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Supra dietary levels of vitamins C and E enhance antibody production and immune memory in juvenile milkfish, *Chanos chanos* (Forsskal) to formalin-killed *Vibrio vulnificus*

I.S. Azad*, J. Syama Dayal, M. Poornima, S.A. Ali

Central Institute of Brackishwater Aquaculture, 75 Santhome High Road, R.A. Puram, Chennai 600028, India

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Abstract

Juveniles of milkfish, *Chanos chanos* (Forsskal), were fed two independent supra dietary levels of vitamins C (500 and 1500 mg kg^{-1} feed, T1 and T2) and E (50 and 150 mg kg^{-1}, T3 and T4). Milkfish fed diets with supra (in addition to the vitamins present in the control diet) and normal levels (T5 containing 90 and 1.2 mg of vitamins C and E, respectively, kg^{-1} of feed) of vitamins were immunized (ip) with formalin-killed *Vibrio vulnificus* (FKVV). Priming and booster antibody responses to the injected bacterin were significantly ($P < 0.05$) better in the milkfish juveniles fed supra dietary levels. Survival response of the experimental fish fed supra dietary levels of vitamins (T1, T2 and T3) was significantly ($P < 0.01$) better than that of the control set. Protective response against virulent bacterial challenge of the vaccinated fish fed vitamin-supplemented diets (T2 and T3) was better than the control (T5) and T1 and T4. Memory factor reflecting immunological memory was superior in the fish fed vitamin-supplemented diets. Diets supplemented with either 1500 mg of Vitamin C or 50 mg of Vitamin E kg^{-1} produced the best antibody responses, final survival and protective response upon challenge. No conclusive inferences could be drawn on the growth responses from the experiment.

Keywords: Milkfish; Immune response; *V. vulnificus*; Vitamins C and E; Memory factor

1. Introduction

The biological role played by vitamins C and E is very vital for the sustained growth and health of many living organisms. These vitamins exhibit antioxidant properties that scavenge reactive oxygen species in membranes [1] and biological fluids [2]. Vitamin deficiencies in fish under aquaculture are known to produce biochemical dysfunction leading to tissue and cellular level clinical manifestations. Several morphological and functional abnormalities have been reported in various fish species deprived of vitamins. Properties of disease resistance in fish fed ascorbic acid and Vitamin E have been reported by several researchers [3–7]. Dietary vitamins were reported to have antibody

* Corresponding author. Present address: Mariculture and Fisheries Department, Kuwait Institute for Scientific Research, P.O. Box No. 1638, Salmiya 22017. Tel.: +965 5711295; fax: +965 5711090.
E-mail address: azadis@rediffmail.com (I.S. Azad).

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enhancement effects in salmon [4,7]. Disease resistance and humoral antibody production in rainbow trout was directly and positively related to the levels of Vitamin C in the trout diet [8]. Interaction between these vitamins is also known to influence the beneficial effects they induce in cultured fish. Vitamin C/E sparing action in channel catfish was studied to explain the variability observed in its sensitivity to Vitamin E deficiency [9]. A dose dependant protection of dietary Vitamin C against dietary deficiency of Vitamin E was demonstrated in Atlantic salmon [10].

With the persistent losses due to diseases in shrimp aquaculture, coastal aquaculture farmers in India are constantly on the look-out for sustainable aquaculture and mixed farming of fish with shrimp. Milkfish is one such fish species that is traditionally harvested from extensive paddy–fish culture systems [11]. Information generated on nutrition and disease management will not only help enhance productivity from milkfish aquaculture but make it possible to tackle the disease problems that are increasingly becoming a part of aquaculture. Private shrimp farms in India use commercial feeds with vitamins C and E supplementations. The present investigation was carried out with an aim of obtaining information on the immune response of milkfish to supra dietary vitamins C and E. This study also is aimed at obtaining the information on the protective response and immunological memory.

2. Materials and methods

2.1. Fish

Fingerlings of milkfish (0.87 ± 0.01 to 1.08 ± 0.04 g) collected from the coastal waters off north Chennai, India, were stocked in 10-tonne cement tanks supplied with filtered aerated seawater (Salinity – 32–34 ppt; DO – 6.2–7.4 ppm) for acclimatisation.

The experiments were conducted in two sets of rearing systems. Set-I was used for evaluating the effect of supra dietary levels of vitamins C and E on the growth and survival after 6 weeks of feeding. Set-II was used to immunize (priming and booster) and evaluate the efficacy of supra dietary vitamins on the antibody production and protective response.

Set-I: Fish were stocked (30 per tank) in fibre glass reinforced plastic (FRP) tanks of 0.5 tonne capacity and conditioned to experimental environment and control diet for a week. Five treatments were randomly laid out each with three replicates.

Set-II: Fish were stocked in 200-l FRP tanks. Duplicates of primed and booster sets ($5 \times 2 \times 2$) containing 12 fish in each tank were immunized and fed as stated above. All the tanks were supplied with filtered aerated seawater with more than 80% daily replenishment.

2.2. Feed preparation

Vitamin incorporated feed was prepared using locally available feed ingredients (Table 1). The ingredients such as the dry fish (*Anchovy* sp.), squid (*Loligo* sp.), mantis shrimp (*Oratosquilla nepa*), Acetes and soya cake were ground in a micropulveriser, passed through a 300-μm mesh screen and mixed with binder (Aquastab) in an electric blender. Fish oil was added into the blender and thoroughly homogenized. Feed ingredients were mixed with additional levels of stable Vitamin C (SD Fine Chemicals, India, T1 and T2 with 500 and 1500 mg kg^{-1} feed, respectively) and Vitamin E (Merck, India, T3 and T4 with 50 and 150 mg kg^{-1} feed, respectively) for the supra dietary supplementation. Control feed, as per the ingredients (Table 1), contained 90 mg of Vitamin C and 1.2 mg of Vitamin E kg^{-1} in the prepared diet. The ingredients were kneaded into a dough (to an approximate moisture level of 30%), steamed at atmospheric pressure for 5 min and pelletized (2 mm diameter) in a bench top pelletizer. The pellet was dried in a hot air oven at 40 °C for 2–3 days to a uniform moisture level of 9–10%.

Proximate composition of the feed was analysed as per the AOAC [12] methods.

2.3. Immunization

V. vulnificus, isolated from diseased wild collections of gray mullet collected from the backwaters of Muttukadu, south of Chennai, India was grown in brain–heart infusion broth (Hi Media, India) with a final salt concentration of 1.5% at 32 °C for 34 h. The bacterium was harvested by spinning the suspension at $13{,}000 \times g$ for 10 min; the process was repeated three times with sterile phosphate buffered saline (PBS, pH 7.2) as the resuspension medium. The final

Table 1
Ingredient and composition of feed used in milkfish feeding trial

Ingredients	% composition	Vitamins C and E (mg kg^{-1} diet)		
		Treatments	C	E
Fish meal	35	T1	0590	01.20
Squid	05	T2	1590	01.20
Squilla	10	T3	0090	50.00
Acetes	05	T4	0090	150.00
Soya cake	15	T5	0090	01.20
Wheat flour	22			
Binder[a]	01			
Fish oil	02			
Lecithin	02			
Vitamin mixture[b]	01			
Mineral mixture[c]	02			

Proximate components	% composition
Moisture	09.65
Crude protein	39.81
Ether extract	06.94
Crude fibre	04.01
Nitrogen free extract[d]	24.91
Ash	14.68

Control feed (T5); T1 and T2 − 500 and 1500 mg kg^{-1}, respectively, of additional stable Vitamin C; T3 and T4 − 50 and 150 mg kg^{-1}, respectively, of additional Vitamin E.

[a] Poly methyl carbomide.

[b] Vitamin mixture (mg/100 g): Vitamin A 2.0, Vitamin D 0.4, Vitamin E 12.0, Vitamin K 6.0, Choline chloride 600.0, Thiamine 18.0, Riboflavin 24.0, Pyridoxine 18.0, Niacin 108.0, Pantothenic acid 72.0, Biotin 0.2, Folic acid 3.0, Vitamin B_{12} 0.015, Inositol 150.0, Vitamin C 900.0.

[c] Mineral mixture (g/kg): $CaCO_3$ − 28.0, $NaHPO_4$ − 22.0, K_2SO_4 − 10.0, $MgSO_4$ − 12.5, $CuSO_4$ − 0.2, $FeCl_3$ − 0.5, $MnSO_4$ − 0.5, KI − 0.01, $ZnSO_4$ − 1.0, $CoSO_4$ − 0.01, Cr_2SO_4 − 0.05, Bread flour − 7.14.

[d] NFE = 100 − (moisture % + Crude protein % + Crude fibre % + Ether extract % + Ash %).

suspension of the bacterium corresponded to a cell density of 10^8 CFU ml^{-1} and the count was confirmed through spread plate enumeration. Formalin inactivation was carried out adjusting the final formalin concentration in the bacterial suspension to 0.5% (v/v of formalin, for 24 h). Inactivated cells were harvested as explained above and the final suspension (in sterile PBS) of formalin-killed *V. vulnificus* (FKVV) was used for immunization and for estimating the antibody levels.

Fish of set-II were conditioned to experimental diet for a week, anaesthetized using 20 ppm crude clove oil (from a local pharmacy) emulsion in filtered sea water, injected intraperitoneally (0.1 ml corresponding to 10^7 CFU) with the stock suspension of FKVV. Set-II had two subsets of experimental fish one each for priming and booster vaccination. The booster sets were injected with FKVV, as stated above, at 21 days post-priming (dpp). All the treatment groups (T1, T2, T3, T4 and T5) of set-II were vaccinated as detailed above and fed respective designated diets for 6 weeks.

2.4. ELISA for anti-FKVV antibodies

Random samples of five fish from different treatments (set-II) were drawn at 0, 7, 14, 21, 28 (7 days post-booster, dpb), 35 (14 dpb) and 42 (21 dpb) dpp. The fish were anaesthetized as explained above and the blood was drawn using 1-ml sterile disposable tuberculin syringe via caudal vein. Blood was allowed to clot at room temperature for 1 h and held overnight at 4 °C, centrifuged at 3500 × *g* for 5 min and the serum was used for the ELISA. Rabbit polyclonal antisera against FKVV bacterin previously produced in the laboratory were used for quantitative titration of antibodies in the fish serum using standard sandwich ELISA protocols. Briefly, 100 μl of test serum from milkfish in 1:100 dilutions with sterile carbonate bicarbonate coating buffer (CBC, pH 9.6) was loaded in 96 well ELISA plates for overnight incubation at 4 °C. The plates were flipped off to remove unbound serum from the wells and washed three times with 1% tween-20 in sterile phosphate buffered saline (T–PBS, pH 7.4). The plates were inverted on paper towels to remove excess moisture and unbound sites of the wells in the plates were blocked using 0.5% BSA in sterile

PBS for 1 h. The blocking solution was flipped off, washed with T–PBS three times and the wells were loaded with 100 μl of FKVV bacterial suspension, incubated for 1 h and washed as above. The wells were then loaded with 100 μl of rabbit anti-FKVV polyclonal serum (1:500 dilutions in sterile PBS, pH 7.4) and incubated for 1 h. After the next washing, 100 μl of goat anti-rabbit HRP conjugated antibody (Bangalore Genei, India) was added, incubated for 1 h, washed and 50 μl of the substrate (TMB–H_2O_2, Bangalore Genei, India) was added. The colour development was stopped after 20 min using 1 N HCl and optical density (OD) of colour developed was measured at 450 nm using an ELISA reader. Negative control consisted of similarly treated wells where un-immunized rabbit serum was used instead of anti-FKVV polyclonal serum. The difference in ODs of treatment sets and that of the negative controls was taken as the anti-FKVV titre of the test serum.

2.5. Challenge and relative percent survival (RPS)

A random sample of eight fish from each replicates of primed and boosted groups of set-II and 8 fish from unvaccinated control (T5) of set-I were challenged, at the end of the experiment using live bacterial culture of *V. vulnificus*.

The bacterium was grown in brain–heart infusion broth (1.5% NaCl) to get a final cell suspension of 10^7 CFU ml^{-1}. Challenge was carried out in 100-l FRP tanks with eight fish from each tank. The fish were maintained for recording mortalities till 10 days post-challenge. Relative percent survival (RPS) was calculated following Amend [13] as:

$$\mathrm{RPS} = \left(1 - \frac{\%\ \text{mortality in vaccinated group}}{\%\ \text{mortality in C-I or C-II}}\right) \times 100$$

where C-I and C-II are control fish (T5) from set-I and set-II.

2.6. Memory factor (MF)

Evaluation of the efficacy of booster response to quantify the immunological memory [14] was carried out using the ELISA titres measured at different time intervals and the memory factor (MF) was calculated as follows:

$$\mathrm{MF} = \frac{\mathrm{Tb}(x) - T(r)}{\mathrm{Tp}(x)}$$

where Tb(x) : titre of boosted fish at x dpb; $T(r)$: titre of primed fish on the day of booster; and Tp(x): titre of primed fish at x dpb.

2.7. Statistical analysis

The data on antibody titres was subjected to ANOVA for testing the significance of difference between treatment parameters. Pair-wise multiple comparisons for final growth and survival were made following Dennet's test using PEPI-404 statistical software. Tukey's HSD test was used for comparing the ELISA titres due to treatments, due to days post-immunization and protective response of different treatment groups.

3. Results

3.1. Growth and survival

Information on the stocking, growth and survival of milkfish juveniles is presented in Table 2a. Growth of milkfish juveniles fed higher levels of Vitamin E (T4) was lower than those of T2 and T3; however, the ANOVA of weight gain data revealed that the parameters did not differ significantly ($P > 0.05$) from one another. It was interesting to note that all the treatments differed significantly ($P < 0.01$) in their survival responses from that of the control (Table 2b). Fish from the control group (T5) showed the lowest survival (80%) followed by those fed higher dietary Vitamin E levels (150 mg kg^{-1}).

Table 2a
Stocking and growth details of juveniles of *Chanos chanos* fed vitamin-supplemented diets

Treatments	Nt (N0)	Wi (g ± SE)	Wf (g ± SE)	Growth (g)	Survival (% ±SE)
T1	79 (90)	0.916 ± 0.138	1.606 ± 0.256	0.69	87.78 ± 1.93
T2	80 (90)	1.082 ± 0.036	1.777 ± 0.118	0.69	88.89 ± 1.93
T3	86 (90)	0.995 ± 0.117	1.682 ± 0.173	0.69	95.56 ± 1.93
T4	84 (90)	0.868 ± 0.011	1.411 ± 0.117	0.54	93.33 ± 3.33
T5	72 (90)	0.952 ± 0.107	1.562 ± 0.217	0.61	80.00 ± 3.33

Nt – number at termination; N0 – Number at start; Wi – initial average weight; Wf – final average weight.

3.2. Antibody response

Antibody titres elicited by the fish fed supra dietary levels of vitamins were significantly ($P < 0.05$) higher than those of the control fish (Tables 3a and 3b). The trends (Fig. 1) in antibody production, with time, under different treatments indicated a clear superior response of fish fed both levels of vitamins C and E. However, the best booster titres were noticed in T2 and T3 followed by T1 and T4.

Memory factor (MF), depicting the efficacy of booster immunization, at 21 dpb was the highest in T2 followed by T3 and T1, while the MF recorded for T4 was significantly ($P < 0.05$) lower than that of T1, T2 or T3 (Fig. 2).

3.3. Protective response

Complete mortality of unvaccinated control fish (T5) from set-I was recorded (100% in all replicates) upon challenge. All the vaccinated groups showed significant ($P < 0.05$) protective response compared to the unvaccinated control fish from set-I. Challenge tests conducted on fish of set-II revealed a significantly ($P < 0.05$) higher protective responses of T2 and T3 compared to that of T1, T4 and T5 (Fig. 3, Table 3c). Though the challenged fish were kept under observation for 10 days, mortality was noticed only up to 5 days post-challenge. The relative protective responses in comparison with unvaccinated and vaccinated control fish (sets I and II, respectively, designated as RPS-I and RPS-II) depicted efficacy of vaccination with different levels of vitamins and efficacy of different levels of vitamins within vaccinated groups. There was no significant ($P > 0.05$) difference between the higher level of Vitamin C (T2) and lower level of Vitamin E (T3) in their protective responses.

4. Discussion

The dietary protein level (39%) used in the present investigation is higher than that suggested by Borlongan and Satoh [15]. They recommended 24% dietary protein for economic grow-out production of milkfish. However, a higher level of protein was used in the present study; keeping in view the commercial shrimp diet used in shrimp production ponds and aquaculture potentials of fish-shrimp mixed farming as done in many of the traditional farms of West Bengal and Kerala in India. Growth responses due to additional supplementation of vitamins C and E, in the present study, did not result in a statistically significant improvement compared to that of the control. Supra dietary levels of Vitamin C in yellow perch (*Perca flavescens*) resulted in better growth and feed efficiency [16]. Similar growth promoting properties of sufficient dietary levels of Vitamin C are well documented [17–20]. Short duration of the experiment was probably responsible for the statistically insignificant difference in growth enhancement between the groups, though, there was an apparent improvement in treatments T1, T2 and T3 compared to that of T5. However,

Table 2b
Multiple comparisons for survival response (Dennett test)

Comparison treatments	Difference	S.E. of difference	Two-tailed *P*
1 vs. 5	7.778	0.667	<0.01
2 vs. 5	8.889		<0.01
3 vs. 5	15.556		<0.01
4 vs. 5	13.333		<0.01

Table 3a
ANOVA of ELISA titres in milkfish fed Vitamins (C and E)-supplemented diets

Source of variation	SS	*df*	MS	*F*	*P*-value	*F* crit
Duration	0.009471	4	0.002368	151.002	1.83E-41	2.462613
Treatments	0.006016	4	0.001504	95.91786	2.41E-33	2.462613
Interaction	0.003905	16	0.000244	15.56677	1.88E-20	1.745647
Within	0.001568	100	1.57E-05			
Total	0.02096	124				

higher levels of Vitamin E (T4) produced growth retardation. Higher levels of vitamins are required by fish in tropical aquaculture due to increased physiological stress [21,22]. Enhanced growth and survival responses of hybrid striped bass juveniles fed vitamins C and E was noticed by Sealy and Galtin [23]. It is evident from the present study that the supra dietary levels of both vitamins C and E resulted in a better survival response of milkfish juveniles compared to the fish in control group.

Role of dietary vitamins in the context of disease resistance of farmed fish has been very well established. Ascorbic acid deficiency in rainbow trout [24], channel catfish [25] and Atlantic salmon [26] was found to increase disease

Table 3b
Multiple comparisons (Tukey's HSD) of mean ELISA titres in different treatments at different time intervals after priming or booster vaccination

Days post-priming/booster	Tukey's HSD '*q*'	Treatments	Difference between mean antibody titres				
			T1	T2	T3	T4	T5
0 dpp	0.00725	T1	0	0.0004	0.002	0.0008	0.0012
		T2		0	0.0016	0.0004	0.0008
		T3			0	−0.0012	−0.0008
		T4					0.0004
		T5					0
7 dpp	0.00738	T1	0	−0.0052	0.0022	0.0104*	0.0128*
		T2		0	0.0074*	0.0156*	0.018*
		T3			0	0.0082*	0.0106*
		T4				0	0.0024
		T5					0
14 dpp	0.00511	T1	0	−0.013*	−0.003	0.0076*	0.0104*
		T2		0	0.01*	0.0206*	0.0234*
		T3			0	0.0106*	0.0134*
		T4					0.0028
		T5					0
21 dpp	0.00579	T1	0	−0.006*	−0.0052	0.003	0.0072*
		T2		0	0.0008	0.009*	0.0132*
		T3			0	0.0082*	0.0124*
		T4				0	0.0042
		T5					0
42 dpp	0.01065	T1	0	−0.006	−0.0066	0.0058	0.007
		T2		0	−0.0006	0.0118*	0.013*
		T3			0	0.0124*	0.0136*
		T4				0	0.0012
		T5					0
7 dpb	0.01045	T1	0	−0.0048	−0.004	0.0104	0.00672
		T2		0	0.0008	0.0152*	0.01152*
		T3			0	0.0144	0.01072
		T4				0	−0.00368
		T5					0
21 dpb	0.003462	T1	0	−0.0112*	−0.006*	0.0203*	0.0238*
		T2		0	0.0052*	0.0315*	0.035*
		T3			0	0.0263*	0.0298*
		T4				0	0.0035*
		T5					0

*Significant at $P = 0.05$.

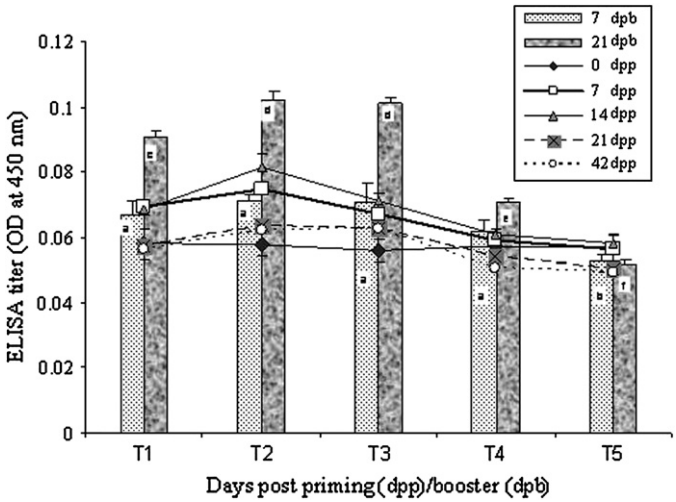

Fig. 1. Immune response (ELISA) of juveniles of *Chanos chanos* fed different vitamin (C and E) enriched diets to *Vibrio vulnificus*.

susceptibility. Supra dietary levels of vitamins C and E were found to have direct influence on the immune response of juvenile milkfish as evident from the enhanced anti-FKVV antibody titres (T1, T2 and T3) in the present study. Supra dietary levels of Vitamin C probably helped in neutralizing the stress responses [4,22] of confinement in the present study and thus, resulted in enhanced antibody production. Similar enhancements in antibody production of channel catfish against *Edwardsiella ictaluri* [25] and in rainbow trout against *V. anguillarum* [4] have been reported. Working on grouper (*Epinehelus awoara*), Wei and co-workers [27] reported higher antibody production in the fish fed supra dietary levels of Vitamin C.

Protective response of milkfish in the present study following vaccination was significant compared to the unvaccinated fish. High protective response and immunological memory in the present study can be attributed to a combination of enhanced specific antibody production and probable elevation of non-specific immune responses as reported in many fish species receiving varying levels of dietary Vitamin C [23,28–31]. High specific antibody response and enhanced protective responses to bacterial challenge exhibited by milkfish juveniles is supported by the enhanced immunological memory. Similar findings were reported by Wei et al. [27] who tested higher levels of dietary Vitamin C in grouper resulting in enhanced specific antibody response against injected formalin-killed *V. vulnificus* and protective response against the bacterium, delivered live via injection/bath.

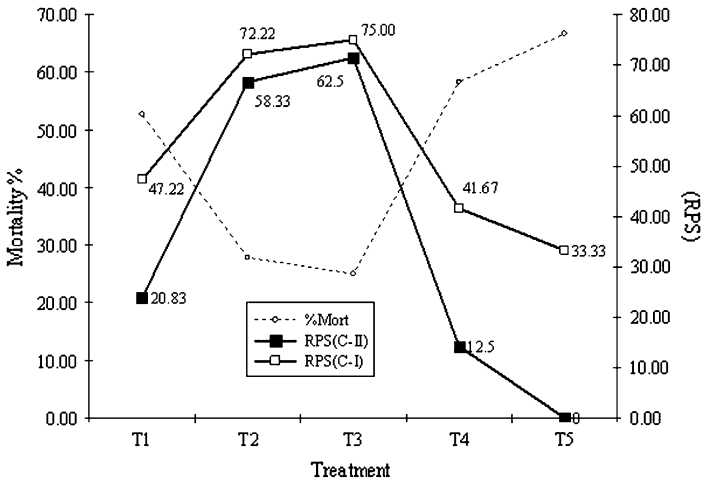

Fig. 2. Memory factor (MF) as an index of specific immunity in vaccinated juveniles of *Chanos chanos* fed vitamin (C and E) enriched diets. Treatment plot points with common letter-lable are not significantly ($P = 0.05$) different from one another.

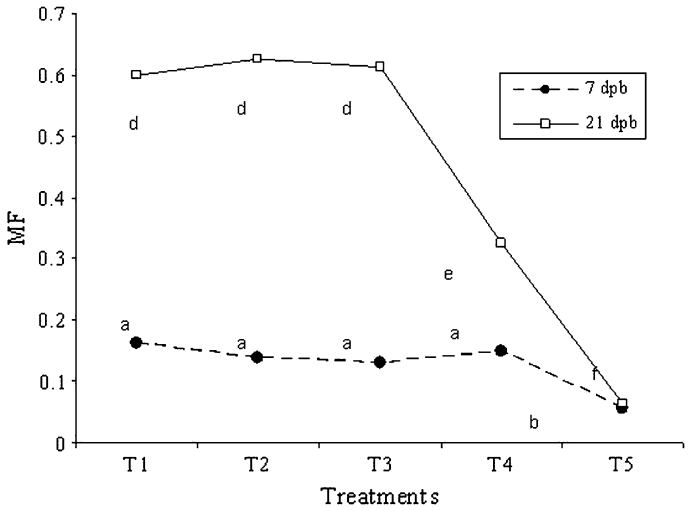

Fig. 3. Protective response of vaccinated juveniles of *Chanos chanos* fed vitamin (C and E) enriched diets to *Vibrio vulnificus* (plot points of treatment means sharing common labels are not significantly ($P = 0.05$) different from one another. RPS (C-I): protective response relative to unvaccinated control. RPS (C-II): protective response relative to the vaccinated control.

Booster vaccination was rendered highly efficient in milkfish juveniles fed high Vitamin C (T2) and low Vitamin E (T3) indicating a probable interaction between the two vitamins. It is widely accepted that Vitamin C, in the water phase, spares Vitamin E and helps in its regeneration from the radical form [32]. Lower antibody titres and protective response of milkfish juveniles fed higher Vitamin E (T4) are probably due to the reduced lymphoproliferation responses as reported in rainbow trout from a feeding trial with varying combinations of vitamins C and E [28].

Protective response of milkfish juveniles followed closely the results of antibody production with high levels of Vitamin C not differing significantly from that of low levels of Vitamin E fed fish. Rainbow trout fed double deficient or double low vitamin (vitamins C/E) diets recorded high mortalities upon challenge with *Yersinia ruckeri* [28]. Low protective response of T5 is probably due to the negligible levels of vitamins C and E (90 mg kg^{-1} and 1.2 mg kg^{-1} of diet, respectively) in the control diet and these levels were not enough to make the fish overcome the vaccination stress. It has been very well shown by previous researchers that sampling and confinement stress can be managed with Vitamin C supplementation [20,21]. Stress is known to reduce the immune response and disease resistance in fish [33]; hence, low levels of vitamins C and E (T5) resulted not only in the reduced antibody production but also in protective response upon challenge with live *V. vulnificus*. High protective response of T2 and T3 in the present study is probably due to both enhanced specific immune response and non-specific immune response.

Results of the present study open up new avenues of making milkfish an alternative for mixed crop species in shrimp aquaculture ponds utilizing the high nutrient feed and supplementing additionally to keep the immune system fit to fight diseases.

Table 3c
Multiple comparisons (Tukey's HSD) of average percentage of mortality in different treatments following challenge with live *Vibrio vulnificus*

Treatments	Calculated difference between means				
	T1	T2	T3	T4	T5
T1	0.00	25.00*	27.78*	5.56	13.89
T2		0.00	2.78	30.56*	38.89*
T3			0.00	33.33*	41.67*
T4				0.00	8.33
T5					0.00
Tukey's HSD at $\alpha = 0.05$	16.48				

*Difference between means significant at $P < 0.05$.

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