietary vitamin E had no significant effect on growth indices due to its interaction with dietary lipid and hence its antioxidant activity (Tables 2 and 5).

Ascorbic acid is an essential coenzyme in tyrosine amino acid oxidation and phenylalanine and can increase growth rate and weight gain through protein synthesis (Faramarzi, 2012), and collagen synthesis (Terova et al., 1998). Kaushik et al. (1998) found a correlation between levels of vitamin C and protein utilization in salmonids. Vitamin E has also been reported to exert its effect on growth performance by preventing muscle atrophy and collagen tissue degradation (Chen et al., 2004; Fracalossi et al., 2001; Montero et al., 1999; Wang et al., 2003). Furthermore, it has been demonstrated that vitamin E improves growth performance by reducing metabolic costs, resulting in reduced tissue damage during stress. The trout fed with diets without vitamins demonstrated significantly less growth than those fed with diets supplemented with vitamins (Miar et al., 2013).

The measurement of biochemical parameters is mainly used to determine the physiological and general health status of aquatic species. The body composition of aquatic animals is essentially influenced by feed nutrients. In the present study, the evaluation of the interaction between dietary lipid and vitamin E (L × E) on muscle protein content showed that the antioxidant effects of vitamin E prevented lipid oxidation. As a result, the energy requirement of the shrimp was provided by non-protein sources and protein played an important role in tissue growth and storage. The results also showed that the shrimp muscle lipid content was higher at high levels of dietary lipid. This could be attributed to the excess lipid storage in the body and the utilization of lipid as an energy source up to 70 g kg^{-1}. The lipid content in the cultured shrimp tissue usually reflects the experimental dietary lipid levels. Shrimp composition was affected by increased dietary lipid levels. Ouraji et al. (2011) showed that the tissues of shrimp fed with 140 g kg^{-1} of dietary lipid had a higher lipid content compared to the tissues of shrimp fed with 90 g kg^{-1} of dietary lipid. According to a report by Glencross et al. (2002), the shrimp could not fully utilize the available lipid to enhance growth. Therefore, part of the lipid remained in the shrimp tissues. This finding is consistent with the results of other studies reporting that lipid content in the muscle and hepatopancreas of shrimp increased with the increase in the dietary lipid levels (Abramo, 1997; Gonzalez-Felix et al., 2002,2003).

Dietary vitamins E and C had a significant effect on tissue vitamin contents in shrimp (Table 4); that is, the highest content of vitamins E and C was observed in shrimp fed with the diet containing high levels of these vitamins. The interaction between vitamins E and C and the interaction between these two vitamins and dietary lipid had a significant effect on the shrimp muscle vitamin content. The increased accumulation of vitamin E in shrimp tissues fed with higher dietary vitamin C supports the hypothesis that vitamin C spares vitamin E by decreasing tocopheroxyl radicals in many animals (Tappel, 1962) and fish (Lee & Dobrowski, 2003; Sealy & Gatlin, 2002).The significant increase in tissue vitamin E at high levels of dietary vitamin C also indicated that vitamin E regeneration depends on vitamin C levels in the diet (Nguyen et al., 2012).

In the present study, the lowest level of vitamin E in shrimp muscle was recorded at 140 g kg^{-1} of dietary lipid level. This finding is in agreement with those of Trenzado et al. (2006) that an increase in dietary HUFA decreased the tissue levels of vitamin E in *Oncorhynchus mykiss*. The results of the present study showed that when dietary lipid levels increased, the antioxidant activity of vitamin E increased, resulting in a decrease in the concentration of vitamin E in shrimp

fed with high dietary lipid levels. In other words, in shrimp fed with high levels of dietary lipid, vitamin E prevented lipid oxidation in the muscle (Ouraji et al., 2011). The most important factors that can affect alpha-tocopherol concentration in carcass are feed duration (Hamre et al., 1997), different forms of tocopherols used in the diet (Sigurgisladottis et al., 1994), the quality and quantity of dietary lipids in the diet, and the amount of antioxidants, such as vitamin C, in the diet (Chaiyapechara et al., 2003). The interaction between vitamin C and vitamin E (C × E) can increase the accumulation of vitamins in shrimp tissues. In this study, increasing the amount of these vitamins in the diet strengthened the shrimp defence against oxidative stress, as reflected by the increased survival rate in shrimp fed with higher levels of vitamins C and E (Table 2).

During the metabolism of normal organisms, the production and elimination of reactive oxygen species (ROS) maintain a dynamic balance. Antioxidant enzymes, such as SOD, CAT and GPX, can remove potentially damaging ROS and reduce the damage by lipid peroxidation (Liang et al., 2015). In the present study, dietary vitamins C and E functioned as antioxidants. As a result, a decrease was observed in the activity of antioxidant enzymes in the hepatopancreas of the experimental shrimp at 1.0 and 0.3 g kg^{-1} of dietary vitamins C and E respectively. According to the results of multivariate analysis of variance, these vitamins (C × E) also had synergistic effects and thus improved the activities of T-AOC, SOD, CAT and GPX at their highest dietary levels. Therefore, the activities of T-AOC, SOD, CAT and GPX could be used as criteria to evaluate the effects of dietary vitamin C and vitamin E on the antioxidant enzyme activity in *L. vannamei*. Increasing the level of dietary lipid in the shrimp diets increased oxidative activities. In this respect, the increased activity of antioxidant enzymes can remove free radicals and is one of the main protective mechanisms against increased dietary lipid levels. These changes in antioxidant enzyme activity follow the immune system response to oxidative stress, induced by an excessive increase in the dietary lipid and ROS production in tissues (Fang et al., 2002). SOD, CAT and GPX are the main antioxidant enzymes in the immune system. O_2^- can be neutralized and converted by SOD to H_2O_2, which can be further metabolized to H_2O by CAT and GPX, thereby preventing the synthesis of hydroxyl radical and reducing the deleterious effects of ROS (Parrilla-Taylor et al., 2013). On the other hand, antioxidant enzymes are the primary enzymes for the inhibition of free radicals. These defence mechanisms and their activity depend on animal condition, diet quality and environmental factors (Li et al., 2008). Increased activity of SOD and CAT is usually indicative of increased production of free radicals. According to the results of this study, a significant increase in SOD and CAT activity in the shrimp fed with higher levels of dietary lipid may indicate the accumulation of free radicals in tissues. Therefore, it could be suggested that the accumulation of ROS in the tissues of the experimental shrimp had a negative impact on the overall growth performance and survival rate.

Based on the observations, it could be concluded that increasing the levels of inclusion of dietary vitamins C and E could enhance the beneficial effects of increased levels of dietary lipid on growth performance and survival rate of shrimp. Finally, under the management conditions of this study, the results of the present study showed that the three-way interaction between 70 g kg^{-1} of dietary lipid, 1.0 g kg^{-1} of vitamin C and 0.3 g kg^{-1} of vitamin E led to the highest growth performance, survival rate and antioxidant enzyme activity in *Litopenaeus vannamei*.

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CONFLICT OF INTEREST

Hajar Ebadi: Running the experiment and lab working, Mohammad Zakeri: Supervisor and design the experiment, Analyzed the data and prepared the manuscript, Seyed Mohammad Mousavi: Analyzed the data and methodology, Vahid Yavari: Analyzed the data and assist to prepared the manuscript, Morteza Souri: Provided the shrimp and other facilities of the experiment.

AUTHOR CONTRIBUTIONS

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data sets generated and/or analysed during the current study are available from the corresponding author on request.

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