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Supplementation of dietary apple cider vinegar as an organic acidifier on the growth performance, digestive enzymes and mucosal immunity of green terror (*Andinoacara rivulatus*)

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Abstract

The present study was designed to investigate the effects of dietary apple cider vinegar (ACV) on digestive enzyme activity and growth performance as well as immune responses and antibacterial activity of skin mucus in green terror (*Andinoacara rivulatus*). Fish were fed diets supplemented with 0%, 1%, 2% and 4% of ACV (40.830 ppm acetic acid concentration) for 63 days. The final weight and weight gain values were observed to be significantly higher in fish fed with 2% of ACV compared to the control group ($p < .05$). ACV inclusion in the diets had significant effects on SGR (%) and FCR values ($p > .05$). ACV treatment resulted in a significant increase in the intestinal protease, α-amylase, lipase and alkaline phosphatase activities compared to control ($p < .05$). The activities of digestive enzymes in fish fed with 2% and 4% of ACV diets were significantly higher than the other groups ($p < .05$). The total protein content, alternative haemolytic complement, alkaline phosphatase, total immunoglobulins and lysozyme activities of skin mucus increased significantly in fish fed with ACV diets ($p < .05$). In conclusion, administration of ACV enhanced digestive enzyme activity, growth performance, immune responses and the immune properties of skin mucus, and it can be used as a natural growth promoter and immunostimulant in green terror culture.

KEYWORDS

antibacterial activity, apple cider vinegar, digestive enzymes, green terror (*Andinoacara rivulatus*), growth performances, immune response

1 | INTRODUCTION

Green terror, *Andinoacara rivulatus*, is one of the most popular aquarium American cichlids characterized by simplicity of the rearing and breeding (Prazdnikov & Shkil, 2019). This fish has many enthusiasts among aquarists because of its hardness and spectacular colours. The total value of ornamental fish industry is estimated at approximately USD 15 billion, and global exports of ornamental fish have grown from USD 181 million to USD 372 million between 2000 and 2011. Currently, approximately 90% of the freshwater traded organisms are cultured in the ornamental fish industry (Ladisa, Bruni, & Lovatelli, 2017). The rapid growth of demand for aquarium fish can be fuelled using intensified aquaculture systems; however, it may lead to stressful conditions and consequently acceleration of the disease outbreaks (Sirimanapong et al., 2015). The overuse of antibiotics and chemotherapeutic to treat diseases has exerted detrimental effects, including antibiotic-resistant bacteria (Teuber, 2001), bioaccumulation in the human consumers (Nawaz & KIRK, 1996), release

to aquatic environments and causing harmful effects on aquatic organisms (Cabello, 2006; Rico et al., 2012). The use of environmentally friendly feed additives such as probiotics, prebiotics, synbiotics, parabiotics, nucleotides, phyto-products (phytogenics) and organic acids as a safe dietary supplement in the aquafeed industry is among the many alternative strategies used to decrease the excessive use of antibiotics and promoting the fish growth and disease resistance (Ahmadnia Motlagh et al., 2019; Safari & Sarkheil, 2018). Short-chain organic acids (C_1–C_7) as acidifiers have been traditionally used in feeds to improve the performance and health of terrestrial livestock. Many of the organic acids are known as antimicrobial agents which exert their effects by reducing the pH of the environment and consequently preventing the growth of acid-sensitive bacteria (Ng & Koh, 2016). Moreover, organic acids included in feeds for terrestrial livestock enhance nutrient bioavailability by lowering gastric pH, thereby increasing pepsin activation and mineral solubility, and acting as chelating agents, which results in an increase in mineral absorption (Ng & Koh, 2016). Recently, the use of organic acids and their salts in aquafeeds have received much attention. Apple cider vinegar (ACV) is the fermented juice made from crushed apples, which is rich in acetic acid. It also contains amino acids and peptides, minerals, vitamins and polyphenolic compounds (Akanksha & Sunita, n.d.; Cocchi et al., 2006; Pazuch, Siepmann, Canan, & Colla, 2015). This substance has been shown to have beneficial properties such as antimicrobial and antioxidant activities, as well as antidiabetic functions in humans and animals (Morgan & Mosawy, 2016). Some studies have revealed the effects of dietary supplements of organic acids and their salts on growth performances and nutrient utilization in rainbow trout, *Oncorhynchus mykiss* (Hernández, Satoh, & Kiron, 2013); red sea bream, *Pagrus major* (Sarker, Satoh, & Kiron, 2005); rohu, *Labeo rohita* (Baruah et al., 2007); beluga sturgeon, *Huso huso* (Khajepour & Hosseini, 2012a, 2012b); and yellowtail, *Seriola quinqueradiata* (Sarker, Satoh, Kamata, Haga, & Yamamoto, 2012, 2012). However, to the best of our knowledge, there is no sufficient evidence regarding the effects of ACV on growth performances and immune response in fish or finfish. Safari et al. (Safari, Hoseinifar, Nejadmoghadam, & Jafar, 2016) reported the immunomodulatory and health-promoting effects of dietary ACV in common carp (*Cyprinus carpio*). They found that combined administration of probiotic and ACV increased total immunoglobulins and lysozyme activities of serum and skin mucus and expression level of GH and IGF1. The study on white shrimp (*Litopenaeus vannamei*) fed with 1%, 2% and 4% of ACV diets for 60 days showed that expression of immune-related genes (prophenoloxidase and lysozyme genes) is significantly up-regulated in the hepatopancreas (Pourmozaffar, Hajimoradloo, & Miandare, 2017). Based on the literature reviewed, there is no study investigating the effects of ACV on ornamental fish; therefore, the present study aimed to evaluate the effects of various levels of dietary ACV on immune parameters (alkaline phosphatase, total IG, lysozyme and ACH50 activities) and antibacterial activity of skin mucus and growth performance as well as digestive enzyme (protease, α-amylase, lipase and alkaline phosphatase) activities in the commercial ornamental fish green terror (*A. rivulatus*) fingerlings.

2 | MATERIALS AND METHODS

2.1 | Experimental diets

A commercial ornamental fish diet (Energy® Ornamental fish feed, Iran) was used as basal diet. The proximate composition (%) was as follows: moisture 30.26 ± 2.09, dry matter 69.74 ± 2.25, crude protein 40.92 ± 1.37, crude lipid 3.72 ± 0.70, ash 2.72 ± 0.59, crude fibre 3.15 ± 0.95 and nitrogen-free extract 19.23 ± 1.12. The experimental diets were prepared by supplementing the basal diet with four levels (0%, 1%, 2% and 4%) of apple cider vinegar (ACV; w/w). The basal diet without inclusion of ACV was considered as the control treatment. The ACV inclusion levels were chosen based on the previous studies on the administration of ACV in aquafeeds (Pourmozaffar et al., 2017). The ACV was purchased from Golchekan Zamani Co., Mashhad, Iran.

In order to prepare the experimental diets, ACV was mixed with gelatin at a concentration of 4 g kg dry diet^{-1} and was sprayed over the basal diet (Ahmadnia & Hajimoradlo, 2017). For preparation of the control treatment, the gelatin (50°C) was sprayed on the basal diet without adding ACV. Diets were isonitrogenous and isoenergetic.

2.2 | Experimental design

A total of 180 fingerlings of green terror (weighing 7.08 ± 0.30 g) were purchased from a local ornamental fish supplier (Topazland, Mashhad, Iran). Fish were acclimatized to laboratory conditions and were fed with the basal diet for 2 weeks. Thereafter, they were randomly stocked into 12 glass aquariums (150 L) at the density of 10 tank^{-1} with 3 replicates for each experimental diet. Fish were fed three times daily for 63 days with experimental diets amounting to 2.5% of body weight. Water of each glass aquarium was exchanged at a rate of 20%, and uneaten feeds were siphoned daily. Fish were maintained under 12:12 (light: dark) of photoperiod condition. Water temperature, pH and dissolved oxygen were measured in each glass aquarium every day, and they were recorded as 22 ± 2.5°C, 7.3 ± 0.5 and 9 ± 0.75 mg/L respectively. All experiments were done according to FUM animal ethics.

2.3 | Growth performance assay

After 63 days of feeding trial, all fish were weighed using an electronic scale (0.01 g, AND, Japan). Growth performance parameters were calculated based on the following equations:

$$\text{Weight gain } (g) = \left(W_f - W_i\right)$$

$$\text{Specific growth rate } \left(\text{SGR; \% body weight day}^{-1}\right) = \left[\left(\ln W_f - \ln W_i\right)/t\right] \times 100$$

$$\text{Feed conversion ratio } (\text{FCR}) = \left(\text{Feed}_{\text{consumed}}/W_{\text{gain}}\right)$$

$$\text{Visceralsomatic Index (VSI)} = 100 \times \text{Weight of the whole digestive tract/Body weight}$$

$$\text{Hepatosomatic Index (HSI)} = 100 \times \text{Liver weight/Body weight}$$

where W_i, W_f, W_{gain}, t and Feed $_{consumed}$ are initial weight, final weight, weight increment (*g*), time period (day) and consumed feed (*g*) respectively.

2.4 | Digestive enzymes

In order to evaluate digestive enzyme activities, fish were starved for 24 hr at the end of feeding trial. Three fish were randomly selected from each glass aquarium and were anaesthetized using ground clove extract (500 mg/L). The intestine was removed and was rinsed with cold distilled water at 4°C according to the method used in the previous studies (Huang, Yan, Mu, & Wang, 1999). Briefly, the intestine was homogenized in phosphate buffer (pH 7.5, at room temperature) using a homogenizer (DI 18 Disperser). Thereafter, the homogenized samples were centrifuged at 15,000 × *g*, 4°C for 15 min and supernatants were stored at −80°C for subsequent enzyme analysis.

Total protein content was detected using a biochemistry kit (Pars Azmun, Iran, 128500). The optical density (OD) was assayed at 540 nm, and protein concentration was expressed as µg/ml. Protease activity was determined based on the casein hydrolysis method described in the study by Hidalgo *et al.* (Hidalgo, Urea, & Sanz, 1999). Briefly, the homogenate supernatant (0.05 ml) was mixed with casein (1% w/v; 0.125 ml) and buffer (0.1 M Tris-HCl, pH 9.0; 0.125 ml) and was incubated at 37°C for 1 hr. Then, 0.3 ml of trichloroacetic acid (TCA) (8% w/v) solution was added to stop the reaction. Next, the samples were kept at 4°C for 1 hr and then were centrifuged at 1,800 × *g* for 10 min. The absorbance of supernatant was recorded at 280 nm. One unit (U) of enzyme activity was defined as the amount of enzyme required to hydrolyse azocasein and produce a change in 0.001 (or 0.01, or 0.1) units of absorbance per minute. Protease was expressed as U/mg protein and as a U/g of tissue.

Lipase activity was analysed based on a modified method described by Gawlicka et al. (Gawlicka et al., 2000) using 0.4 mM of p-nitrophenyl myristate as a substrate at 25°C, and the absorbance was measured at an optical density of 405 nm. One unit (U) of enzyme activity was defined as the amount of enzyme required to hydrolyse p-nitrophenyl myristate and produce a change in 0.001 (or 0.01, or 0.1) units of absorbance per minute. Lipase was expressed as U/mg protein and as a U/g of tissue.

The α-amylase content of samples was determined according to the 3,5-dinitrosalicylic acid method, using 1% starch (diluted in a buffer at pH 6.9, 0.02 M Na_2HPO_4 and 0.006 M NaCl) as a substrate, and absorbance was measured at 540 nm (Worthington, 1988). One unit (U) of enzyme activity was defined as the amount of enzyme required to hydrolyse starch and produce a change in 0.001 (or 0.01, or 0.1) units of absorbance per minute. α-amylase was expressed as U/mg protein and as a U/g of tissue.

Alkaline phosphatase activity was determined using a commercial kit (Pars Azmun Company, Iran). The optical density (OD) was measured at 405 nm. Digestive enzyme activities were evaluated in 3 replicates for each dietary treatment using a microplate scanning spectrophotometer (HACH DR/4000, USA). This formula was used to calculate ALP activity: U/ml = OD/18.8 × 10^3×enzyme volume/Total tube volume. ALP was expressed as U/mg protein and as a U/g of tissue.

2.5 | Immunological assays

Immune parameters including lysozyme (LYZ) activity, total immunoglobulins (IG), alkaline phosphatase and alternative complement pathway haemolytic activity (ACH_{50}) were investigated in the skin mucus of treated fish. For this purpose, fish were starved for 24 hr at the end of feeding trial. Three fish were sampled from each glass aquarium. Fish were anaesthetized as reported above and were placed in polyethylene bags containing NaCl (50 mM; 5 ml/g fish; Merck, Germany), for approximately 1 min. Skin mucus samples were collected by slow shaking the fish inside the plastic bag for 2 min. Then, fish were removed and transferred to the glass aquariums to recover. The mucus samples were instantly transferred to 15-ml sterile centrifuge tubes and were centrifuged at 1,500 × *g*, at 4 °C, for 10 min. The supernatants were collected and were stored in 2-ml tubes at −80°C until further use (Safari & Sarkheil, 2018; Subramanian, MacKinnon, & Ross, 2007).

Lysozyme activity was determined according to the turbidimetric method described previously (Hoseinifar, Zoheiri, & Caipang, 2016). Briefly, 50 µl of a lysozyme-sensitive Gram-positive bacterium *Micrococcus luteus* (Sigma, USA) suspension was prepared and placed in a 96-well plate, and equal amount of mucus sample was added to each wall. Afterwards, the prepared samples were incubated at 30°C for 15 min. The change in absorption was recorded for 50 min using a spectrophotometer (HACH DR/4000, USA). The lysozyme activity was calculated based on the reduction in the absorbance 0.001 min^{-1} at 450 nm and was expressed as U/ml.

Total immunoglobulins (IG) in the skin mucus were determined according to the method described in the study by Siwicki and Anderson (1994). Briefly, the total protein content of the skin mucus samples was measured based on the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). Thereafter, the IG molecules were precipitated using 12% solution of polyethylene glycol and were incubated under constant agitation at room temperature for 2 hr, and then, the total protein content was analysed. The total IG of the skin mucus samples was calculated based on the difference in protein content measured before and after the precipitation.

The skin mucus alternative complement pathway haemolytic activity (ACH_{50}) was evaluated according to the method proposed by Yano (Yano, 1992). Briefly, a range of diluted skin mucus samples from 50 to 250 µl was prepared and was distributed into test tubes. A mixture of the barbitone buffer, ethylene glycol-bis(2-aminoethoxy)-tetraacetic acid (EGTA) and Mg^{2+} was added to make up the total volume of 250 µl. Then, 100 µl of New Zealand rabbit red blood cells (RaRBC) was added to each tube and was incubated in a shaker incubator at 20°C for 90 min. 3.15 ml of NaCl was added to each

tube and then was centrifuged at 1,600 × *g* for 5 min to remove the unlysed RaRBC. The supernatant was read at 414 nm. The number of ACH_{50} units/ml was calculated based on the skin mucus volume, producing 50% haemolysis (ACH_{50}).

Alkaline phosphatase activity was assayed in the skin mucus using a commercial kit (Pars Azmun Company, Iran). The optical density (OD) was read at 405 nm using a spectrophotometer (HACH DR/4000, USA).

2.6 | Mucus antibacterial test

Two pathogenic bacteria including *Aeromonas hydrophila* (ATCC 7966) and *Streptococcus iniae* (ATCC 29178) were used to evaluate the antibacterial activity of the skin mucus based on the standardized single disc method (Bauer, Kirby, Sherris, & Turck, 1966). Skin mucus samples were collected as described in the previous section. For this experiment, bacteria were cultured in the nutrient broth medium (Merck, Germany) and was incubated in a shaking incubator at 200 rpm (JSSI-200CI; JSR, Gongju City, Korea) at 37 °C for 24 hr. After the incubation, aliquots (100 µl) of bacterial suspension (1.5 × 10^8 CFU/ml) were cultured on nutrient agar (Merck, Germany). Then, paper discs (6 mm diameter) were impregnated with 150 ul of the mucus sample for 20 min and were placed over solidified agar gel and then were incubated at 37°C 4 hr. Eventually, the diameter of the growth inhibition zone created around the paper disc was measured using a digital calliper (Zone Guilin, China).

2.7 | Analysis of acetic acid in ACV sample

In order to measure the concentration of acetic acid in ACV sample, 4 ml of ACV was mixed with 1 ml of meta-phosphoric acid (25%) and was centrifuged at 2,800 *g* for 5 min at 4°C. The supernatant was analysed by GC-FID (Varian, Model CP-3800) using a column Teknokroma TRB-FFAP with the following dimensions: 30m × 0.32mm × 0.5µm. Helium was used as carrier gas, inlet temperature was equal to 220°C, oven temperature was raised from 100°C to 160°C at a rate of 5C/min (held for 2 min), and detector temperature was set at 250°C. Acetic acid standards (Sigma, USA) with concentrations of 5,000, 2,500, 1,250 and 625 ppm were employed for preparation of the calibration curves (Erwin, Marco, & Emery, 1961; Mojtahedi & Mesgaran, 2011).

2.8 | Statistical analysis

The data were expressed as mean ± Standard Deviation (*SD*). Statistical analysis was conducted using SPSS software (version, 19, IBM SPSS, Armonk, NY, USA), and the *p*-value of <0.05 was considered as statistically significant. Normality assumption was determined using the Kolmogorov–Smirnov test. The significant differences between the means were analysed using one-way analysis of variance (ANOVA) followed by Duncan's new multiple range test.

3 | RESULTS

3.1 | Growth performance

Both the growth performance and feed utilization of green terror fingerlings were influenced by different levels of dietary ACV after 63 days of feeding trial (Table 1). The results showed no significant difference in the initial weight of fish between the experimental groups ($p > .05$). The final weight and weight gain values in fish fed with 2% of dietary ACV were significantly higher than the control group ($p < .05$), whereas these values did not significantly increase in fish fed with 1% and 4% of ACV diets compared to the control ($p > .05$). There was a significant difference in the specific growth rate (SGR %) between fish fed with diet supplemented with 4% ACV and the control group ($p > .05$).

The liver index (HIS) showed no significant difference between fish fed with ACV diets and the control group ($p > .05$). There was a significant difference in the Visceral Index (VIS) of fish fed with dietary ACV and controls ($p < .05$). The VIS value significantly decreased in fish fed with 2% of dietary ACV compared to the controls ($p < .05$). The highest value of VIS was recorded in fish fed with 1% of ACV diets and control group ($p < .05$).

TABLE 1 Growth performance parameters of green terror (*Andinoacara rivulatus*) fingerling fed different levels of dietary apple cider vinegar (ACV) for 63 days (Mean ± *SD*, *n* = 3)

	Dietary apple cider vinegar (ACV) levels (%)			
	0 (Control)	1	2	4
Initial weight (*g*)	6.91 ± 0.31^{a}	7.29 ± 0.17^{a}	7.06 ± 0.34^{a}	7.08 ± 0.40^{a}
Final weight (*g*)	11.61 ± 0.85^{a}	11.75 ± 0.21^{a}	12.38 ± 0.78^{ab}	13.04 ± 0.51^{b}
Weight gain (*g*)	4.70 ± 1.14^{a}	4.46 ± 0.06^{a}	5.32 ± 0.86^{ab}	5.96 ± 0.30^{b}
Specific growth rate (% BW/day)	0.82 ± 0.18^{a}	0.75 ± 0.01^{a}	0.89 ± 0.13^{a}	0.97 ± 0.05^{a}
FCR	2.50 ± 0.13^{b}	2.42 ± 0.075^{b}	2.29 ± 0.15^{ab}	2.14 ± 0.11^{a}
HSI (%)	1.38 ± 0.25^{a}	1.50 ± 0.48^{a}	1.04 ± 0.48^{a}	1.46 ± 0.82^{a}
VSI (%)	2.45 ± 0.10^{bc}	2.51 ± 0.19^{c}	2.11 ± 0.18^{a}	2.14 ± 0.17^{ab}

Note: Means with different letters in the same row are significantly different (ANOVA, $p < .05$).

3.2 | Digestive enzyme activities

The variations in the levels of digestive enzyme activities (U/g intestine per treatment) for green terror fingerlings fed with different levels of ACV are shown in Table 2. The total protein content, protease, α-amylase, lipase and alkaline phosphatase specific activities (U/mg protein) of fish fed with dietary ACV significantly increased compared to the control group ($p < .05$). The total protein content and the protease specific activity increased by increasing ACV percentage in the diet ($p < .05$). The highest α-amylase specific activity was observed in 2% of ACV, followed by 4% of ACV diets ($p < .05$). The lipase and alkaline phosphatase specific activities were significantly higher in fish fed with 2% of dietary ACV compared to other groups ($p < .05$).

3.3 | Skin mucus immune parameters

The effects of different levels of dietary ACV on the skin mucus immune parameters for green terror fingerlings are shown in Figure 1a–d. The total protein content, alkaline phosphatase, total IG, lysozyme and ACH_{50} activities significantly increased in fish fed with ACV diets compared to controls ($p < .05$). The highest level of these immune parameters was observed in the group fed with 2% of ACV diet, followed by the group fed with 4% of ACV diet ($p < .05$).

3.4 | Skin mucus antibacterial activity

Figure 2 shows the antibacterial activity of the skin mucus for green terror (*A. rivulatus*) against 2 pathogenic bacteria, *A. hydrophila* and *S. iniae*. The antibacterial activity of the skin mucus obtained from fish fed with dietary ACV was significantly higher than the control group ($p < .05$). The highest antibacterial activity of the skin mucus against *A. hydrophila* was observed in fish fed with 2% of dietary ACV ($p < .05$). In the case of *S. iniae*, the skin mucus of fish fed with 2% and 4% of ACV diets had higher antibacterial activity compared to other groups ($p < .05$).

3.5 | The concentration of acetic acid in ACV samples

A linear response was obtained in the interval between 5,000 and 625 ppm ($R^2 = 0.996207$). The results obtained from GC-FID showed that the concentration of acetic acid in ACV samples was equal to 40.830 ppm.

4 | DISCUSSION

The effects of different organic acids and their salts on the activity of digestive enzymes in fish and shellfish species have been evaluated only in few studies. Castillo et al. (Castillo, Rosales, Pohlenz, & Gatlin, 2014) reported that dietary calcium lactate, citric acid and potassium diformate increased the specific activities of the pancreatic (trypsin and lipase) and intestinal (leucine aminopeptidase and phosphatase) enzymes in juvenile red drum (*Sciaenops ocellatus*). Li et al. (Li, Li, & Wu, 2009) also demonstrated a significant increase in the protease specific activity of tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) fed with 10 g/kg (1%) of citric acid. They also found that the activity of amylase in hepatopancreas and gut increased because of changes in the gut pH induced by citric acid and subsequently the release of cholecystokinin and exocrine secretions of the pancreas. In contrast, Su et al. (Su et al., 2014) showed that the intestinal amylase activity of the white shrimp fed with 2 g/kg (2%) of citric acid had no significant difference compared to the control group. Silva et al. (Silva et al., 2016) also reported that the activities of trypsin and chymotrypsin enzymes decreased in white shrimp fed with diets supplemented by sodium lactate or citrate. The results of the current study revealed that dietary ACV significantly increased the activity of the intestinal digestive enzymes including protease, α-amylase, lipase and alkaline phosphatase. The highest incremental effect on the measured intestinal enzyme activities (protease, α-amylase, lipase and alkaline phosphatase) was observed in the green terror (*A. rivulatus*) fingerling fed with 2% and 4% of the dietary ACV. To the best of our knowledge, there is not sufficient evidence on the effect of dietary ACV on the digestive enzyme activities but some researchers believe that decrease in intestinal pH (Lückstädt 2008), increase in the number of beneficial gut bacteria (Ahmadnia Motlagh et al., 2019) and gut morphology changes (Hamer et al. 2008; Gao et al. 2011) can increase the activity of digestive enzymes.

Recently, some studies have investigated the effects of dietary organic acids and their salts on growth performance in different commercial fish and shellfish species (Castillo et al., 2014; Hoseinifar et al., 2016; Pourmozaffar et al., 2017; Su et al., 2014). The positive

TABLE 2 Digestive enzyme activity (U/mg protein/min) of green terror (*Andinoacara rivulatus*) fingerling fed different levels of dietary apple cider vinegar (ACV) for 63 days (Mean ± *SD*, $n = 3$)

	Dietary apple cider vinegar (ACV) levels (%)			
	0 (Control)	1	2	4
Total protein	1.24 ± 0.015^a	1.33 ± 0.010^b	1.48 ± 0.057^c	1.62 ± 0.066^d
Protease	0.54 ± 0.026^a	0.79 ± 0.010^b	1.17 ± 0.025^c	1.33 ± 0.010^d
α-amylase	0.17 ± 0.020^a	0.47 ± 0.020^b	0.54 ± 0.021^c	0.55 ± 0.026^c
Lipase	0.87 ± 0.015^a	0.96 ± 0.015^b	1.34 ± 0.011^d	1.09 ± 0.015^c
Alkaline phosphatase	0.11 ± 0.025^a	0.23 ± 0.020^b	0.38 ± 0.030^c	0.26 ± 0.026^b

Note: Means with different letters in the same row are significantly different (ANOVA, $p < .05$).

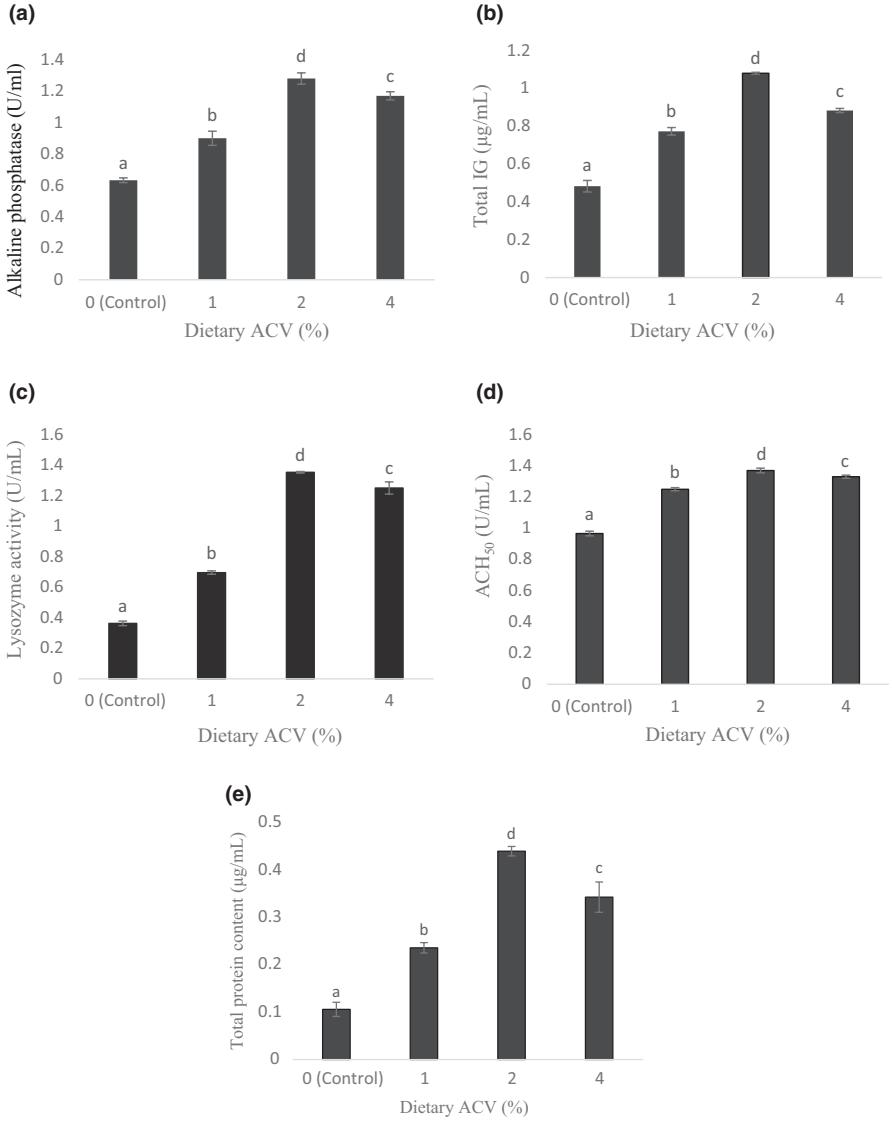

FIGURE 1 Alkaline Phosphatase (U/ml) (a), total immunoglobulin (IG) (µg/ml) (b), lysozyme (µg/ml) (c), alternative haemolytic complement activity (ACH50) (U/ml) (d) and total protein (µg/ml) (e) levels in the skin mucus of green terror (*Andinoacara rivulatus*) fingerling fed different levels of dietary apple cider vinegar (ACV) for 63 days (Mean ± SD). Bars with different letters are significantly different ($p < .05$)

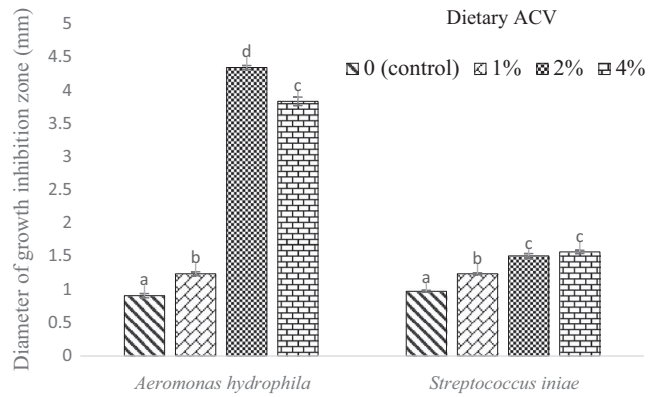

FIGURE 2 Diameter of the growth inhibition zone (mm) created around the paper disc inoculated with skin mucus of fish (*Andinoacara rivulatus*) fed on different levels of dietary apple cider vinegar (ACV) for 63 days against two bacterial strains, *Aeromonas hydrophila* and *Streptococcus iniae* (Mean ± *SD*, $p < .05$). Bars with different letters in each bacterial strain are significantly different ($p < .05$)

effect of organic acids on growth performance of aquatic animals is attributed to the changes in population and composition of gastrointestinal microbiota, improvement of gastrointestinal morphology, and enhancement of nutrient digestibility and digestive enzyme activity (Ng & Koh, 2016; Scheppach, 1994). For example, supplementation of diet with organic acid blend (OAB) (containing 10 g/kg (1%) of acetic acid + 10 g/kg (1%) of formic acid, 10 g/kg (1%) of benzoic acid + 10 g/kg (1%) of sorbic acid and 10 g/kg (1%) of Na-benzoate + 10 g/kg (1%) of K-sorbate) improved growth performance in South African abalone (*Haliotis midae*) but had no significant effect on FCR and feed intake (Goosen, Görgens, De Wet, & Chenia, 2011). Baruah et al. (Baruah et al., 2007) also showed that growth performance, FCR and *p* availability significantly improved in rohu (*Labeo rohita*) fed with a diet supplemented using 30 g/kg (3%) of citric acid. However, another study demonstrated that feeding white shrimp, *Litopenaeus vannamei* (10–14 g), with diets supplemented with 1%, 2% and 4% of ACV had no significant effect on growth performance (Pourmozaffar et al., 2017). The results of the present study revealed

that the growth performance parameters, including final weight and weight gain, significantly increased in fish fed with 2% of ACV compared to the controls, whereas SGR (%) and FCR showed significant differences in fish fed with 4% of ACV compared to the controls. Safari et al. (Safari, Hoseinifar, Nejadmoghadam, & Khalili, 2017) showed that the expression of growth hormone (GH) gene was up-regulated in the common carp (*C. carpio*) fed with diets supplemented with 1% and 2% of ACV. Furthermore, the enhancement of growth performance in fish fed with dietary ACV may be due to the existence of acetic acid in ACV, which may increase the growth of lactic acid bacteria (Najdegerami et al., 2015).

ACV contains polyphenols and organic acids such as acetic acid, citric acid, formic acid, lactic acid, malic acid and succinic acid (Morgan & Mosawy, 2016). These constituents have been reported to have immunomodulatory effects (Pourmozaffar et al., 2017; Safari et al., 2016) by adjusting the innate immune response through binding to G protein-coupled receptor (mainly expressed on inflammatory cells) (Maslowski & Mackay, 2010). In the present study, the evaluation of the skin mucus immune parameters revealed that dietary ACV influenced total protein content, alkaline phosphatase, total IG, lysozyme and ACH_{50} activities. The highest immune response was observed in fish fed with 2% of dietary ACV. According to the review of the literature, there is no enough available information on the effects of apple cider vinegar on the immune parameters of fish skin mucus. The results of a study showed a significant increase in the total IG and lysozyme activities in the serum of the common carp (*C. carpio*) fed with diet supplemented with high (2%) and low (1%) levels of ACV (Safari et al., 2017). Furthermore, modulatory effects of organic acids on the immune response of the skin mucus have been reported for the zebrafish, *Danio rerio* (Hoseinifar, Safari, & Dadar, 2017); Caspian white fish, *Rutilus frisii kutum* (Hoseinifar et al., 2016); and Nile tilapia, *Oreochromis niloticus* (Reda, Mahmoud, Selim, & El-Araby, 2016).

Apple cider vinegar exerts its antimicrobial activity by limiting the microbial growth and inhibiting mononuclear cytokine and phagocytic activities. Antimicrobial mechanisms of ACV include destroying cell integrity, structural proteins, metabolic enzymes and nuclear compounds (Yagnik, Serafin, & Shah, 2018). The results of a study revealed that ACV has these antimicrobial properties against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* (Yagnik et al., 2018). In the present study, the skin mucus samples obtained from fish fed with different levels of dietary ACV had antibacterial activity against *A. hydrophila* and *S. iniae*. The highest antibacterial activity against *A. hydrophila* was observed in fish fed with 2% of ACV, while the elevation of ACV level to 4% in fish diet resulted in a significant reduction in antibacterial activity of the skin mucus. For *S. iniae*, the same results were observed in fish fed with 2% and 4% of ACV diets. Yagnik et al. (Yagnik et al., 2018) showed that, based on the width of inhibition zone, the antimicrobial activity of ACV against methicillin-resistant *Staphylococcus aureus* (MRSA) and resistant *E. coli* varied in a dose-dependent manner. For MRSA, the highest antibacterial effect was observed in 100 mg/ml of ACV, whereas the concentration of 200 mg/ml was more effective against resistant *E. coli*.

5 | CONCLUSIONS

The results of the present study showed that dietary ACV had beneficial effects on the immune response (alkaline phosphatase, total IG, lysozyme, ACH50 activities and the antibacterial activity of the skin mucus) and growth performance (final weight, weight gain and SGR), as well as digestive enzyme (protease, α-amylase, lipase and alkaline phosphatase) activities in the green terror (*A. rivulatus*) fingerling. Immune parameters (alkaline phosphatase, total IG, lysozyme and ACH50 activities) and antibacterial activity of the skin mucus improved particularly in fish fed with 2% and 4% of ACV diets. The highest digestive enzyme activities and the best growth performance were also observed in fish fed with 2% and 4% of ACV diets. Based on these results, the administration of ACV as an immunostimulant can be considered in aquaculture, although further studies are required to determine a fully safe concentration of ACV for this species.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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