



Effects of dietary ascorbic acid on growth, hematology, intestinal histology, and resistance to *Vibrio harveyi* in early life stage of milkfish

Thirugnanamurthy Sivaramakrishnan^{1,2} · Thangaraj Sathish kumar² ·
Kizhakkekarammal Puthiyedathu Sandeep² · Aritra Bera² · Ramalingam Ananda Raja² · Sujeet kumar² ·
Muniyandi Kailasam² · Nathan Felix³ · Kondusamy Ambasankar²

Received: 10 January 2023 / Accepted: 31 March 2023

© The Author(s), under exclusive licence to Japanese Society of Fisheries Science 2023

Abstract

A 45-day feeding experiment was conducted to examine the effect of dietary ascorbic acid (AA) supplementation on the growth of milkfish larvae. Five isonitrogenous and isolipidic (580 g protein/kg and 120 g lipid/kg) experimental diets were prepared with varying levels of AA supplementation at 0 (AA₀), 250 (AA₂₅₀), 500 (AA₅₀₀), 1000 (AA₁₀₀₀), and 2000 (AA₂₀₀₀) mg ascorbic acid (L-ascorbyl-2-polyphosphate) equivalent/kg diet. The experiment results revealed that milkfish fed with 500 and 1000 mg/kg of AA showed significantly better ($p < 0.05$) performance in terms of final body weight, weight gain, specific growth rate and survival rate. The highest level of red blood cells count in pre- and post-challenge groups was recorded in AA₅₀₀ group. Intestinal histology revealed that the length of villi, the thickness of lamina propria, and the concentration of lymphoid cells in the lamina propria were observed to be more in AA₅₀₀ and AA₁₀₀₀ compared with control, AA₂₅₀ and AA₂₀₀₀. AA supplementation at 1000 level (AA₁₀₀₀) had the best survival (93.3%) against *Vibrio harveyi* challenge (5×10^5 CFU/fish) with significantly higher survival compared with control. Based on the curvilinear (polynomial) regression analyses, the optimum final body weight, weight gain, specific growth rate, feed efficiency ratio, protein efficiency ratio, and survival were obtained at dietary ascorbic acid levels of 1175, 1174, 1238, 1262, 1246 and 1174 mg/kg diet, respectively. Accordingly, the optimum dietary ascorbic acid required for milkfish in a range of 1174 mg/kg to 1262 mg/kg is beneficial for better health, survival, and growth. The findings of this study can be used as the baseline scientific information on the ascorbic acid requirement for the formulation of a cost-effective diet for milkfish larvae.

Keywords Larval feed · *Chanos chanos* · Vitamin C · Optimum requirement · Immune response

Introduction

Nutritional needs are described as the ‘requirements for maximal growth and survival’, where the relationship between fish diet and feeding significantly impacts the determination of the quantitative demands (Izquierdo and Lall 2004). Vitamins are essential trace dietary components for robust larval growth, reproduction, and well being (Gasco et al. 2018; Gouda et al. 2020). Ascorbic acid (AA) is an essential nutrient for fish larval development and immune response (Xu et al. 2016; Daniel et al. 2018). The AA significantly promotes growth, collagen formation, iron metabolism, reproduction, stress physiology, wound healing, and immunological response in fish (Halver et al. 1975; Albrektsen et al. 1988; Roberts et al. 1995; Anbarasu and Chandran 2001). Most farmed fish species, including ictalurids,

This research work is part of the thesis to be submitted to the Tamil Nadu Dr. J. Jayalalithaa Fisheries University for award of Ph.D degree.

✉ Thirugnanamurthy Sivaramakrishnan
sivaraman.fish@gmail.com; sivaramakrishnan.t@icar.gov.in

¹ TNJFU-Institute of Fisheries Post Graduate Studies (IFPGS), OMR Campus, Vaniyanchavadi, Chennai 603 103, India

² ICAR-Central Institute of Brackishwater Aquaculture, MRC Nagar, RA Puram, 75-Santhome High Road, Chennai 600028, Tamil Nadu, India

³ Tamil Nadu Dr. J.Jayalalitha Fisheries University (TNJFU), Vettar River View Campus, Nagapattinam 611002, Tamil Nadu, India

cichlids, and cyprinids, lack the enzyme L-gulonolactone oxidase; hence, the nutritional requirement of vitamin C needs to be supplemented (Xie et al. 2006; Daniel et al. 2018). Though it is believed that fish larvae require higher concentrations of micronutrients than juvenile or adult fish, there is a lack of studies on the nutritional requirement of AA (Daniel et al. 2018).

According to the reports, AA is unstable in its natural state because throughout the feed manufacturing process, 50% of the AA will be destroyed. As a result, a major issue was the correct estimate of dietary AA for fish. Because phosphate groups are easily hydrolyzed by digestive enzymes such as phosphatases, L-ascorbyl-2 polyphosphate is the phosphate derivative, an excellent supply and the most stable form of AA (Daniel et al. 2018; Abdelghany 1996) that is better utilized by animals (Matusiewicz et al. 1995). L-ascorbyl-2 polyphosphate always has a better bioavailability of AA than other AA sources (Abdelghany 1996).

Although the global production of *Chanos chanos* is reaching one million tonnes annually, the production in India has not yet achieved the full potential (FAO 2020). The availability of hatchery-produced milkfish fry in adequate numbers is the prerequisite for increasing milkfish farming in India (Bera et al. 2019; Bera et al. 2021). This situation necessitates understanding the critical elements of larval nutrition and optimal feeding strategies (Bera et al. 2019; Sivaramakrishnan et al. 2021, 2023a). The prime objective, while formulating larval feeds is to meet its known nutritional requirements, especially those vital nutrients, which are critical during the early larval stages (Sivaramakrishnan et al. 2023a, 2023b). Moreover, infectious diseases are the foremost reason behind economic losses in commercial aquaculture (Lovell 1996). The application of chemotherapeutics and antibiotics have been criticised for antimicrobial resistance and drug residues. Therefore, preventive strategy using a proper diet has been considered necessary to improve animal health and maintain their ability in disease resistance (Lall and Olivier 1993). Keeping these points in mind, the present study was planned to investigate the effect of AA supplementation on the growth, survival, and nutrient utilization of *C. chanos* larvae and to determine the optimum dietary AA supplementation level. In addition, the effects of AA on histology and hematology were also assessed.

Materials and methods

Experimental diets and proximate composition

Five isoproteic (580 g/kg) and isolipidic (120 g/kg) micro-diets were prepared to comprise different levels of ascorbic acid supplementation at 0 (AA₀), 250 (AA₂₅₀), 500 (AA₅₀₀), 1000 (AA₁₀₀₀) and 2000 (AA₂₀₀₀) mg ascorbic

acid (L-ascorbyl-2-polyphosphate) equivalent per kg diet using practical ingredients (Table 1). The dry solid ingredients were powdered and mixed with fish oil, lecithin and water, followed by steam cooking. Once the dough is cooled (60 °C), vitamin and mineral mixture were added and mixed homogeneously. Dough was further pressed by a micro extruder (MG-55 Multi-xtruder™, Japan) and grounded (QJ-230 T Laboratory marumerizer, Japan) (MeM). Diets were prepared in 300, 500 and 800 µg, resulting in feed sizes suitable for feeding milkfish larvae at 18–33, 34–47 and 48–63 days post hatch (dph), respectively. Diets were oven dried at 40 °C to less than 10% moisture and stored under refrigeration in airtight containers until use.

The composition of experimental diets were done using standard methods of AOAC (2005). Dry matter was detected by drying the samples at 105 °C in a hot-air oven till a constant weight was achieved. The crude protein content was determined by analyzing the nitrogen content and multiplying by a factor of 6.25 (micro-Kjeldhal method). Fat content was determined after extraction with petroleum ether (boiling point: 40–60 °C) in Soxhlet's apparatus. Total ash content was determined by incinerating the moisture-free sample at 550 °C temperature in a muffle furnace. The crude fiber of feed was determined by acid digestion (H₂SO₄–1.25%) followed by alkali digestion (NaOH–1.25%). Feed gross energy values were measured using a semi-micro oxygen bomb calorimeter (Parr Instrument Company, USA Model 1425).

Experimental fish, design and feeding trial

The 18 dph larvae of milkfish were procured from a fin-fish hatchery, ICAR-Central Institute of Brackishwater Aquaculture (CIBA). A total of 1500 larvae with a mean weight of 25.18 mg were randomly distributed into five experimental groups in triplicate according to a completely randomized design. The experimental setup consisted of 15 fiber-reinforced plastic (FRP) rectangular tanks (100 L capacity) stocked at one larvae/L (100 larvae/tank) covered with perforated lids. Larvae in the tanks were reared with continuous aeration and water exchange to provide optimum water quality requirements. Feeding rate was calculated on the basis of the demand for satiation. The total amount of feed was divided into three equal portions and fed at 08:00, 13:00, and 17:00 h.

Sampling and analysis

Growth performance, nutrient utilization, and survival

For growth indices, twenty-five larvae from each tank were fortnightly sampled and weighed. The larvae were starved overnight before sampling, where they were individually weighed in an electronic balance with an accuracy of 0.001 g

Table 1 Ingredient * and chemical composition of the experimental diets

Ingredient composition (g·kg ⁻¹ feed)	AA ₀	AA ₂₅₀	AA ₅₀₀	AA ₁₀₀₀	AA ₂₀₀₀
Fish meal	550	550	550	550	550
Acetes, dried	150	150	150	150	150
Squid meal	50	50	50	50	50
Wheat gluten	90	90	90	90	90
Yeast	25	25	25	25	25
Fish oil	35	35	35	35	35
Soy lecithin	35	35	35	35	35
Spirulina	10	10	10	10	10
Fish hydrolysate	10	10	10	10	10
Vitamin mix ^a	15	15	15	15	15
Mineral mix ^b	15	15	15	15	15
Binder	10	10	10	10	10
Astaxanthin	1	1	1	1	1
Choline chloride	2	2	2	2	2
Ascorbic acid	0	0.25	0.5	1	2
Chemical composition of feed (g·kg ⁻¹ dry matter basis)					
Moisture	77.2	77	76.3	82.3	76
Crude protein	584.2	581.6	584	589.3	586.4
Ether extract	128.2	126.6	121.5	123.7	124.9
Crude fibre	7.9	8.1	15.8	10.6	8.5
Gross energy, MJ/g DM	20.5	20.6	20.6	20.5	20.4
Protein to energy ratio, g CP/MJ	28.5	28.2	28.2	28.7	28.6

^aComposition of vitamin premix (quantity/kg); vitamin A 500,000 IU, vitamin D3 250,000 IU, vitamin E 2000 mg, vitamin K 1000 mg, thiamin 100 mg, riboflavin 2000 mg, pyridoxine 500 mg, cyanacobalamin 400 mg, calcium pantothenate 2500 mg, niacin 4000 mg, biotin 4000 mg, folic acid 200 mg

^bComposition of mineral premix (quantity/kg); manganese oxide 500 mg, potassium iodide 500 mg, ferrous sulphate 10 g, zinc oxide 1000 mg, copper sulphate 250 mg, cobalt carbonate 2 mg, sodium selenite 10 mg, chromium chloride 100 mg, calcium lactate 250 g, calcium phosphate (monobasic) 350 g, magnesium sulphate 50 g

*Same ingredients source details described (Sivaramakrishnan et al. 2022)

(Shimadzu, BL220H). Growth performance, nutrient utilization, and survival of the experimental fish were calculated on the basis of the formulae mentioned below.

Weight gain (mg) = Final weight/ initial weight.

Specific growth rate (%/day) = (In final weight/ In initial weight)/(number of days) × 100.

Feed conversion ratio (FCR) = Total feed given/total weight gain.

Feed efficiency ratio (FER) = Total wet weight gain/total feed given.

Protein efficiency ratio (PER) = Total weight gain/total protein intake.

Survival (%) = (Total number of survived larvae)/(total number of larvae stocked) × 100.

Hematological analysis

Blood samples were collected in heparinized vials in triplicates for hematological parameters on the 45th day

of treatment from the randomly selected fish population, either through gill arch or intracardiac puncture, which was employed only wherever the blood sample could not be collected from the gill arch. Approximately 33.3 µL of blood from each fish was collected in 66.6 µL of heparin preloaded 1 mL sterile disposable syringe with 23 gauge needle and mixed gently and stored in heparin vials for hematological parameters. The blood was diluted 1/1000 in Natt–Herrik staining solution to count red blood cells (RBCs) and 1/100 for white blood cells (WBCs) and counted in a Neubauer hemocytometer using a light microscope. Differential WBC counts were carried out by binding the smears with Leishman–Giemsa stain, and the cells were identified on the basis of characteristic morphology. Hemoglobin (Hb) was estimated by hematin conversion method using Sahli's hemoglobinometer. Hematocrit (HCT) value was calculated by micro hematocrit capillary method. Erythrocyte indices were calculated by the standard formulae as mentioned by Ananda Raja et al. (2020).

Histology of intestine

Milkfish larvae ($n=3$) per treatment were ice killed and dissected under a stereo-microscope to remove the intestine. The intestinal tissue ($n=3$) was collected in 10% neutral buffered formalin (NBF) and processed for histological examination following the standard procedures (Ananda Raja and Jithendran, 2015). Briefly, the intestinal tissue was subjected to dehydration in graded level of alcohol series (70%, 90%, and 100%), and clearing by xylene. The fixed tissue samples were embedded in a paraffin block. Three sections per tissue sample at 4- μ m thick were made and positioned on the glass slides coated with 2% APTES (3-aminopropyltriethoxysilane) (Sigma–Aldrich, USA). After rehydration, the sections were stained with hematoxylin and eosin (H&E) and were viewed under a light microscope (Eclipse NIU, Nikon microscope with DS Fi3 camera and NIS BR Software). Then the sections were assessed as described earlier (Segner et al. 1987).

Challenge study

After 45 days of the feeding trial, milkfish was challenged with a virulent strain of *Vibrio harveyi* LMG19643. The challenge was carried out as per our earlier method (Kumar et al. 2021) with 1 LD₅₀ dose of this strain equivalent to 5×10^5 CFU for milkfish. In brief, the bacterium was revived on freshly prepared Zobell Marine agar (ZMA) (HiMedia, Mumbai, India) and incubated overnight at 30 °C. The colonies were resuspended in normal saline solution (NSS, 0.9% NaCl). After washing, the OD₆₀₀ was set at 1.0 ($\sim 10^8$ CFU/mL) using UV–VIS spectrophotometer. The culture was further diluted four fold (2.5×10^7 CFU/mL). A 20 μ L of diluted culture provided the challenge dose of 5×10^5 CFU/fish and was intramuscularly injected near the dorsal fin of the fish using a tuberculin syringe (U-40, 31 gauge). The challenge trial was conducted in 100 L FRP tanks, with five fish/replicate, resulting in 15 fish/treatment. Mortality recorded within 2 h of injection was regarded as death due to injection shock, and dead fish were discarded from the study and replaced to the challenge with fresh fish. Postinjection, mortality was recorded at 6, 12, 24, 48, 72, 96, 120, 144, and 168 h. No water was exchanged during that period. The survival at the end of the 7th day was analyzed with analysis of variance (ANOVA) and presented as survival percentage.

Statistical analysis

Before statistical analysis, the data were tested for normality and homogeneity of variance using the Shapiro–Wilk test and Levene test, respectively. Growth performance, hematological parameters and survival post challenge were analyzed among the treatments by one-way analysis of variance

(ANOVA) using SPSS 16.0. Duncan's multiple range test was used for post hoc comparison of means among the treatments ($p < 0.05$) (Duncan 1955). Orthogonal polynomial regression analysis were used to optimum dietary ascorbic acid supplementation for maximum growth and nutrient utilization. All the data presented in the text, figures, and tables are mean $\pm$ standard deviation (SD), and statistical significance for all statistical tests was set at $p < 0.05$ and $p < 0.01$.

Result

Proximate compositions of the experimental diets

All experimental diets contained similar proximate compositions (Table 1), with approximately 58% protein, 12% lipid, and 8% of moisture content. The gross energy content of experimental diet is 20.5, MJ g/DM, and the protein to energy ratio was 28.8 g CP/MJ.

Effect of AA supplementation on the growth performance of milkfish larvae

The effect of dietary supplementation of ascorbic acid on weight gain, specific growth rate, protein efficiency ratio, feed efficiency, and survival were calculated and presented in Table 2. Dietary supplementation of AA at different concentrations showed significantly ($p < 0.05$) higher weight gain in AA₁₀₀₀ and AA₅₀₀ group than those without supplementation of AA (AA₀). Also, significantly ($p < 0.05$) higher specific growth rate (SGR) was recorded in AA₁₀₀₀ than other AA-supplemented diets. In addition, there was a significant difference observed between AA supplemented diet and the control diet in nutrient utilization parameters, FER and PER. The AA supplemented groups showed significant difference ($p < 0.05$) and 20% higher survival compared with the control diet (AA₀).

Dietary ascorbic acid requirement

Based on the curvilinear (orthogonal polynomial) regression analyses, optimum final body weight (FBW), weight gain (WG), SGR, FER, PER, and survival were obtained at dietary ascorbic acid levels of 1175, 1174, 1238, 1262, 1246 and 1174 mg/kg diet, respectively (Fig. 1). Accordingly, the optimum dietary ascorbic acid required for milkfish in a range of 1174 to 1262 mg/kg diet.

Hematology

Statistically significant differences ($p < 0.01$) in hematological parameters, such as RBC, WBC, Hb, packed-cell volume (PCV), mean corpuscular cell volume (MCV), mean

Table 2 Growth performance of *Chanos chanos* larvae fed with graded level of dietary ascorbic acid (AA)

Parameters	AA ₀	AA ₂₅₀	AA ₅₀₀	AA ₁₀₀₀	AA ₂₀₀₀
IBW (mg)	25.18 ± 3.23	25.18 ± 3.23	25.18 ± 3.23	25.18 ± 3.23	25.18 ± 3.23
FBW (mg)	145.3 ^a ± 10.39	275.6 ^c ± 14.52	275.7 ^c ± 12.80	315.0 ^d ± 17.64	255.0 ^b ± 10.32
WG (mg)	120 ^a ± 10.40	250 ^c ± 20.38	250 ^c ± 20.80	290 ^d ± 30.65	230 ^b ± 20.43
SGR	3.75 ^a ± 0.17	5.03 ^b ± 0.17	5.18 ^b ± 0.17	5.46 ^c ± 0.13	5.22 ^b ± 0.16
FI (g)*	0.82 ± 0.06	0.91 ± 0.5	1.08 ± 0.02	0.83 ± 0.10	0.82 ± 0.07
FER	0.18 ^a ± 0.03	0.20 ^b ± 0.03	0.20 ^b ± 0.03	0.20 ^b ± 0.03	0.20 ^b ± 0.03
PER	0.31 ^a ± 0.03	0.34 ^b ± 0.03	0.34 ^b ± 0.03	0.35 ^b ± 0.03	0.34 ^b ± 0.03
Survival (%)	69.56 ^a ± 3.19	90.22 ^{bc} ± 3.55	93.78 ^{bc} ± 0.96	95.34 ^c ± 2.85	88.01 ^b ± 4.85

Data are presented as mean ± SD ($n=3$). Values in the same row with different superscript differ significantly ($p < 0.05$). *IBW* initial body weight, *FBW* final body weight, *WG* weight gain, *SGR* specific growth rate, *FER* feed efficiency ratio, *PER* protein efficiency ratio

*NS: not significant

corpuscular hemoglobin concentration (MCHC), total thrombocytes, mature neutrophils, myelocytes, and monocytes, were observed among the groups fed with different levels of AA. Among the treatments, the highest RBC count in pre- and post-challenge groups were recorded in AA₅₀₀ group, while the highest level of hemoglobin and PCV level was observed in AA₁₀₀₀ group. Furthermore, most of the hematological parameters had a higher level in the pre-challenge groups compared with the post-challenge groups (Table 3). However, the post-challenge groups also showed a higher level of hematological indices in AA fed groups compared with that of the control. Microscopically, no morphological abnormalities were observed in RBCs among the different treatment groups.

Histological examination of intestine

In the histological examination, intestinal villi height, villi density, mucous membrane thickness, and lamina propria were examined for evaluating the effect of AA supplementation (Fig. 2). Histological examination of foregut revealed largely normal architecture in all the treatments with sufficient number of villi, goblet cells, and lymphoid cells in the lamina propria. However, the length of villi, thickness of lamina propria, concentration of lymphoid cells in the lamina propria were observed to be more in AA₅₀₀ and AA₁₀₀₀ compared with other treatments. Also, the gap between the villi was observed to be the smallest in the groups AA₅₀₀ and AA₁₀₀₀.

Survival analysis post challenge

The influence of AA supplementation on its protective effect against pathogens was evaluated by a challenge study using *V. harveyi* LMG19643 and is presented in Fig. 3. The results suggest that AA supplementation at 1000 level (AA₁₀₀₀) had the best protective effect as it resulted in significantly higher

survival (93.3%) compared with the control ($p < 0.01$), AA₂₅₀ ($p < 0.01$), and AA₅₀₀ ($p < 0.05$). Moreover, although the difference was insignificant ($p > 0.05$), the highest dose of AA (AA₂₀₀₀) showed a lower level of protection compared with AA₁₀₀₀.

Discussion

AA is attributed to play an essential role as an antioxidant scavenger and as an immune stimulant (Ibrahim et al. 2020). Their deficiency in fish diet causes spinal deformation, retarded growth, and a lower survival rate (Zhou et al. 2013). Dietary supplementation of AA in Nile tilapia *Oreochromis niloticus* fingerlings significantly increased the growth performance of fish in comparison with the control diet (Ibrahim et al. 2020). In the present study, dietary AA significantly affected the growth and feed intake of milkfish in a dose-dependent manner. The experimental group provided with AA dietary supplementation has proven to have a higher survival rate than that of the control group. This indicated that the different levels of AA supplementation had a prominent effect on weight gain by improving the feed intake, PER, and survival rate. Furthermore, increased disease resistance and hematological indices of AA were also observed in this study. The deficiency of AA in the diet slows down the growth rate, immunocompetence (Lin and Shiau 2005), causes spinal deformities, hemorrhages, anemia, hypertyrosinemia, and increases the exposure to bacterial disease risks (Lakani 2015). AA deficiency induces excessive production of toxic lipid peroxides, leading to a decrease in weight gain and feed efficiency in fishes (Daniel et al. 2018). The relevance of AA in fish nutrition was first reported by McLaren et al. (1947), who noted the fact that deficiency of AA can arrest the growth in rainbow trout. Merchie et al. (1995) observed that high levels of AA as a dietary supplement increased growth rate

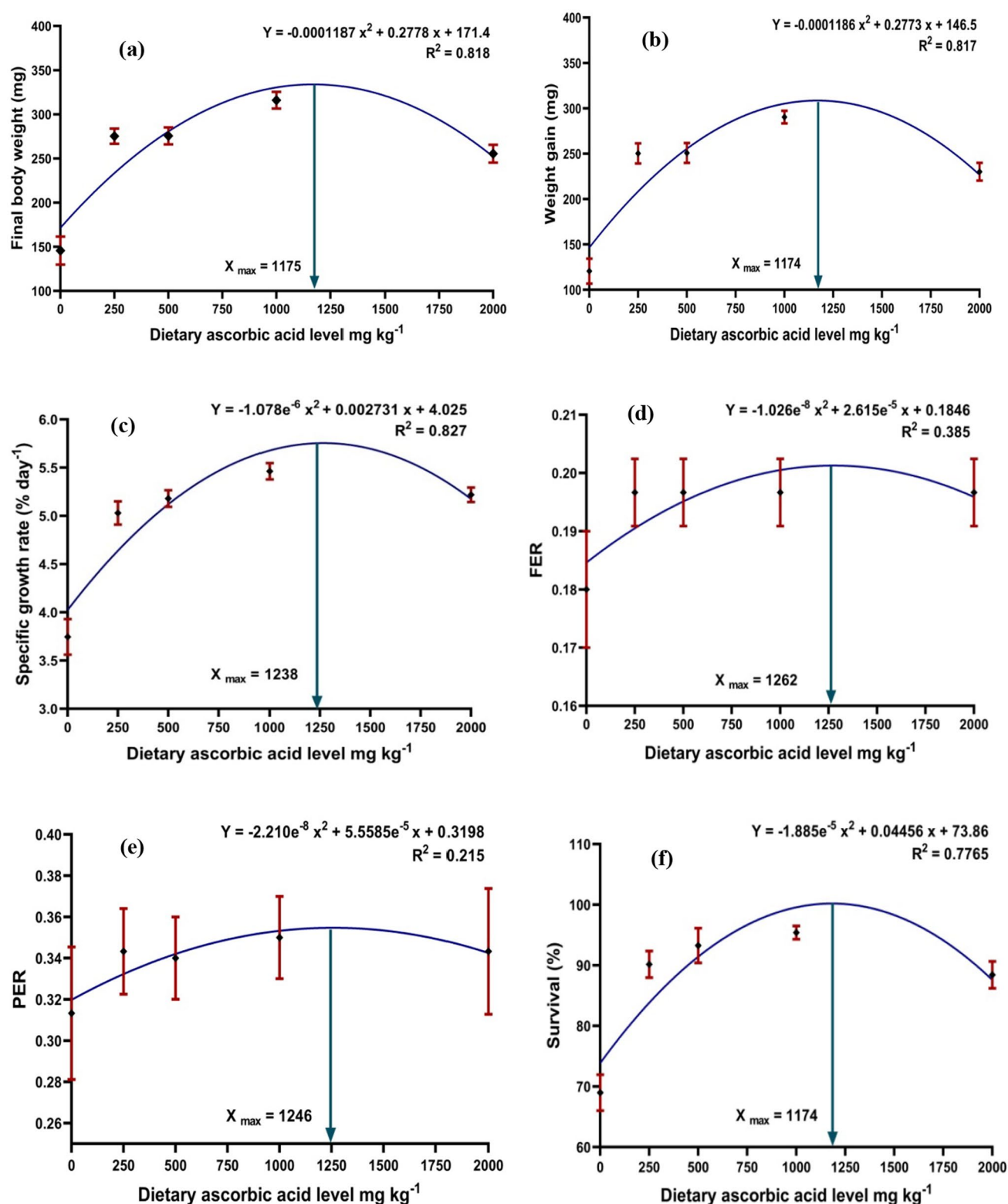


Fig. 1 Orthogonal polynomial regression analysis for the relationship between final body weight (FBW) **a**, weight gain (WG) **b**, specific growth rate (SGR) **c**, feed efficiency ratio (FER) **d**, protein efficiency

ratio (PER) **e**, and survival **f** against varying levels of dietary ascorbic acid in milkfish, *Chanos chanos*

Table 3 Hematological parameters of milkfish (*Chanos chanos*) fry fed with different levels of vitamin C concentrations

Parameters	AA ₀	AA ₂₅₀	AA ₅₀₀	AA ₁₀₀₀	AA ₂₀₀₀
Pre-challenge					
Red blood cells ($\times 10^6 \text{ mm}^3$)**	9.77 ^c $\pm$ 0.12	10.03 ^{cd} $\pm$ 0.33	10.40 ^d $\pm$ 0.21	10.03 ^{cd} $\pm$ 0.18	9.87 ^{cd} $\pm$ 0.33
White blood cells ($\times 10^3 \text{ mm}^3$)**	159.03 ^{cd} $\pm$ 1.13	154.87 ^{abc} $\pm$ 2.22	160.70 ^d $\pm$ 0.67	160.20 ^d $\pm$ 1.99	158.20 ^{cd} $\pm$ 2.48
Hemoglobin (g/dL)**	7.47 ^d $\pm$ 0.09	7.30 ^{cd} $\pm$ 0.10	7.60 ^d $\pm$ 0.15	8.03 ^e $\pm$ 0.12	7.73 ^{de} $\pm$ 0.12
Packed-cell volume (%)**	22.20 ^{de} $\pm$ 0.20	21.87 ^d $\pm$ 0.32	22.43 ^{de} $\pm$ 0.33	24.27 ^f $\pm$ 0.23	23.20 ^{ef} $\pm$ 0.35
Mean corpuscular volume (fl)**	22.83 ^{bc} $\pm$ 0.48	21.83 ^{ab} $\pm$ 0.42	21.63 ^{ab} $\pm$ 0.35	24.30 ^d $\pm$ 0.56	23.57 ^{cd} $\pm$ 0.44
Mean corpuscular hemoglobin (pg)**	7.67 ^{bcd} $\pm$ 0.20	7.27 ^{ab} $\pm$ 0.13	7.30 ^{ab} $\pm$ 0.10	8.03 ^d $\pm$ 0.20	7.87 ^{cd} $\pm$ 0.13
Mean corpuscular hemoglobin concentration (g/dL)**	33.63 ^{ab} $\pm$ 0.13	33.37 ^{ab} $\pm$ 0.67	33.87 ^{ab} $\pm$ 0.20	33.13 ^a $\pm$ 0.20	33.33 ^{ab} $\pm$ 0.15
Elongated thrombocytes (%)**	23.00 ^b $\pm$ 0.58	23.00 ^b $\pm$ 0.58	23.00 ^b $\pm$ 1.16	22.33 ^b $\pm$ 1.20	22.00 ^b $\pm$ 1.73
Oval thrombocytes (%)	7.00 $\pm$ 1.52	6.33 $\pm$ 0.88	6.33 $\pm$ 0.33	4.67 $\pm$ 1.76	7.33 $\pm$ 0.88
Total thrombocytes (%)**	30.00 ^{bc} $\pm$ 1.00	29.33 ^{bc} $\pm$ 0.33	29.33 ^{bc} $\pm$ 1.20	27.00 ^b $\pm$ 0.58	29.33 ^{bc} $\pm$ 2.60
Large lymphocytes (%)	9.67 $\pm$ 1.20	9.33 $\pm$ 2.60	5.00 $\pm$ 1.73	7.33 $\pm$ 1.20	9.00 $\pm$ 0.58
Small lymphocytes (%)*	21.33 ^{abc} $\pm$ 0.67	22.00 ^{abc} $\pm$ 1.73	22.00 ^{abc} $\pm$ 1.16	24.33 ^{bc} $\pm$ 0.67	20.33 ^{ab} $\pm$ 0.88
Total lymphocytes (%)	31.00 $\pm$ 1.73	31.33 $\pm$ 0.88	27.00 $\pm$ 2.52	31.67 $\pm$ 1.20	29.33 $\pm$ 1.33
Juvenile neutrophils (%)	4.33 $\pm$ 0.33	2.67 $\pm$ 1.20	2.67 $\pm$ 1.20	3.00 $\pm$ 0.58	4.33 $\pm$ 0.88
Mature neutrophils (%)**	6.33 ^{abc} $\pm$ 0.88	4.00 ^a $\pm$ 1.16	5.33 ^{ab} $\pm$ 1.45	7.00 ^{abc} $\pm$ 1.52	6.00 ^{ab} $\pm$ 2.65
Total neutrophils (%)*	10.67 ^{abc} $\pm$ 0.67	6.67 ^a $\pm$ 1.45	8.00 ^{ab} $\pm$ 2.65	10.00 ^{abc} $\pm$ 2.00	10.33 ^{abc} $\pm$ 2.91
Myelocytes (%)**	11.67 ^{abc} $\pm$ 0.33	15.00 ^{cd} $\pm$ 0.58	15.00 ^{cd} $\pm$ 1.73	13.67 ^{bcd} $\pm$ 0.88	11.67 ^{abc} $\pm$ 2.73
Monocytes (%)**	16.67 ^b $\pm$ 1.20	17.67 ^b $\pm$ 1.20	20.67 ^{bc} $\pm$ 1.45	17.67 ^b $\pm$ 2.33	19.33 ^{bc} $\pm$ 0.88
Parameters	AA ₀	AA ₂₅₀	AA ₅₀₀	AA ₁₀₀₀	AA ₂₀₀₀
Post-challenge					
Red blood cells ($\times 10^6 \text{ mm}^3$)**	8.53 ^a $\pm$ 0.13	9.10 ^{ab} $\pm$ 0.15	9.53 ^{bc} $\pm$ 0.30	9.07 ^{ab} $\pm$ 0.07	8.53 ^a $\pm$ 0.09
White blood cells ($\times 10^3 \text{ mm}^3$)**	156.63 ^{bcd} $\pm$ 0.44	150.97 ^a $\pm$ 0.17	159.77 ^d $\pm$ 1.79	156.97 ^{bcd} $\pm$ 0.88	153.37 ^{ab} $\pm$ 0.52
Hemoglobin (g/dL)**	5.90 ^a $\pm$ 0.16	6.70 ^b $\pm$ 0.1	6.93 ^{bc} $\pm$ 0.20	6.67 ^b $\pm$ 0.03	6.03 ^a $\pm$ 0.23
Packed-cell volume (%)**	17.76 ^a $\pm$ 0.30	18.57 ^{ab} $\pm$ 0.33	20.00 ^c $\pm$ 0.81	19.10 ^{bc} $\pm$ 0.42	18.23 ^{ab} $\pm$ 0.26
Mean corpuscular volume (fl)**	20.87 ^a $\pm$ 0.47	20.43 ^a $\pm$ 0.28	21.07 ^a $\pm$ 0.19	21.10 ^a $\pm$ 0.50	21.47 ^{ab} $\pm$ 0.49
Mean corpuscular hemoglobin (pg)**	6.93 ^a $\pm$ 0.19	7.40 ^{abc} $\pm$ 0.17	7.30 ^{ab} $\pm$ 0.06	7.37 ^{abc} $\pm$ 0.03	7.10 ^a $\pm$ 0.29
Mean corpuscular hemoglobin concentration (g/dL)**	33.20 ^a $\pm$ 0.15	36.10 ^c $\pm$ 0.61	34.70 ^{abc} $\pm$ 0.38	34.93 ^{bc} $\pm$ 0.62	33.10 ^a $\pm$ 1.15
Elongated thrombocytes (%)**	17.00 ^a $\pm$ 0.58	22.00 ^b $\pm$ 0.58	24.67 ^b $\pm$ 0.88	23.67 ^b $\pm$ 0.88	16.67 ^a $\pm$ 0.88
Oval thrombocytes (%)	5.00 $\pm$ 0.58	8.00 $\pm$ 0.58	9.33 $\pm$ 1.20	8.67 $\pm$ 0.88	6.00 $\pm$ 0.58
Total thrombocytes (%)**	22.00 ^a $\pm$ 1.16	30.00 ^{bc} $\pm$ 1.16	34.00 ^d $\pm$ 0.58	32.33 ^{cd} $\pm$ 1.67	22.67 ^a $\pm$ 0.33
Large lymphocytes (%)	7.33 $\pm$ 1.20	9.67 $\pm$ 0.88	9.67 $\pm$ 0.88	10.67 $\pm$ 1.20	7.33 $\pm$ 0.88
Small lymphocytes (%)*	24.33 ^{bc} $\pm$ 1.20	20.33 ^{ab} $\pm$ 0.88	22.00 ^{abc} $\pm$ 2.31	25.33 ^c $\pm$ 0.88	19.33 ^a $\pm$ 0.88
Total lymphocytes (%)	31.67 $\pm$ 1.20	30.00 $\pm$ 1.16	31.67 $\pm$ 3.18	36.00 $\pm$ 1.16	26.67 $\pm$ 1.76
Juvenile neutrophils (%)	3.67 $\pm$ 0.33	3.00 $\pm$ 0.58	3.33 $\pm$ 0.67	4.67 $\pm$ 0.33	2.33 $\pm$ 0.67
Mature neutrophils (%)**	8.67 ^{bcd} $\pm$ 0.33	9.33 ^{bcd} $\pm$ 0.67	11.33 ^d $\pm$ 0.33	10.33 ^{cd} $\pm$ 0.88	7.00 ^{abc} $\pm$ 0.58
Total neutrophils (%)*	12.33 ^{bc} $\pm$ 0.67	12.33 ^{bc} $\pm$ 0.33	14.67 ^c $\pm$ 0.88	15.00 ^c $\pm$ 0.58	9.33 ^{ab} $\pm$ 1.20
Myelocytes (%)**	13.67 ^{bcd} $\pm$ 1.20	11.33 ^{abc} $\pm$ 0.88	9.67 ^{ab} $\pm$ 1.20	7.33 ^a $\pm$ 1.45	17.00 ^d $\pm$ 1.00
Monocytes (%)**	20.33 ^{bc} $\pm$ 2.91	16.33 ^b $\pm$ 1.33	10.00 ^a $\pm$ 3.01	9.33 ^a $\pm$ 2.03	24.33 ^c $\pm$ 2.40

Data are presented as mean $\pm$ SD ($n=3$). Values bearing different superscripts in a row differ significantly (** $p < 0.01$, * $p < 0.05$)

and stress resistance in the larval stages of African catfish (*Clarias gariepinus*). In addition, in the present study, dietary AA supplementation increased the feed efficiency, which is an essential factor and visual index to evaluate the feed quality, and the results of the present study were consistent with the findings of the earlier studies quoted above.

Depending on the fish species, size, the form of AA, and experimental settings, varying amounts of ascorbic acid may be needed in the food. The amount of dietary ascorbic acid had an impact on feed consumption as well. The amount of total feed consumed correlated with the diet's ascorbic acid content. In fish fed with diet deficient in ascorbic acid, both

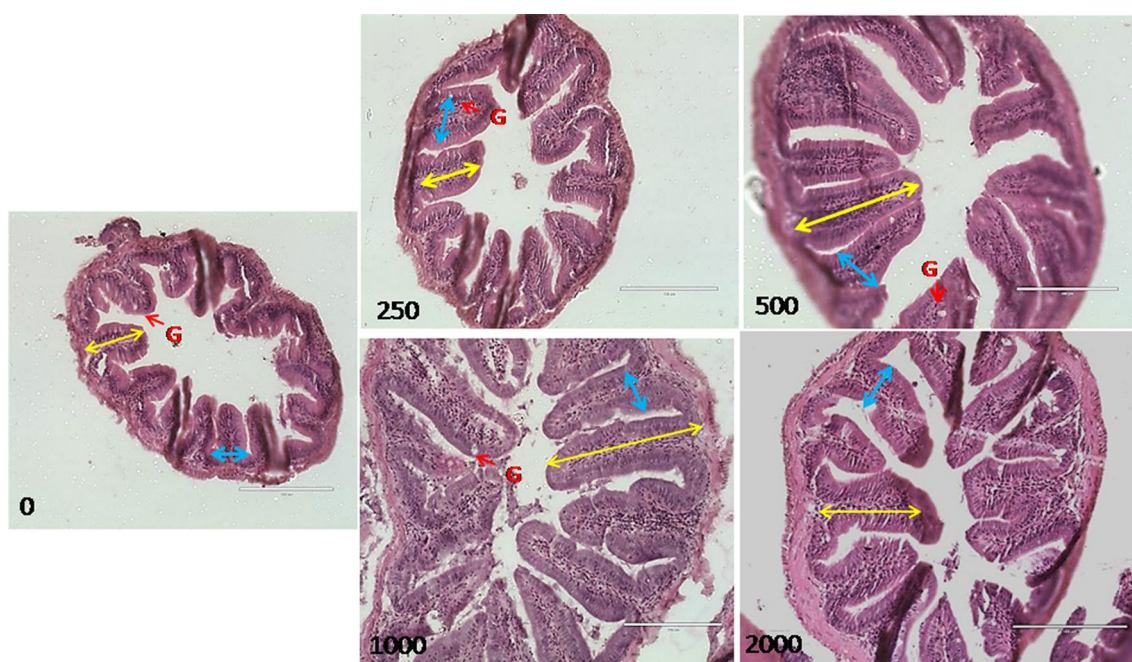


Fig. 2 Histology sections showing the cross section of foregut of control and treatment. Yellow arrow indicates the length of villi and blue arrow indicates the thickness of lamina propria. Goblet cells are represented by “G”

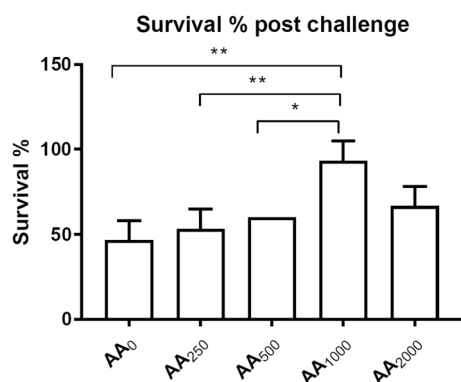


Fig. 3 Survival percentage of milkfish larvae challenged with *Vibrio harveyi*

the protein conversion ratio and the feed conversion ratio were lower. This showed that through increasing feed intake and PER in the group AA₁₀₀₀, AA had a notable impact on specific growth rate. The difference in the requirement of dietary ascorbic acid is likely related to fish species, size, the form of AA, and different experimental conditions. The feed utilization was also affected by the dietary ascorbic acid level. The total feed consumed was proportional to the ascorbic acid concentration in the diet. Both protein conversion ratio and feed conversion ratio were lower in fish fed with diet without ascorbic acid. This indicated that AA has a remarkable effect on specific growth rate by improving the feed intake and PER in the group AA₁₀₀₀.

The hematological parameters are an important characteristic tool used to monitor the physiological and pathological characters in fishes (Fazio et al. 2012). AA plays a principal role in protecting membranes from oxidation (Narra 2017). Several studies have reported that AA supplemented diets had a positive effect on the hematological parameters. A considerable increase was observed in *Cirrhinus mrigala* (Zehra and Khan 2012). In this study, the values of hemotological parameters were higher in pre-challenge groups when compared with post-challenge groups. The RBC count was the highest in challenge group AA₅₀₀, while the WBC count was higher in AA₅₀₀ and AA₁₀₀₀ groups. Hb and PCV also showed similar trend with the highest values in pre-challenge groups. However, among the post-challenge groups, AA fed groups showed higher values compared with that of the control. It is pertinent to note that hematological parameters, particularly the RBCs, seem to significantly increase with the increase in the concentration of AA supplementation. This result implies that AA could protect the red blood cell membranes from oxidation (Yu et al. 2020).

The intestine has a dedicative role in nutrient absorption and digestion, and the digestive function directly relates to intestinal tract development. The intestinal morphology, including the heights of the intestinal villi and the number of goblet cells, is a significant indicator of the digestive and absorptive efficiency of the gut (Pirarat et al. 2011). Instead of having an intestinal villus as in terrestrial organisms, the intestinal folds present in fishes directly reflect the digestive and absorptive capacity (Olli and Kroghdahi

1994). In the current study, the inclusion of dietary AA increased the villi heights and the number of goblet cells. The histological examination of the intestine revealed that the length of the villi, thickness of the lamina propria, and the concentration of lymphoid cells in the lamina propria were higher in AA₅₀₀ and AA₁₀₀₀ groups compared with the control, AA₂₅₀, and AA₂₀₀₀ groups. Also, the gap between the villi was minimal in groups AA₅₀₀ and AA₁₀₀₀. The above findings stipulate that the absorptive function of the intestine is enhanced because of the availability of greater surface area for the absorption of nutrients and a better immune defensive mechanism against pathogens (Rahman et al. 2018; Pirarat et al. 2011).

The addition of vitamins as supplements in the diets has proven to improve the growth rate, immunity, disease resistance, and survival. AA is one of the most proven nutrients for its immunomodulatory and antioxidant functionalities (Oscoii et al. 2012). In this study, the immunogenicity of the milkfish was determined by exposing it to a challenge study against *V. harveyi* LMG19643 pathogen. The results imply that the AA supplementation at the 1000 level (AA₁₀₀₀) had the best defensive mechanism against the above pathogen, as it had a significantly higher survival rate compared with the control ($p < 0.01$), AA₂₅₀ ($p < 0.01$), and AA₅₀₀ ($p < 0.05$). Several reports from the literature have reported to improve growth performance, and immune robustness of fish fed on AA supplemented diets (Zhou et al. 2013; Liua et al. 2016). However, the highest dose of AA (AA₂₀₀₀) had the lowest level of protection compared with AA₁₀₀₀; hence, these results required further studies to confirm the toxic potential of AA at higher doses in the fish.

Acknowledgements The authors thank the Indian Council of Agricultural Research for providing financial support to carry out this research program. Authors are thankful to the director of ICAR-CIBA for support and guidance. The first author is thankful to the Tamil Nadu Dr. J. Jayalalithaa Fisheries University for giving the opportunity to pursue a Ph.D. We also appreciate the fish hatchery and feed mill staff at Muttukadu Experimental Station for their help in conducting the experiments and laboratory analysis.

Author contributions T.S.—conceptualization, methodology, data analyzed, and writing—original draft, T.S.—histology and review, Sandeep, K.P.—methodology and data analyzed, A.B.—methodology and live animal maintenance, R.A.R.—hematology analysis and review, S.K.—challenge study, M.K.—conceptualization, supervision, project administration, and review and editing of manuscript, N.F.—conceptualization, supervision, project administration, and review and editing of manuscript, K.A.—conceptualization, supervision, project administration, and review and editing of manuscript.

Funding Indian Council of Agricultural Research, FISHCIBASIL 201800800136.

Data availability The data that support the findings of this study are available within this manuscript.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The institute's animal ethical committee clearance was obtained for this study at the Central Institute of Brackishwater Aquaculture, Chennai, India. Milkfish larvae used in the experiment were obtained from a hatchery, and they are not an endangered fish as per the provisions of the Government of India's Wildlife Protection Act of 1972.

Human and animal rights Not applicable.

Consent for publication All the authors agree to publish in this journal.

References

- Abdelghany AE (1996) Growth response of Nile tilapia *Oreochromis niloticus* to dietary L ascorbic acid, L-ascorbyl 2sulfate, and L-ascorbyl-2-polyphosphate. *J World Aquac Soc* 27(4):449–455
- Albrektsen S, Lie Ø, Sandnes K (1988) Ascorbyl palmitate as a dietary vitamin C source for rainbow trout (*Salmo gairdneri*). *Aquaculture* 71(4):359–368
- Ananda Raja R, Jithendran KP (2015) Aquaculture disease diagnosis and health management. In *Advances in marine and Brackishwater aquaculture*, Springer, New Delhi
- Ananda Raja R, Patil PK, Avunje S, Aravind RP, Alavandi SV, Vijayan KK (2020) Biosafety, withdrawal and efficacy of anti-parasitic drug emamectin benzoate in Asian Seabass (*Lates calcarifer*). *Aquaculture*. <https://doi.org/10.1016/j.aquaculture.2020.735335>
- Anbarasu K, Chandran MR (2001) Effect of ascorbic acid on the immune response of the catfish, *Mystus gulio* (Hamilton), to different bacterins of *Aeromonas hydrophila*. *Fish Shellfish Immunol* 11(4):347–355
- AOAC (Association of Official Agricultural Chemists) (2005) Official methods of analysis of AOAC International, volume 1. Agriculture chemicals, contaminants, drugs, 16th edition. AOAC International, Arlington, Virginia
- Bera A, Kailasam M, Mandal B, Sukumaran K, Makesh M, Hussain T, Vijayan K (2019) Effect of tank colour on foraging capacity, growth, and survival of milkfish (*Chanos chanos*) larvae. *Aquaculture*. <https://doi.org/10.1016/j.aquaculture.2019.734347>
- Daniel N, Muralidhar AP, Srivastava PP, Jain KK, Pani Prasad K, Manish J, Sivaramakrishnan T (2018) Dietary ascorbic acid requirement for growth of striped catfish, *Pangasianodon hypophthalmus* (Sauvage, 1878) juveniles. *Aquac Nutri* 24(1):616–624
- Duncan DB (1955) Multiple range and multiple F tests. *Biometrics* 11(1):1–42
- FAO (2020) The state of world Fisheries and Aquaculture: Opportunities and challenges. Food and Agriculture organizations of United Nations, Rome, pp 188–191
- Fazio F, Filiciotto F, Marafioti S, Di Stefano V, Assenza A, Placenti F, Buscaino G, Piccione G, Mazzola S (2012) Automatic analysis to assess haematological parameters in farmed gilthead sea bream (*Sparus aurata* Linnaeus, 1758). *Mar Freshw Behav Physiol* 45(1):63–73
- Gasco L, Gai F, Maricchiolo G, Genovese L, Ragonese S, Bottari T, Caruso G (2018) Supplementation of vitamins, minerals, enzymes and antioxidants in Fish feeds. In: Gasco L, Gai F, Maricchiolo G, Genovese L, Ragonese S, Bottari T, Caruso G (eds) *Feeds for the Aquaculture Sector: Current Situation and Alternative Sources*. Springer International Publishing, Cham, pp 63–103

- Gouda A, Amer SA, Gabr S, Tolba SA (2020) Effect of dietary supplemental ascorbic acid and folic acid on the growth performance, redox status, and immune status of broiler chickens under heat stress. *Trop Anim Health Prod* 1–10.
- Halver JE, Smith RR, Tolbert BM, Baker EM (1975) Utilization of ascorbic acid in fish. *Ann N Y Acad Sci* 258(1):81–102
- Ibrahim RE, Ahmed SA, Amer SA, Al-Gabri NA, Ahmed AI, Abdel-Warith AWA, Younis ESM, Metwally AE (2020) Influence of vitamin C feed supplementation on the growth, antioxidant activity, immune status, tissue histomorphology, and disease resistance in Nile tilapia. *Oreochromis niloticus* Aquac Rep 18:100545
- Izquierdo MS, Lall S (2004) May. Experimental design for lipid research. In Workshop on Methodologies in Fish Nutrition Research, pp 2–7.
- Kumar S, Kumar CB, Rajendran V, Abishaw N, Anand PS, Kannapan S, Nagaleekar VK, Vijaya KK, Alavandi SV (2021) Delineating virulence of *Vibrio campbellii*: a predominant luminescent bacterial pathogen in Indian shrimp hatcheries. *Sci Rep* 11(1):1–16
- Lakani FB (2015) Dietary vitamin C requirements in fish larvae. In Journal First International Larviculture Conference in Iran.
- Lall SP, Olivier G (1993) Role of micronutrients in immune response and disease resistance of fish. In: Fish Nutrition in Practice (Kausshik, S.J. Luquet, P. eds), INRA edn. National Institute for Agricultural Research, Paris, France.
- Lin MF, Shiau SY (2005) Dietary L-ascorbic acid affects growth, nonspecific immune responses and disease resistance in juvenile grouper. *Epinephelus Malabaricus* Aquaculture 244(1–4):215–221
- Liua B, Wana J, Gea X, Xiea J, Zhou Q, Miaob L, Renb M, Panb L (2016) Effects of dietary Vitamin C on the physiological responses and disease resistance to pH stress and *Aeromonas hydrophila* infection of *Megalobrama amblycephala*. *Turk J Fish Aquat Sci* 16:421–433
- Lovell RT (1996) Feed deprivation increases resistance of channel catfish to bacterial infection. *Aquac Mag* 6:65–67
- Matusiewicz M, Dabrowski K, Volker L, Matusiewicz K (1995) Ascorbate polyphosphate is a bioavailable vitamin C source in juvenile rainbow trout: tissue saturation and compartmentalization model. *J Nutr* 125(12):3055–3061
- McLaren BA, Keller E, Donnell DJ, Elvehjem CA (1947) The nutrition of rainbow trout. I. Studies of vitamin requirements. *Arch Biochem Biophys* 15:169–178
- Merchie G, Lavens P, Dhert Ph, Pector R, Mai Soni AF, Abbes M, Nelis H, Ollevier F, De Leenheer A, Sorgeloos P (1995) Live food mediated vitamin C transfer to *Dicentrarchus labrax* and *Clarias gariepinus*. *J Appl Ichthyol* 11:336–341
- Narra MR (2017) Haematological and immune upshots in *Clarias batrachus* exposed to dimethoate and defying response of dietary ascorbic acid. *Chemosphere* 168:988–995
- Olli JJ, Krogdahi A (1994) Nutritive value of four soybean products as protein sources in diets for rainbow trout (*Oncorhynchus mykiss*, Walbaum) reared in fresh water. *Acta Agric Scand A Anim Sci* 44:185. <https://doi.org/10.1080/09064709409410896>
- Oskoi SB, Kohyani AT, Parseh A, Salati AP, Sadeghi E (2012) Effects of dietary administration of *Echinacea purpurea* on growth indices and biochemical and hematological indices in rainbow trout (*Oncorhynchus mykiss*) fingerlings. *Fish Physiol Biochem* 38(4):1029–1034
- Pirarat N, Pimpimai K, Endo M, Katagiri T, Ponpornpisit A, Chansue N, Maita M (2011) Modulation of intestinal morphology and immunity in Nile tilapia (*Oreochromis niloticus*) by *Lactobacillus rhamnosus* GG. *Res Vet Sci* 91(3):92–97
- Rahman AN, Khalil AA, Abdallah HM, ElHady M (2018) The effects of the dietary supplementation of *Echinacea purpurea* extract and/or vitamin C on the intestinal histomorphology, phagocytic activity, and gene expression of the Nile tilapia. *Fish Shellfish Immunol* 82:312–318
- Roberts ML, Davies SJ, Pulsford AL (1995) The influence of ascorbic acid (vitamin C) on non-specific immunity in the turbot (*Scophthalmus maximus* L). *Fish Shellfish Immunol* 5(1):27–38
- Segner H, Burkhardt P, Avila EM, Juario J V, Storch V (1987) Nutrition-related histopathology of the intestine of milkfish *Chanos chanos* fry. *Dis Aqua Org* 2 (2) 99–107. <http://hdl.handle.net/10862/1281>
- Sivaramakrishnan T, Ambasankar K, Kumaraguru Vasagam KP, SyamaDayal J, Sandeep KP, Bera A, Makesh M, Kailasam M, Vijayan KK (2021) Effect of dietary soy lecithin inclusion levels on growth, feed utilization, fatty acid profile, deformity and survival of milkfish (*Chanos chanos*) larvae. *Aquac Res* 52(11):5366–5374. <https://doi.org/10.1111/are.15406>
- Sivaramakrishnan T, Ambasankar K, Felix N, Sandeep KP, Bera A, Suresh E, Kamalam BS, Kailasam M, Shanmugam SA (2023a) Effect of dietary lipid sources and their combinations on growth and fatty acid composition of milkfish (*Chanos chanos*) larvae. *Indian J Ani Res.* <https://doi.org/10.18805/IJAR.B-5072>
- Sivaramakrishnan T, Syama Dayal J, Ambasankar K, Felix N, Sandeep KP, Bera A, Kumaraguru Vasagam KP, Thiagarajan G, Kailasam M (2023b) Amino acid and fatty acid compositions of various stages of *Chanos chanos* larvae: Implications for early life feed formulation†† : Amino acids and fatty acid profile of milkfish larvae. *J Indian Fish* 1–6.
- Xie Z, Niu C, Zhang Z, Bao L (2006) Dietary ascorbic acid may be necessary for enhancing the immune response in Siberian sturgeon (*Acipenser baerii*), a species capable of ascorbic acid biosynthesis. *Comp Biochem Physiol A Mol Integr Physiol* 145(2):152–157
- Xu HJ, Jiang WD, Feng L, Liu Y, Wu P, Jiang J, Kuang SY, Tang L, Tang WN, Zhang YA, Zhou XQ (2016) Dietary vitamin C deficiency depresses the growth, head kidney and spleen immunity and structural integrity by regulating NF-κB, TOR, Nrf2, apoptosis and MLCK signaling in young grass carp (*Ctenopharyngodon idella*). *Fish Shellfish Immunol* 52:111–138
- Yu YB, Park HJ, Kang JC (2020) Effects of dietary ascorbic acid on growth performance, hematological parameters, antioxidant and non-specific immune responses in starry flounder. *Platichthys stellatus* Aquac Rep 18:100419
- Zehra S, Khan MA (2012) Dietary vitamin C requirement of fingerling, *Cirrhinus mrigala* (Hamilton), based on growth, feed conversion, protein retention, hematological indices, and liver vitamin C concentration. *J World Aquac Soc* 43(5):648–658
- Zhou Q, Wang L, Wang H, Xie F, Wang T (2013) Effect of dietary vitamin C on the growth performance and innate immunity of juvenile cobia (*Rachycentron canadum*). *Fish Shellfish Immunol* 32(6):969–975

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.