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Ascorbic acid enhances the treatment efficacy of tetracycline against experimentally infected *Aeromonas hydrophila*

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ABSTRACT: Combinations of two or more pharmaceuticals are sometimes used to achieve efficient treatment of an ailment. The objectives of this study were to evaluate the synergistic action of ascorbic acid combined with tetracycline in the treatment of *Aeromonas hydrophila* infection. A total of 210 apparently healthy 2-month-old uniformly sized *Clarias gariepinus* with a mean weight of 24.65±2.78 g were used in the study. The fish were randomly assigned to seven treatment groups (A, B, C, D, E, F and G), each in triplicate. The fish in group A were the uninfected controls, while those in groups B to G were infected with the bacterium and were either treated or not treated with tetracycline, and their diets were either supplemented with ascorbic acid or not. Growth, survival, hematological parameters, serum chemistry responses were investigated as bioindicators of health status. Infected fish deprived of L-ascorbic acid presented lower growth and survival rates. Hemorrhages, skin and fin erosions and skeletal deformities were some of the lesions observed. There were significantly ($P<0.05$) lower red blood cell counts and hemoglobin; significantly higher ($P<0.05$) total leucocyte counts; total protein, globulin and albumin; and elevated A/G ratios, also significantly higher alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and urea levels when compared with the group without ascorbic acid supplementation. This study suggested that ascorbic acid supplementation at 100 and 200 mg/kg is recommended in the diet of *C. gariepinus* to potentiate the action of tetracycline in the treatment of *A. hydrophila* infection.

Keyword: Antibiotics; condition factor; hematology; septcaemia; serum biochemistry.

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INTRODUCTION

African sharp toothed catfish (*Clarias gariepinus*) is a species of catfish of the family *Clariidae* and is an air-breathing catfish (Weyl *et al*., 2016). It is found mostly in Africa and some parts of the Middle East. *Clarias gariepinus* has a high fecundity rate, grows fast, and tolerates high stocking density and environmental extremes (Dunham and Elaswad, 2018). The economic viability of catfish culture depends on the fine balance between the maximum utilization of inputs and the avoidance of disease conditions (Rodger, 2016). The economic impact of fish loss because of disease outbreaks has been severe in recent years. The prevention and control of the introduction and multiplication of these disease agents are very important for profitable fish culture and as a measure to reduce production losses. Approximately one-third of fishes are lost to diseases, 60% of which are caused by pathogenic microorganisms such as bacteria, fungi and viruses (Peterman and Posadas, 2019). Diseases caused by bacteria are the most common in both wild and cultured fishes. Bacterial diseases, especially those caused by gram-negative organisms, are responsible for mass mortality in both wild and cultured aquatic organisms (Hamid *et al*., 2017). *Aeromonas hydrophila* and other motile aeromonads are among the most common bacteria in freshwater habitats worldwide, and these bacteria frequently cause disease among cultured and feral fishes (Dias *et al*., 2016). *Aeromonas hydrophila* is a gram-negative enterobacterium widely distributed in aquatic environments (Chenia and Duma, 2017). The bacterium causes diseases in fish known as "hemorrhagic septicaemia", "motile Aeromonas septicaemia", "ulcer disease" or "red sore disease", which results in heavy mortalities in farmed and wild fishes (Stratev and Odeyemi, 2017). The disease has caused substantial economic loss to fish farmers and the fisheries sector. Oxytetracycline (terramycin) is a drug used for the control of *A. hydrophila* infections in fishes (Semwal *et al*., 2023). Tetracycline and oxytetracycline are broad spectrum antibiotics and are active against both Gram negative and positive cocci. The drugs are also effective against spirochetes, actinomycetes and even some viruses. They are indicated for the treatment of furunculosis caused by *Aeromonas salmonicida,* haemorrhagic septicaemia caused by *Aeromonas hydrophila* (Pakravan and Akbarzadeh, 2017) and other susceptible bacteria species. Sulfadimethoxine plus or trimethoprim (Romet®) is also approved for controlling *A. hydrophila* infections in fish (Julinta *et al*., 2017). However, the indiscriminate and frequent use of antibiotics in aquaculture as preventive and control measures has been questioned because of the development and spread of antibiotic resistance (Tulaby Dezfuly, 2019). Resistant bacteria transfer their resistance gene (R-plasmid) to other bacteria that have never been exposed to antibiotics, ultimately leading to public health hazards (Ben *et al*., 2019). Studies have shown that fishes, especially those from culture facilities, may acquire resistance to oxytetracycline because of the frequent use of this antibiotic by fish farmers (Omeje *et al*., 2019).

Ascorbic acid is likely the most important vitamin in aquaculture production because it is a powerful antioxidant and helps the immune system of fish (Faramazi, 2012; Khara *et al*., 2016). Ascorbic acid belongs to the water-soluble group of vitamins and has been reported to stimulate immune responses such as natural killer cell activity, lysozyme activity and antibody levels (Khara *et al*., 2016). Gulonolactone oxidase is the enzyme responsible for the synthesis of ascorbic acid, and because many fish species lack the enzyme in their liver and kidney, dietary supplementation with ascorbic acid becomes an essential dietary requirement of fish (Trichet *et al*., 2015). However, there is a paucity of information on the prophylactic effects of the inclusion of ascorbic acid in the diet on the immune status of cultured *C. gariepinus*. This study was designed to establish the possible enhancement of disease resistance by the use of vitamin C in the diet of *Clarias gariepinus* and the possibility of the use of ascorbic acid to potentiate the therapeutic effect of tetracycline in the treatment of *A. hydrophila*-infected *C. gariepinus*.

MATERIALS AND METHODS

Ethics declaration

Ethical approval for the protocols reported in this work was granted by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine University of Nigeria (IACUC, FVM-UNN), with approval number FVMUNNIACUC202403/149. The methods used in this work are reported in line with the ARRIVE guidelines (https://arriveguidelines.org).

Fish and experimental design

Two hundred and ten (210) apparently healthy (were examined thoroughly to ascertain their health status) 2-month-old uniformly sized *Clarias gariepinus* juveniles (with a mean weight of 24.65±2.78 g and

mean total and standard lengths of 14.84±0.52 cm and 13.12±0.28 cm, respectively) were procured from a reliable fish farm in Nsukka, Enugu State. Upon arrival, they were acclimatized for two weeks at the experimental fish ponds of the Department of Veterinary Medicine, University of Nigeria, Nsukka, during which period they were fed a 2 mm compounded fish basal diet provided at a rate of 3% of their body weight twice daily (Sarka and Rahid, 2012). The experiment was carried out in 21 tarpaulin fish ponds constructed with a metallic framework. Each of the ponds had a capacity of 120 L. The 210 African catfish (*C. gariepinus*) used for the study were randomly assigned to seven treatment groups (A, B, C, D, E, F and G) of 30 catfish per group, each in triplicate (30 fish/group, 10/replicate).

Experimental layout

The experimental layout and the inclusion level of the ascorbic acid are as shown in Table 1.

The culture water was changed weekly by siphoning 80–90% of its volume and replacing it with fresh water to ensure adequate oxygenation. Before siphoning the water, the sides of the tarpaulin fish pond were brushed to prevent fungal and algae growth, which may serve as a source of ascorbic acid. Water quality parameters such as dissolved oxygen, temperature and pH were monitored weekly. A digital YSI ProODO instrument (Model: EC300, YSI Inc., Yellow Springs, USA) was used to monitor the disolved oxygen and water temperature while a Crison ICR12502 pH meter fitted with an ICR15053 electrode (HACH, Lange GmbH, Germany) was used to monitor the pH of the culture water. The recorded water quality parameters included dissolved oxygen (5.04 ± 0.62), temperature (25.44 ± 1.38) and pH (6.08 ± 0.12), which are within the tolerable limits for fish culture (Li *et al*., 2022).

Bacterial strains

The *Aeromonas hydrophila* isolates used for the study were isolated from diseased fish samples collected from a fish farm that experienced mortality caused by bacteria in Enugu State, Nigeria. Pure cultures of the organisms were isolated following a method adopted from Bose (2006). Pure cultures of the bacteria isolated were subjected to standard morphological, physiological and biochemical tests. The general methods used for visual inspection of the growth, size, color, shape, elevation, edge characteristics, surface presentation, consistency and translucence of the colonies followed those of Song *et al*. (2017). Standard biochemical assays, such as Gram-staining, citrate, H_2S, methyl red, catalase, oxidase, hydrogen sulfide and indole tests, were performed as described by Cheesebrough (2002) to confirm the bacteria. The isolates were also subjected to polymerase chain reaction (PCR) and molecular studies to confirm the identity of the organism. The isolates were also subjected to antibiotic sensitivity tests to select appropriate antibiotics to be used for the experimental treatment. The characterized *A. hydrophila* was homogenized in sterile phosphate-buffered saline, and the turbidity was adjusted

Table 1. Inclusion of ascorbic acid in the diet of C. gariepinus experimentally infected with Aeromonas hydrophila and treated with tetracycline

S/No	Treatment	Designation	Inclusion level of ascorbic acid and tetracycline
I	Basal diet only (BD)	A	No Inclusion and uninfected and no tetracycline treatment
II	Basal diet only (BDIU)	B	No inclusion but infected and untreated
III	Basal diet only (BDIT)	C	No inclusion but infected and treated
IV	BD+100mg ascorbic acid (BD+100mg IT)	D	100mg ascorbic acid (low level) infected and treated
V	BD+100mg ascorbic acid (BD+100mg IU)	E	100mg ascorbic acid (low level) infected and untreated
VI	BD+200mg ascorbic acid (BD+200mg IT)	F	200mg ascorbic acid (high level) infected and treated
VII	BD+200mg ascorbic acid (BD+200mg IU)	G	200mg ascorbic acid (high level) infected and untreated

*where BD: basal diet, I: infected with *Aeromonas hydrophila*, T: treated with tetracycline, U: untreated

to correspond to 0.5 McFarland's turbidity standard (equivalent to 1×10^8 colony-forming units/mL). The fish in **groups B** to **G** were infected by intraperitoneal injection of 0.2 mL of the bacterial inoculum on day0 of the experimental period.

Ascorbic acid supplementation and dietary inclusion of tetracycline

The basal diet was formulated with readily available ingredients to contain 41.3% crude protein. The proximate composition of the basal diet is shown in Table 2. The diet was supplemented with ascorbyl-2-monophosphate Mg^{2+} (S.D. America, New York, NY). The ascorbic acid was incorporated into the basal diet at the indicated rate for each treatment group according the method adopted from Ibiyo *et al*. (2007). Tetracycline used for the study was manufactured by Alben healthcare IND. LTD, Nigeria. Each capsule contain tetracycline hydrate B.P. equivalent to tetracycline 250mg. The Tetracycline was added at a rate of 750 mg/100 g of the basal diet, as indicated for the treatment group according to method adopted from Lundström *et al*. (2016) and fed for 7 days beginning from day0. After the 7th day, the fish were fed the experimental diet without tetracycline which was continued throughout the experimental period of 6 weeks (42 days). The infected fish were monitored daily for signs of poor health, such as sluggishness, off feed, morbidity, mortality and skin lesions.

Growth

The morphometric parameters, including the total length, standard length and weight, of all the fish in each treatment group and the replicates were recorded on days 0 and 42. The data obtained were used to determine growth performance with reference to the condition factor (K) and specific growth rate (SGR). Additionally, the mortality in each treatment was recorded to calculate the survival rate.

The condition factor, growth rate and mortality rate were determined as follows:

The condition factor (K) of each fish was calculated using the formula

$$K = \frac{100w}{L^3}$$ (Froese, 2006)

Where L = standard length (cm) and W = body weight (g)

The specific growth rate was calculated via the fol-

$$SGR = \frac{L_n w_f - L_n w_i}{T_{(days)}} \; x \; 100$$

(Tran-duy *et al*. 2008)

$$Survival\ rate = \frac{Final\ number\ of\ fish}{Initial\ number\ of\ fish} \; x \; 100$$ ght

Table 2. Ingredients and proximate composition of the basal diet

Ingredients	Inclusion levels (%)
Fish meal	20.00
Soybean meal	62.25
Maize bran	14.75
Vitamin C- free premix	1.00
Vegetable oil	2.00
Proximate composition	
Crude protein %	41.3
Crude fat %	10.85
Crude fiber %	1.5
Ash %	8.25
NFE %	28.5
Moisture content %	9.6

Blood and serum collection for heamatology and biochemistry

The experiment lasted for 42 days (6 weeks). Blood samples were collected from 4 randomly selected fish per replicate for hematology and blood chemistry analyses. Blood samples were collected on day 0 and at the end of the experimental period through the caudal venipuncture technique (Affonso *et al.,* 2002) without anesthesia. The collected blood was put into sample bottles containing 10% ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The blood was gently rocked and used for hematological studies. The erythrocyte count and total white blood count were determined via hemocytometer methods, whereas the packed cell volume (PCV) was determined via the microheamatocrit method (Docan *et al*., 2018). The Hb concentration was determined via the cyanometmoglobin method (Witeska *et al*., 2022). Erythrocyte indices such as the mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular volume (MCV) were calculated via the standard formula described by Witeska *et al*. (2022):

$$MCH = \frac{Hb\ x\ 10}{RBC\ (per\ mm^3)}$$

$$MCV = \frac{PCV(\%)\ x\ 10}{RBC\ (per\ mm^3)}$$

$$MCHC = \frac{Hb\ x\ 100}{PCV}$$

Blood for serum biochemistry parameter determination was obtained by collecting 1.5–2.0 mL of blood into sample bottles, into which no anticoagulant was added. The bottles were placed in slanting positions for 2–3 hours to allow the blood to clot. The bottles containing clotted blood were placed in a bench centrifuge and centrifuged for 5 minutes at 5,000 rpm. The serum was then gently decanted into Eppendorf tubes and stored at -20°C until it was used for serum biochemical analysis. Serum biochemical assays were carried out using test kits. The following serum biochemistry parameters were measured: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein, albumin, serum urea nitrogen and creatinine. Serum AST and ALT levels were determined via the standard colorimetric method of Huang *et al*. (2006). This was an in vitro method of determining AST and ALT levels via Randox Glutamic-Oxaloacetic transaminase test kits and Randox Glutamic-Pyruvic transaminase test kits, respectively. Serum alkaline phosphatase levels were determined via the phenolphthalein monophosphate method, an in vitro method of determining ALP levels via a Quimica Clinica test kit. Serum urea and creatinine levels were determined via Randox urea and creatinine colorimetric kits, respectively (Stoskopf, 1993). The total protein content was determined via the direct biuret method (Chaijan and Undeland, 2015), whereas the albumin content was determined via the bromocresol green method (Garcia Moreira *et al*., 2018). Globulin was obtained from the difference between total protein and albumin concentrations. The globulin concentration (g/dL) = total protein – albumin.

Data analysis

Data on body weight variations, condition factor, specific growth rate, hematology and serum biochemistry among the seven experimental groups were compared using two way analysis of variance (ANOVA). The ANOVA was carried out using the IBM Statistical package for social sciences (SPSS version 21.0). Variant means were separated using Duncan Multiple range test. The significant difference were accepted at (P<0.05)

RESULTS

Physical changes

On the 5th day post infection, some of the infected fish were off feed, and by the evening of the 8th day, mortality, especially among the fish in Treatment B, which were infected with the bacterium but received neither antimicrobial treatment nor ascorbic acid supplementation in the basal diet, was observed. Mortality continued to increase gradually and then regressed. The last mortality was recorded on the 22nd day post infection, although some fish with signs of disease were observed on day 42, when the experiment was terminated. Hemorrhages on the body surface, ulcers of various sizes on the skin, fin erosions, inflamed vents, abdominal distension, exophthalmia and skeletal deformity are the signs observed among the infected and untreated fish, as shown in Fig. 1.

Morphometric parameters

The morphometric parameters measured on the day of stocking (day 0 post infection) were not significantly different (Table 3).

However, the results of the morphometric parameters measured on the 42nd day post infection revealed that the standard length and weight of the fish in treatment group B were significantly (P<0.05) lower than those of the control (group A). The total length and Fulton's condition factor of group B were also lower than those of group A, although the differences were not statistically significant (P>0.05). The specific growth rate (SGR) and survival rate of group B were equally lower than those of the control group. Whereas the group A (control), groups D and F that were treated with tetracycline and diet supplemented with 100g and 200g of ascorbic acid respectively recorded 100% survival, group B that was not treated with tetracycline nor the diet supplemented with ascorbic acid had 56.7% survival

Figure 1. Skeletal deformity among *Clarias gariepinus* infected with *Aeromonas hydrophila* that were not treated.

Table 3. Morphometric parameters (mean ± SE) of *Clarias gariepinus* at the start of the experimental infection with *A. hydrophila* and treatment with tetracycline supplemented with ascorbic acid

Treatment	Total length (cm)	Standard length (cm)	Weight (g)	Condition factor
A (control)	14.35 ±0.43	12.36 ±0.43	23.41 ±1.73	1.13 ±0.05
B	14.36 ±0.48	13.26 ±0.66	23.52 ±2.00	1.05 ±0.08
C	15.06 ±0.51	13.32 ±0.39	25.17 ±1.98	1.05±0.03
D	15.07 ±0.58	13.27 ±0.52	24.62 ±2.58	1.02±0.03
E	14.78 ±0.72	13.14 ±0.66	25.53±3.51	1.06 ±0.03
F	15.16 ±0.66	13.30±0.51	25.27±3.24	1.07±0.03
G	15.10 ±0.33	13.21 ±0.27	25.00 ±1.75	1.07±0.03

Treatment A = 0 mg of ascorbic acid /kg of basal diet uninfected; **B**= 0 mg of ascorbic acid /kg of basal diet infected and untreated; Treatment **C**= 0 mg of ascorbic acid /kg of basal diet infected and treated with tetracycline; **D** = 100 mg of ascorbic acid /kg of basal diet infected and treated with tetracycline; **E**=100 mg of ascorbic acid /kg of basal diet infected and untreated; **F** = 200 mg of ascorbic acid /kg of basal diet infected and treated with tetracycline; **G** = 200 mg of ascorbic acid /kg of basal diet infected and untreated.

rate. Also whereas the group that their diet were supplemented with ascorbic acid had 100% survival, group C that was treated with tetracycline without inclusion of ascorbic acid recorded 86.7% survival rate. However, there was a high survival rate in all the treatment groups except group B, whose survival rate was 56.67%, as shown in Table 3. Infected fish deprived of ascorbic acid presented reduced feed intake. The total length, standard length, weight and Fulton's condition factor of group C were lower than those of groups D and F; however, the differences were not statistically significant (P>0.05). The specific growth rate of group C was also lower than those of groups D and F. Additionally, whereas groups D and F presented 100% survival, group C presented an 86.67% survival rate, as shown in Table 4. It seems that % of survival is the index which differentiates clearly the various treatments among them. In treatments D & F the inclusion level of Ascorbic acid (100 / 200mg) seems to have NO differential effect on length, weight, Condition Factor or SGR, but only on survival, which however was equally high among treatments. On the contrary, in treatments E & G (which have not received antibiotic therapy), better survival has been recorded in the treatment G with the high level of Ascorbic acid (200mg) which clearly indicates the beneficial effect of Ascorbic acid.

Hematological parameters

The red blood cell (RBC) counts, white blood cell (WBC) counts, packed cell volume (PCV), hemoglobin concentration (Hb), and derived erythrocyte indices (MCV, MCH and MCHC) of the different treatment groups did not differ significantly (P>0.05) at the start of the experiment (day 0 pi), as presented in Table 5. However, the hematological parameters assayed on day 42 post infection revealed that the red blood cell count and hemoglobin concentration of the control group (group A) were significantly greater than those of group B, which were experimentally infected with bacteria and were neither treated with

Table 4. Morphometric parameters (mean ± SE) of *Clarias gariepinus* infected with *A. hydrophila* and treated with tetracycline supplemented with ascorbic acid at the end of the experimental period of 42 days

Treatment	Total length (cm)	Standard length (cm)	Weight (g)	Condition factor	SGR	Survival rate %
A (control)	20.13 ±0.31^{a,b}	18.58 ±0.33^{a}	68.56±3.91^{a}	1.06 ±0.04^{a,b}	3.09	100
B	18.74 ±0.45^{b}	16.92 ±0.42^{b}	50.30±4.12^{b}	1.01 ±0.03^{b}	2.17	56.67 (13)
C	19.19 ±0.70^{a,b}	17.45 ±0.64^{a,b}	60.28 ±6.66^{a,b}	1.08 ±0.02^{a,b}	2.51	86.67 (4)
D	20.78 ±0.60^{a}	18.76 ±0.54^{a}	73.21 ±6.48^{a}	1.07 ±0.03^{a,b}	3.11	100
E	20.29 ±0.84^{a,b}	18.35 ±0.78^{a,b}	71.94 ±8.02^{a}	1.11 ±0.05^{a}	2.97	80 (6)
F	20.97 ±0.60^{a}	18.90 ±0.52^{a}	74.51 ±5.54^{a}	1.08 ±0.0.02^{a,b}	2.97	100
G	19.45 ±0.73^{a,b}	17.69±0.70^{a,b}	63.92 ±6.03^{a,b}	1.10 ±0.03^{a,b}	2.69	90 (3)

$^{a, b}$ Columns with different superscripts differ significantly (P< 0.05)

Table 5. Hematological parameters (mean±SE) of *Clarias gariepinus* fed ascorbic acid -supplemented diet, infected with *Aeromonas hydrophila* and treated with tetracycline on day 0.

Parameter	Grp A	Grp B	Grp C	Grp D	Grp E	Grp F	Grp G
RBC (10^{12}/L)	2.09±0.08	2.11±0.09	2.11±0.06	2.16±0.12	2.09±0.08	2.07±0.04	2.05±0.03
HGB (g/dL)	8.70±0.19	8.85±0.40	8.55±0.23	8.15±0.17	8.90±0.29	8.90±0.17	8.48±0.27
HCT (%)	23.25±0.85	22.75±0.85	22.75±0.63	23.25±1.11	24.75±0.85	23.75±0.48	23.50±1.04
MCV (fL)	111.52±2.93	108.39±5.38	107.97±1.34	108.20±4.97	118.41±2.52	115.15±4.66	114.93±5.01
MCH (pg)	41.91±2.13	42.05±1.45	40.75±2.24	40.85±1.37	42.70±2.01	43.05±0.09	41.44±1.19
MCHC (g/dL)	37.60±1.75	38.89±0.82	37.76±2.10	35.21±1.16	36.14±1.99	37.56±1.42	36.13±0.59
WBC (10^9/L)	1.55±0.12	1.52±0.11	1.58±0.02	1.53±0.14	1.52±0.11	1.56±0.16	1.57±0.12

antibiotics nor given a diet supplemented with ascorbic acid. The packed cell volume of group B was also lower than those of the other treatment groups. The erythrocyte indices (MCV, MCH, MCHC) were not significantly different (P>0.05) among the different treatment groups. The white blood cell counts of group B were significantly greater than those of the control group.

The effects of ascorbic acid supplementation on the hematological parameters indicated that the RBC of group C was lower than those of groups D and F, although the differences were not statistically significant (P>0.05). Furthermore, the hemoglobin level of group C was significantly (P<0.05) lower than that of group D but not that of group F, as shown in Table 6. The WBC counts of group C were greater than those of groups D and F, although the differences were not statistically significant (P>0.05). In a related experiment, Oluyemi (2021) reported significantly higher red blood cell count among *Clarias gariepinus* fed diet supplemented with ascorbic acid when compared with the control whose diet were devoid of ascorbic acid. However, Adewolu and Aro (2009) recorded non significant differences in RBC, WBC, PCV and haemoglobin concentration of *Clarias gariepinus* fed diet containing Vitamin C.

Serum biochemical parameters

The results of the serum biochemistry assay at the end of the experimental period (42 days pi) revealed that group B infected with *A. hydrophila* and not treated with antibiotics or a diet supplemented with

Table 6. Hematological parameters (mean±SE) of *Clarias gariepinus* fed a ascorbic acid -supplemented diet, infected with *Aeromonas hydrophila* and treated with tetracycline on day 42 post infection.

Parameter	Group A	Group B	Group C	Group D	Group E	Group F	Group G
RBC (10^{12}/L)	2.20 ± 0.12^{a}	1.87 ± 0.13^{b}	$2.00\pm0.19^{a,b}$	2.19 ± 0.09^{a}	$2.03\pm0.07^{a,b}$	$2.11\pm0.05^{a,b}$	$2.07\pm0.05^{a,b}$
HGB (g/dL)	9.00 ± 0.15^{a}	$7.80\pm0.22^{b,c}$	8.03 ± 0.41^{b}	8.85 ± 0.41^{a}	$8.23\pm0.16^{b,c}$	$8.45\pm0.22^{a,b,c}$	8.20 ± 0.11^{b}
HCT (%)	$24.25\pm1.38^{a,b}$	22.00 ± 0.41^{b}	$23.50\pm1.32^{a,b}$	25.25 ± 0.48^{a}	$23.25\pm1.03^{a,b}$	$24.00\pm0.58^{a,b}$	$23.75\pm0.85^{a,b}$
MCV (fL)	110.54±2.49	118.97±7.05	119.78±7.24	115.53±2.89	114.41±3.60	114.41±5.44	114.84±4.28
MCH (pg)	41.41±2.77	42.14±2.40	41.22±3.70	40.54±2.27	40.54±0.85	40.24±1.65	39.65±0.65
MCHC (g/dL)	37.45±2.23	35.45±0.65	34.40±2.21	35.09±1.76	35.55±1.40	35.30±1.49	34.62±0.98
WBC (10^9/L)	1.65 ± 0.07^{b}	2.01 ± 0.03^{a}	$1.87\pm0.0.10^{a,b}$	1.73 ± 0.13^{b}	$1.80\pm0.05^{a,b}$	1.72 ± 0.15^{b}	$1.84\pm0.07^{a,b}$

[a, b] Rows with different superscripts differ significantly (P< 0.05)

ascorbic acid had a significantly (P<0.05) lower concentration of total protein than did group F, which was infected and treated with antibiotics and a diet supplemented with 200 mg/kg ascorbic acid. Additionally, the albumin concentration of group B was significantly (P<0.05) lower than that of group D, which was infected with *A. hydrophila* and treated with a diet supplemented with 100 mg/kg ascorbic acid. The liver function test results revealed that the globulin concentrations of group B were also significantly (P<0.05) lower than those of group A (control) and group F. Furthermore, the albumin/globulin ratio of group B was significantly (P<0.05) elevated compared with those of groups A and F. The liver function test results revealed that there were significantly (P<0.05) elevated AST and ALT levels in group B compared with those of the control group (group A). ALP in group B was significantly (P<0.05) elevated compared with that in groups A, D and F. Kidney function test results also revealed significantly elevated urea concentrations in the serum of groups B and C compared with those in groups A and D. The results also revealed significantly higher creatinine levels in groups B and C than in group D, as shown in Table 7.

Table 7. Serum biochemical profile (mean±SE) of *Clarias gariepinus* fed a ascorbic acid -supplemented diet, infected with *Aeromonas hydrophila* and treated with tetracycline on day 42 post infection

Parameter	Group A	Group B	Group C	Group D	Group E	Group F	Group G
T. protein(g/dl)	$2.73\pm0.24^{a,b}$	2.17 ± 0.11^{a}	$2.53\pm0.20^{a,b}$	2.83 ± 0.10^{b}	$2.53\pm0.09^{a,b}$	2.78 ± 0.06^{b}	$2.53\pm0.05^{a,b}$
Albumin(g/dl)	$1.55\pm0.12^{a,b}$	1.45 ± 0.07^{a}	$1.60\pm0.17^{a,b}$	1.78 ± 0.08^{b}	$1.58\pm0.11^{a,b}$	$1.60\pm0.15^{a,b}$	$1.60\pm0.04^{a,b}$
Globulin(g/dl)	1.18 ± 0.16^{b}	0.72 ± 0.05^{a}	0.93 ± 0.03^{a}	$1.05\pm0.15^{a,b}$	$0.95\pm0.09^{a,b}$	1.18 ± 0.17^{b}	$0.93\pm0.03^{a,b}$
Alb/Glo ratio	1.38 ± 0.18^{a}	2.01 ± 0.02^{b}	$1.72\pm0.14^{a,b}$	$1.79\pm0.14^{a,b}$	$1.72\pm0.26^{a,b}$	1.50 ± 0.31^{a}	$1.73\pm0.06^{a,b}$
AST (IU/L)	18.25 ± 2.02^{a}	21.75 ± 0.48^{b}	$20.25\pm1.11^{a,b}$	$19.50\pm0.87^{a,b}$	$20.75\pm2.32^{a,b}$	$19.50\pm0.65^{a,b}$	$20.5\pm0.96^{a,b}$
ALT (IU/L)	9.25 ± 0.75^{b}	11.25 ± 0.48^{a}	$11.00\pm0.71^{a,b}$	$9.75\pm0.63^{a,b}$	$9.75\pm0.63^{a,b}$	$9.50\pm0.65^{a,b}$	$9.75\pm0.48^{a,b}$
ALP (IU/L)	4.50 ± 1.30^{b}	8.95 ± 1.31^{a}	$6.90\pm0.81^{a,b}$	4.48 ± 0.65^{b}	$5.53\pm1.12^{a,b}$	4.98 ± 0.69^{b}	$5.03\pm0.33^{a,b}$
Urea (mg/dL)	8.45 ± 2.31^{b}	12.13 ± 0.48^{a}	12.30 ± 1.37^{a}	8.45 ± 0.43^{b}	$10.20\pm1.16^{a,b}$	$9.53\pm0.25^{a,b}$	$9.28\pm1.03^{a,b}$
Creatinine (mg/dL)	$0.14\pm0.02^{a,b}$	0.19 ± 0.03^{a}	0.18 ± 0.05^{a}	0.10 ± 0.01^{b}	$0.15\pm0.01^{a,b}$	$0.14\pm0.01^{a,b}$	$0.14\pm0.02^{a,b}$

^{a,b}Rows with different superscripts differ significantly (P < 0.05)

DISCUSSION

Aeromonas hydrophila and other motile aeromonads are among the most common bacteria in freshwater habitats worldwide, and these bacteria frequently cause disease among cultured and feral fishes (Zhang *et al*., 2016). Vitamin supplementation in fish feeds is a strategy to improve immune responses against a myriad of pathogenic microorganisms that the fish are in contact with regularly. Among vitamins, ascorbic acid supplementation has been credited with an improved immune response and resistance to diseases (Khara *et al*., 2016). Fish do not synthesize ascorbic acid and therefore have to be supplied in their diet for optimal growth, development and disease resistance (Youssef *et al*., 2021). It is also an alternative to antibiotic use, which, in the long run, can promote the development of pathogen resistance to various antibiotics. The physiological and health importance of ascorbic acid in the diets of different fish species has been shown in various studies (Adham *et al*., 2000; Okhionkpamwonyi and Edema, 2017; Youssef *et al*., 2021). Hemorrhages on the body surface, ulcers of various sizes on the skin, fin erosions, inflamed vents, abdominal distension and exophthalmia were observed in the group deprived of ascorbic acid in this study, which is in agreement with the reports of Adham *et al*. (2000).

Fulton's condition factor (CF) and specific growth rate (SGR) are indicators of the health status of fish. In the present study, fish fed a diet containing ascorbic acid presented improved growth performance, such as increased specific growth rates, weight gain, survival rates and condition factors. These findings are in agreement with those of Okhionkpamwonyi and Edema (2017), who reported that production parameters such as growth performance, survival rate and nutrient utilization were increased with ascorbic acid supplementation in the catfish diet. Ascorbic acid is essential for bone development, and research has shown that deficiencies in ascorbic acid in fish manifest as deformities of the bone and skeletal system, leading to lordosis and even death in extreme cases (Ibiyo *et al*., 2007). In this study, ascorbic acid deprivation in *A. hydrophila*-infected fish resulted in spinal deformities in some of the fish samples, and these findings are in agreement with the findings of Ibiyo *et al*. (2007). In a related study, Eya (1996) reported "broken-skull disease" in *Clarias gariepinus* subjected to dietary deficiency of ascorbic acid. However, such skeletal deformities mostly occur in fingerlings and juveniles deprived of dietary ascorbic acid since such abnormalities rarely occur in adult fishes (Adham *et al*., 2000). Aeromonas hydrophila have been shown to modulate fish skeletal muscle growth (Elbialy et al., 2023) therefore can lead to skeletal curvature especially in growing fish exacerbated by ascorbic acid deficiency.

Changes in hematological parameters are indicators of metabolic disorders caused by toxicants (Akter *et al*., 2020), microbial pathogens (Reyes and Aliasas, 2018) and other stressors. In stressed fish, resistance to disease decreases, and metabolic processes and the assimilation of food are disrupted. These changes lead to morphological, biochemical and physiological changes in response to stressful conditions, which implies profound disturbances in metabolism and in the functioning of enzymatic, nervous and other systems. When inadequate, ascorbic acid supplementation is presumed to impair erythrocyte synthesis, thereby leading to anemia (Adham *et al*., 2000). The results of the hematological parameters indicated a significant increase in RBC and hemoglobin in the ascorbic acid -supplemented group compared with the treatment group, whose diet was not supplemented with ascorbic acid. This may be due to the increase in erythropoiesis and osmo-regulatory function in hematopoietic organs (Adham *et al*., 2000). The reported increase in RBC, HGB and PCV in the ascorbic acid -supplemented group may also be attributed to its role in the absorption of iron from the gastrointestinal tract, which is subsequently utilized in erythropoiesis. Additionally, as an antioxidant, ascorbic acid protects fish against oxidative damage to various tissues, including RBCs (Faramarzi, 2012). Antioxidants protect red blood cell membranes against hemolysis, thereby leading to increased RBC counts (Gharaei *et al*., 2020). The results of this study are in agreement with those of Khara *et al*. (2016), who reported significantly higher RBC, HGB, PCV and WBC in a ascorbic acid -supplemented diet of Caspian brown trout (Salmo truttacaspius). Harikrishnan *et al*. (2003) reported decreased red blood cells and hematocrit in carp (*Cyprinus carpio*) experimentally infected with *A. hydrophila*. However, Rafiq *et al*. (2001) did not observe any alterations in white blood cells in tilapia challenged with *A. hydrophila*. Conversely, the decreased RBC, HGB and PCV recorded among the groups that were experimentally infected with *A. hydrophila* and were not treated with antibiotics or their diet supplemented with ascorbic acid may be due to the hemolytic activity of the bacterial isolate. The hemolytic activity of *A. hydrophila* is a function of its pathogenicity; therefore, the higher the pathogenicity of an isolate is, the greater the hemolytic activity (Omeje *et al*., 2019). White blood cells play a key role in controlling the immune functions of an organism, and a change in count may indicate a reduction in nonspecific immunity. The increase in the WBC of the group infected with *A. hydrophila* without ascorbic acid supplementation may be due to a generalized immune response to the infection, suggesting that the immune system has been compromised. An increase in WBCs is expected in microbial infection since they are vital in the innate defense of the organism. According to Adhan *et al*. (2000), ascorbic acid deficiency predisposes *C. gariepinus* to leukopenia, resulting in depression of the phagocytic activities of the WBC against pathogenic agents. The increase in WBC count may be indicative of the fish immune response in the presence of ascorbic acid (immune stimulant) against infection.

Alterations in serum enzymes are indicators of stress; therefore, the activities of AST, ALT and ALP have been employed in the detection of tissue damage due to environmental toxicants and in the diagnosis of fish diseases. An increase in AST, ALT and ALP may indicate degenerative changes in the liver (Muthusamy *et al*., 2018). The effects of toxicants on hepatocytes can lead to tissue damage with consequent leakage of liver enzymes into the

bloodstream (Muthusamy *et al*., 2018). The decreases in the levels of AST and ALT among the groups whose diets were supplemented with ascorbic acid compared with those whose diets were not supplemented with ascorbic acid obtained in this study indicate hepato-protective activity and a reduction in organ damage resulting from ascorbic acid supplementation. Elevated levels of creatinine and urea are a consequence of damage to the kidney, leading to the inability of the excretory organs to eliminate them from the blood circulation. In fish, urea is produced by the liver and excreted mainly through the gills. Toxicants and pathogens that cause gill damage and dysfunction ultimately predispose individuals to elevated urea. Additionally, renal damage induced by the exposure of fish to toxicants and pathogens can lead to elevated creatinine, which is used as a kidney function test.

The results of this study revealed that total protein, albumin and globulin increased significantly in fish fed a diet supplemented with ascorbic acid. These findings may indicate immune system stimulation and a stronger innate immune response. Conversely, the hypoproteinaemia and hypoalbuminemia recorded among the group deprived of ascorbic acid can be attributed to decreased food intake, conversion and utilization or even liver and kidney damage. The importance of globulin in maintaining a healthy immune system has been reported (Jagruthi *et al*., 2014). All blood proteins necessary for immune responses are products of gamma globulin. The stimulatory effects of ascorbic acid on the immune system have been reported in other fish species, such as Caspian brown trout (Khara *et al*., 2016), *Catla catla* (Reddy, 2018), and common carp (Faramarzi, 2012). Like other vertebrates, albumin, whose main function is the transport of nutrients, metabolites and xenobiotics, is the most abundant protein in the blood plasma of fish (Chernyavskikh *et al*., 2019). In this study, the differences in the albumin concentration between the treatment groups were not significant, which showed that the diet did not predispose the treated fish to osmoregulatory dysfunction.

In conclusion, the results of the present study suggested that ascorbic acid supplementation might be useful in ameliorating the deleterious effects of *A. hydrophila* infection on RBC, hemoglobin, PCV, and WBC counts and serum biochemical parameters. Also the results of the study shows that ascorbic acid supplementation enhanced the treatment potential of tetracycline against *A. hydrophila* infection of *C. gariepinus*. Furthermore, there were no adverse effects of ascorbic acid supplementation on the different parameters evaluated.

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Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

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