



Biological and histological changes and DNA damage in *Oreochromis niloticus* exposed to oxytetracycline: a potential amelioratory role of ascorbic acid

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Abstract

Antibiotics are crucial in the control of bacterial infections on fish farms. However, the influence of licensed antibiotics on DNA damage, histological changes, and protein differentiation in some farmed fish species is unidentified. The current study aimed to investigate the effect of therapeutic (80 mg/kg body weight/day) and high doses (160 mg/kg body weight/day) of oxytetracycline (OTC) on some biological indices, protein differentiation, histological changes, and DNA damage in *Oreochromis niloticus* ($n = 360$; 40 ± 3.5 g) and the potential positive effects of dietary ascorbic acid (AA) and whether it is capable of reducing the deleterious impacts of OTC and working synergistically to increase fish disease resistance and decrease morbidity and mortality rates. In the current study, fish were fed diets with or without OTC (80 or 160 mg/kg body weight/day) and AA (100 mg/kg diet/day) for 28 days, followed by a bacterial stress test with two different virulent strains for 14 days. Treatments include CTR (control; without additives), AA, OTC80, OTC160, OTC80 + AA, and OTC160 + AA. The results revealed that the AA group had the best antioxidative (significant increase in SOD, CAT, and GPx) and immunomodulatory effects (an increase in IgM, nitroblue tetrazolium, and total leucocytic count). Furthermore, the hematological parameters, immune indices (NBT), and antioxidant enzymes were significantly reduced by OTC160 ($p \leq 0.05$). The results also showed that OTC160 notably augmented the activity of liver (ALT, AST, and LDH) and kidney (creatinine and urea) damage markers ($p \leq 0.05$). OTC160 revealed considerable hepatic and renal tissue damage and significantly higher hepatocyte DNA damage. Pathological changes and DNA damage were directly correlated to the dosage of OTC exposure, where they were more severe in OTC160. OTC80 showed insignificant changes in most parameters. Compared to CTR, the inclusion of AA in the *O. niloticus* diet significantly restored the biological indices, histological changes, and DNA damage in OTC-treated groups. The morbidity and mortality percentages after bacterial challenge were significantly lower in the OTC80 + AA group, followed by the OTC160 + AA group than in the CTR. These results corroborate the use of AA as a potent antioxidant and immune stimulant and a potential co-adjuvant to OTC in the diet of *O. niloticus* to reach optimal resistance to disease.

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Keywords *Oreochromis niloticus* · Oxytetracycline · Ascorbic acid · DNA damage · Antioxidants · Protein electrophoresis

Introduction

Intensive aquaculture techniques make fish much more susceptible to infectious diseases, which causes financial losses due to the cost of chemotherapy and fish mortality (Pepi and Focardi 2021). Bacterial diseases may result in significant losses in tilapia production, particularly during the winter season. Farmed fish may have a lower disease risk by improving their resistance to infections through better nutritional formulations, vaccinations, and immunostimulants (Al-Khalaifah et al. 2020). Antibiotic therapy is crucial in aquaculture for maintaining fish health and controlling bacterial infections. To date, the US Food and Drug Administration has only permitted three antibiotics for use in aquaculture: florfenicol, oxytetracycline (OTC), and ormetoprim and sulfadimethoxine (USFWS 2015).

OTC is one of the antibiotics authorized for usage in aquaculture and is frequently used as an effective therapy for fish bacterial diseases, for instance, *Vibrio anguillarum*, *Aeromonas hydrophila*, *Aeromonas salmonicida*, and *Pseudomonas* species (Nakano et al. 2018; Leal et al. 2019). The oral route is the most popular method of administering antibiotics in aquaculture since there is little chance of ecological contamination (Leal et al. 2019). The OTC therapeutic dose for specific fish species or diseases was between 5 and 82.5 mg/kg body weight/day for 10 successive days (USFWS 2015). The excessive and improper use of antibiotics in aquaculture contributes to the spread of antibiotic resistance and residues, which can remain long in water, tissues, or sediments after drug use is stopped (Bojarski et al. 2020).

The use of immunomodulators in fish feeds is an approach for not only improving defense responses to threats to which fish are exposed but also for finding alternatives to antibiotics and chemotherapeutics used for treating fish diseases (Oliva-Teles 2012). Vitamins are organic compounds required for life since normal growth, reproduction, and health all depend on trace amounts of them. Ascorbic acid (Vitamin C) modulates immune functions such as cell proliferation, macrophage infiltration, phagocytic activity, lysozyme and cytokines levels, and antibody production (Ibrahim et al. 2020). It is also an effective natural antioxidant that is essential in scavenging reactive oxygen species (ROS) and protecting the fish from oxidative damage (Hajirezaee et al. 2020). Additionally, AA plays a vital role in recovery from toxicant and environmental stressor exposure (Hajirezaee et al. 2020). Fish are deficient in the L-gluconolactone oxidase enzyme, which is responsible for the de novo synthesis of AA, so they must get their AA requirements from external sources (Fracalossi et al. 2001).

Several markers are used for assessing the health status of fish and the therapeutic efficacy of the drug used. Hematological parameters are good tools for assessing physiological and pathological alterations in fish. The changes in hematology could be caused by feeding habits, pollutants, and environmental factors. There is a delicate balance called redox balance between the antioxidant defenses and pro-oxidant forces. Oxidative stress arises when ROS increases in the absence of oxidant defense. To combat ROS damage, fish employ a variety of defense mechanisms, including antioxidant enzymes like catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GRx) (Ahmed et al. 2017). Comet assays are used to review and assess the health and genetic condition of many fish species because of their high sensitivity in determining

DNA damage in a single cell (Kousar et al. 2022). The most genotoxic compounds have been found to produce ROS, which interacts with DNA and disrupts it. Protein electrophoresis is a blood test that measures specific proteins. The test uses electrical charges to separate proteins in the blood.

NBT is a fast and low-cost test that focuses on phagocytes' capacity to reduce the dye by producing ROS which are responsible for pathogen elimination *in vivo*. One of the most effective methods of protection against fish diseases is the capability of macrophages to destroy pathogens. As a result, the bacterial stress test is the most reliable test of immune function with the maximum level of biological significance because it evaluates an integrated immune reaction at the organismal level (Köllner et al. 2002). *Aeromonas hydrophila* and *Vibrio parahaemolyticus* are significant public health concerns and the primary reason for bacterial disease in *O. niloticus*. *A. hydrophila* is the main cause of MAS (motile *Aeromonas* septicemia) in a broad variety of fish species. Vibriosis is a potentially fatal bacterial disease that affects fish globally, leading to high mortalities and severe financial losses.

To our knowledge, no data on the role of dietary AA in OTC-induced stress in *O. niloticus* is currently available. Thus, this study was designated to evaluate the potential immunostimulatory and antibacterial effects of AA when given alone or combined with OTC to assess its role in modifying the deleterious impacts of OTC through investigating the changes in biological indices, protein differentiation, DNA damage, histological changes, and disease resistance against different bacterial infections. To the best of the authors' knowledge, it is the first study to investigate the effect of OTC, AA, and their combination on serum protein differentiation. So far, only a few studies have been conducted on the genotoxicity of antibiotics on aquatic animals.

Material and methods

Feed formulation

A commercial fish feed (30% protein, 5.4% fiber, 7.93% ether extract, 8.17% Ash, 48.5% non-protein nitrogen, and digestible energy equals 3373 (kcal/kg)³, 2mm in diameter) was used for the preparation of experimental diets. Six experimental diets were formulated; one diet served as a control without any additives, and the other five diets were prepared to contain 100 mg AA/kg diet, 80 mg OTC/kg biomass/day, 160 mg OTC/kg biomass/day, 80 mg OTC/kg biomass/day + 100 mg AA/kg diet, and 160 mg OTC/kg biomass/day + 100 mg AA/kg diet. OTC (oxytetracycline hydrochloride 20%, ADWIA Co., Egypt) and ascorbic acid (99.8%, CAT: sA178, Piochem, Egypt). OTC was given following the manufacturer's instructions. OTC contains 200 mg/g of powdered oxytetracycline hydrochloride, so 400 or 800 mg of powdered oxytetracycline hydrochloride per 1 kg of fish mass was used to achieve the recommended dose of 80 or 160 mg/kg Bwt/day. Briefly, a pre-weighed quantity of pellets (2% Bwt) was soaked in approximately 1 mL of vegetable oil containing 400 or 800 mg of powdered oxytetracycline hydrochloride, gently mixed, and air-dried following the method of Trushenski et al. (2018) and Julinta et al. (2019) for OTC oral administration. The medicated feed was freshly prepared daily. The control diet without additives and AA diet were also prepared with the same method.

Experimental fish and design

Only one set of healthy juvenile *O. niloticus* ($n=360$, 40 ± 3.5 g) was purchased from a private farm in Kafrelsheikh province, Egypt. All fish, regardless of sex, were distributed after 2 weeks of adaptation to lab conditions in six groups in 18 glass aquaria ($50 \times 40 \times 40$ cm), each in triplicate ($n=60$ /group, 20/tank), as described in Table 1. The debris and wastes were siphoned daily, and about 50% of the aquarium water was changed with dechlorinated 24-h stored water every 2 days. During the adaption period, fish were fed until satiation on a commercial diet of 30% protein. Throughout the experiment, fish were fed 2% of their body weight for 28 days. The feed intake was visually evaluated after each feeding. Fish were weighed biweekly, the variations in weight gain were calculated, and the increase in feed intake was determined by the biomass in each tank. Uneaten food was gathered on each feeding day, collected tank-wise, dried, and weighed. Water parameters during acclimation and the experimental period were regularly checked. Dissolved oxygen was measured using an oxygen meter (HI76407-Hanna Instruments Inc., RI, USA) and recorded at 7.1 ± 0.4 mg/L. Temperature and pH were measured using the digital waterproof pHep® pH/temperature tester (HI98128-Hanna Instruments Inc., RI, USA) and recorded at 25 ± 2 °C and 7.3 ± 0.3 , respectively. Observations of external signs, feed intake, and mortality were recorded daily.

Sampling

At the end of the feeding trial (28 days), all fish were starved for 24 h before sampling. Blood was collected from caudal veins with anticoagulant (EDTA) for hematological parameters and NBT (nitro blue tetrazolium reduction test), which was done within 24 h. Another set of blood was collected without anticoagulant for serum purposes. The serum was obtained by centrifugation at 4000 rpm/5 min, collected in Eppendorf tubes, and stored at -20 °C for further analysis (done within 14 days).

The fish were sacrificed via spinal cord transection after being anesthetized by immersion in freezing water and a saturated benzocaine solution. Tissue samples (liver and kidney) were kept either in 10% formalin till their histological examination or frozen at -20 °C until comet assay.

Bacterial inoculum preparation and experimental challenge

The therapeutic efficacy of OTC and AA was assessed at the end of the feeding period (28 days), by challenging *O. niloticus* with a pathogenic strain of *A. hydrophila* and *V. parahaemolyticus* which were kindly provided by Microbiological Unite, Kafrelsheikh Provincial Lab, Animal Health Research Institute, Egypt. The strains were previously isolated from diseased *O. niloticus* and were identified using phenotypic and molecular techniques. The isolates were stored in glycerol at -20 °C until use. The bacterial suspensions were prepared using McFarland standard turbidity tubes, following El Latif et al. (2019). Loopfuls from *A. hydrophila* and *V. parahaemolyticus* aliquots were inoculated into Tryptic soya broth (incubated at 25 °C for 24 h) and alkaline saline peptone water (incubated at 41.5 °C for 18 h), respectively. A loopful from each broth was inoculated into *Aeromonas* medium base for *A. hydrophila* (incubated at 25 °C for 24 h) and TCBS (thiosulfate citrate bile salts sucrose agar) for *V. parahaemolyticus* (incubated at

Table 1 Experimental design of fish groups

Fish group	No. fish	Type of treatment	Treatment code	Tested concentration of Oxy-tetracycline (OTC)	Ascorbic acid (AA)
1	60	Control	CTR	×	×
2	60	Treated with AA	AA	×	100mg/kg feed
3	60	Treated with a therapeutic dose of OTC	OTC80	80mg/kg biomass/day	×
4	60	Treated with a high dose of OTC	OTC160	160mg/kg biomass/day	×
5	60	Treated with a therapeutic dose of OTC plus AA	OTC80+AA	80mg/kg biomass/day	100mg/kg feed
6	60	Treated with a high dose of OTC plus AA	OTC160+AA	160mg/kg biomass/day	100mg/kg feed

37 °C for 24 h). The required turbidity degree was achieved by matching them against McFarland standard turbidity tubes (0.5 McFarland, equals 1.5×10^8). The required bacterial dose was then prepared by dilution with normal saline.

After the feeding trial, each group was divided into two subgroups of 20 fish each. Subgroup A challenged intraperitoneally with 0.2 ml of 1×10^7 CFU *A. hydrophila* and subgroup B was intraperitoneally challenged with 0.2 ml of 1×10^6 CFU *V. parahaemolyticus*. During the challenging time, fish were fed control diets alone, including AA and/or OTC. Fish were observed for 14 days to track clinical signs, morbidity, and mortality rates (MR%) daily. MR% and RPS (relative percent of survival) are evaluated according to the following equations:

$$\text{MR\%} = \frac{\text{No fish mortalities}}{\text{Total no population}} \times 100$$

$$\text{RPS\%} = 1 - \left(\frac{\text{mortality\% in the treated group}}{\text{mortality\% in the control group}} \right) \times 100 \text{ (Amend 1981)}$$

Hematological indices

The hematological indices were all measured according to Faggio et al. (2014). RBCs (red blood cell count; $\times 10^6/\text{mm}^3$), TLC (total leukocyte count; $\times 10^3/\text{mm}^3$), and PLT (platelets count; $\times 10^3/\text{mm}^3$) were counted using a modified Neubauer’s hemocytometer. Hb concentration (hemoglobin; g/100ml) was determined by the cyanmethemoglobin method and PCV (packed cell volume; %) was assessed using a micro-hematocrit centrifuge. Thin blood films were prepared on clean slides for the assessment of differential leucocyte count. The slides were allowed to dry before being stained with a modified Wright’s stain.

Immune-related indices

Following the manufacturer’s procedures, the serum IgM and IgE levels were determined using the sandwich ELIZA (Table 2). OD (optical density) is measured spectrophotometrically at wavelength 450 nm. The OD value is relative to the quantity of specific Ig.

NBT was estimated as described by Esteban et al. (2001). Briefly, 1×10^6 peripheral blood leucocytes/well were treated with an equivalent NBT volume of 0.2% for 2 h at 28 °C. The supernatants were disposed of, and the cells were fixed for 5 min in 100% (v/v) methanol. Each well received two washes with 125 mL of 70% (v/v) methanol. The fixed cells were left to dry. Per well, 120 ml of 2 N potassium hydroxide (KOH) and 140 ml of dimethyl sulfoxide (Sigma-Aldrich, USA) were used to dissolve the reduced NBT (in the

Table 2 Fish-specified ELISA kits (Sunlong Biotech Co., LTD, China)

Parameter	Cat no	Sensitivity	*Product spec
IgM	SL0048FI	14ng/ml	96T
IgE	SL0070FI	0.01ng/ml	96T

*Specificity: There was no evidence of significant interference or cross-reactivity between fish parameters and analogs. *IgM*, immunoglobulin M; *IgE*, immunoglobulin E

form of the blue precipitate formazan). Using an ELISA assay or reader, the turquoise-blue solution was evaluated at 630 nm.

Oxidative stress biomarkers

Serum antioxidant capacity was evaluated by detecting the levels of SOD (superoxide dismutase), GPx (glutathione peroxidase), and CAT (catalase) activities. Using diagnostic kits from Bio-diagnostic CO., Egypt. SOD, GPx, and CAT were colorimetrically determined at 560, 340, and 510 nm, respectively, according to Nishikimi et al. (1972), Paglia and Valentine (1967), and Aebi (1984).

Serum biochemical parameters

Serum biochemical markers for liver function (ALT, AST, and LDH) and kidney function (creatinine and urea) were determined by a colorimetric method (Tietz 1995). Total protein and albumin were estimated according to Doumas et al. (1981). Globulin was calculated by subtracting albumen from total protein. Kits used for the estimation of serum biochemical parameters are shown in Table 3.

Protein electrophoresis was done using the CAPI 3 PROTEIN (E) 6 kit by the SEBIA CAPILLARYS 3 TERA automated instrument in an alkaline buffer (pH 9.9), following the manufacturer's instructions.

Histological examination and single-cell gel electrophoresis

Paraffin blocks including fixed tissues of the hepatopancreas and posterior kidney were cut into sections (3–7 mm thick), continuously sliced, and stained with H&E (hematoxylin and eosin). The stained tissues were microphotographed using a digital camera (Leica EC3) linked to a light microscope (Leica DM 5000).

DNA damage was detected in the hepatocyte via the comet assay (single-cell gel electrophoresis), as described by Singh et al. (1988). In brief, tissues were placed on microscope slides in 0.6% normal melting point and low melting point agarose gel, and then immersed in lysing solution (2.5mol NaCl, 100 Mm Na₂EDTA, newly added 1% Triton- \times 100, and 10% DMSO) at 4 °C for 1 h for denaturation and unwinding of DNA. Slides were immersed in electrophoresis buffer (300 mM NaOH, 1 mM Na₂EDTA, PH. 13.0) for 30 min at 4 °C, and then neutralized with Tris–HCl buffer (400 Mm Tris–HCl, PH 7.4) before final staining with a fluorescent dye (20ug/ml ethidium bromide). The DNA damage was observed using a fluorescent microscope (Nikon Microscope-Eclipse, E600 with Y-FL EPI-Fluorescence attachment, Japan) outfitted with a 515–560-nm excitation filter,

Table 3 Details on the serum biochemical markers tested and kits used

Parameters	Kits used
ALT, AST	Human, Germany
LDH	Roche Diagnostic, Indianapolis, IN, USA
Creatinine	Biosystems S.A., Spain
Urea	Diamond Diagnostics, Egypt
Total protein, Globulin	Spinreact, Spain

a 590-nm barrier filter, and an automatic digital imaging system running Comet assay™ software (perceptive Instrument, UK).

Statistical analysis

The data is expressed as mean \pm standard deviation (SD). For testing the data, a one-way ANOVA (SPSS® version 22, SPSS Inc., IL, USA) was used, followed by Duncan's post hoc test to compare the means, considering the significance of the treatment effects at a probability level of $p \leq 0.05$.

Method validation

To assess biological variation and repeatability, each treatment was conducted in triplicates, consisting of three samples obtained from different fish for each triplicate ($n=9$ samples/treatment).

Results

Hematological parameters

Table 4 shows the variations in hematological parameters (Hb, RBCs, PCV, TLC, and PLT) and differential leukocyte counts after feeding fish diets treated with AA and/or OTC. The best enhancement was observed in the AA group, followed by the OTC + AA groups. The OTC160 group exhibited a significant decrease in Hb, RBCs, and PCV% compared to CTR, except for platelet counts, which were significantly higher than CTR. Likewise, TLC, lymphocytes, and MID counts were noticeably higher in either AA or OTC + AA groups than in OTC (OTC80/OTC160) and CTR groups. The latter groups exhibited insignificant differences between their TLC and DLC, except MID counts were significantly higher in the OTC160 group than in the other two groups (OTC80 and CTR).

Immune response and antioxidant activity

Effects of dietary AA and/or OTC on immune response are presented in Fig. 1a. The obtained data showed a significantly higher IgM and NBT in fish fed AA alone or combined with OTC, with the highest levels in AA, followed by AA+OTC as compared to CTR. OTC80 showed a significant increase in IgM levels without significant NBT activity changes. The OTC160 group substantially decreased NBT level compared to the CTR group. OTC160 showed a considerable increase in IgE levels compared to other groups.

The results of antioxidant activity are presented in Fig. 1b. AA and OTC80 + AA groups had significantly higher antioxidant enzyme (GPx, CAT, and SOD) activities than CTR. The OTC80 + AA group improved slightly compared to the AA group, while insignificant differences were found between OTC80 and CTR. OTC160 displayed significant decreases in all the measured antioxidant enzymes. OTC160 + AA showed improvement toward control values.

Table 4 Erythrogram and leukogram of *O. niloticus* experimental groups

Parameter	CTR	AA	OTC80	OTC160	OTC80 + AA	OTC160 + AA	<i>p</i> -value
Hg (g/100ml)	6.37 ± 0.96 ^c	9.6 ± 0.75 ^a	6.8 ± 1.47 ^c	4.37 ± 0.76 ^d	7.8 ± 1.3 ^b	7.83 ± 0.55 ^b	0.038
RBCs (× 10 ⁶ /mm ³)	1.92 ± 0.39 ^b	3.06 ± 0.18 ^a	1.75 ± 0.58 ^b	1.38 ± 0.53 ^c	2.47 ± 0.53 ^a	1.72 ± 0.19 ^b	0.04
PCV%	23.33 ± 1.55 ^b	26.4 ± 2.51 ^a	21.77 ± 2.38 ^b	11.70 ± 1.56 ^c	22.13 ± 1.96 ^b	21.37 ± 2.57 ^b	0.011
PLT (× 10 ³ /mm ³)	239 ± 46.18 ^c	395.33 ± 53.00 ^a	285 ± 18.33 ^c	317.33 ± 62.32 ^b	347.33 ± 26.39 ^{ab}	378 ± 36.50 ^{ab}	0.04
TLC (× 10 ³ /mm ³)	40.78 ± 9.26 ^b	65.05 ± 9.11 ^a	38.08 ± 6.60 ^b	44.10 ± 2.25 ^b	57.68 ± 2.28 ^a	57.90 ± 3.80 ^a	0.045
Lymphocyte (× 10 ² /mm ³)	37.40 ± 8.32 ^b	59.78 ± 8.58 ^a	34.74 ± 4.42 ^b	39.62 ± 1.64 ^b	53.12 ± 2.04 ^a	52.93 ± 3.79 ^a	0.045
Granulocytes (× 10 ³ /mm ³)	0.82 ± 0.51	1.05 ± 0.07	1.18 ± 1.38	1.07 ± 0.45	1.28 ± 0.66	1.41 ± 0.69	0.17
MID (× 10 ³ /mm ³)	2.41 ± 0.73 ^b	4.22 ± 0.52 ^a	2.16 ± 0.83 ^b	3.41 ± 0.97 ^a	3.29 ± 0.42 ^a	3.57 ± 0.58 ^a	0.044

Values are expressed as means ± SD (*n* = 9/group). Different subscripts in the same row differ significantly at *p* ≤ 0.05. *PLT* platelets; *TLC* total leukocytic count; granulocytes indicate neutrophils, basophils, and eosinophils; *MID* total value of all WBCs, neither lymphocytes nor granulocytes.

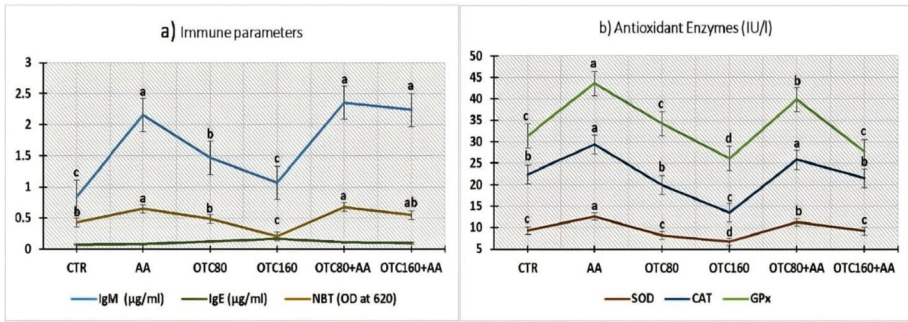


Fig. 1 a Serum immune parameters of *O. niloticus* experimental groups, b serum antioxidant enzymes of *O. niloticus* experimental groups, values are means; the error bars represent SD ($n=9/\text{group}$). Significant differences between treatments are indicated by different subscripts above the bars ($p \leq 0.05$)

Biochemical parameters

Compared to CTR, OTC160 had significantly higher liver enzymes (ALT, AST, and LDH), followed by OTC160 + AA (Table 5). In contrast, all the other groups showed insignificant differences. Again, high doses of OTC with or without AA (OTC160 / OTC160 + AA) exhibited a significant increase in urea and creatinine concerning the levels of CTR. Therapeutic doses of OTC (OTC80 / OTC80 + AA) also significantly increased the urea levels, while AA showed a significant decrease in urea levels (Table 5). Fish were actively feeding, except OTC160 which showed decreased feed intake % ($84.67 \pm 3.06\%$). No mortalities were recorded during the experiment in the treatment or control groups except for 3 mortalities in OTC160.

Protein profile and protein electrophoresis

Figure 2 shows the impact of dietary AA and/or OTC on protein profiles. The obtained data showed significantly higher serum levels of total protein and globulin in fish fed AA alone or combined with OTC, with the highest levels in AA and OTC80 + AA, followed by the OTC160 + AA group compared to CTR. Albumen level and percent increased in all OTC-treated groups except OTC80 + AA, which showed only an increase in albumen level with an insignificant increase in albumen percent.

The protein electrophoresis results (Table 6) revealed that AA caused a significant increase in beta1% and gamma %, while OTC80 revealed a significant increase in beta1%. OTC + AA groups showed a substantial decrease in alpha 2%. Alpha 1% and beta 2% showed insignificant changes compared to CTR in all fish groups (Fig. 3).

Internal and histological examination

Internal examination of OTC-treated groups (Fig. 4) revealed hemorrhagic spots on the liver, hemorrhagic abdomen, and enlarged spleen. The severity of signs was less in the

Table 5 Biochemical parameters, feed intake, and survival rate of *O. niloticus* experimental groups

Parameter	CTR	AA	OTC80	OTC160	OTC80+AA	OTC160+AA	<i>p</i> -value
ALT (U/L)	40.92 ± 1.29 ^c	38.93 ± 1.66 ^c	39.08 ± 8.32 ^c	76.41 ± 12.48 ^a	39.91 ± 2.08 ^c	47.26 ± 2.25 ^b	0.28
AST (U/L)	62.2 ± 2.83 ^c	65.95 ± 15.92 ^c	72.69 ± 8.85 ^c	152.67 ± 19.55 ^a	76 ± 11.53 ^c	90.9 ± 5.24 ^b	0.007
LDH (U/L)	265.25 ± 44.30 ^c	281.23 ± 6.03 ^c	272.22 ± 22.53 ^c	842.93 ± 132.73 ^a	298 ± 44.23 ^c	489.67 ± 122.46 ^b	0.022
Urea (mg/dl)	9.94 ± 0.90 ^c	6.98 ± 0.34 ^d	10.67 ± 1.00 ^b	12.70 ± 0.33 ^a	11.09 ± 0.81 ^b	11.72 ± 0.45 ^{ab}	0.017
Creatinine (mg/dl)	0.30 ± 0.06 ^b	0.25 ± 0.06 ^b	0.30 ± 0.04 ^b	1.01 ± 0.38 ^a	0.31 ± 0.05 ^b	0.71 ± 0.08 ^a	0.008
Feed intake%	100	100	100	84.67 ± 3.06 [*]	100	100	0.009
Survival rate%	100	100	100	95 [*]	100	100	0.007

Values are expressed as means ± SD (*n* = 9/group). Different subscripts in the same row differ significantly at *p* ≤ 0.05. Values (mean ± SD) bearing asterisks differ significantly (*p* ≤ 0.05).

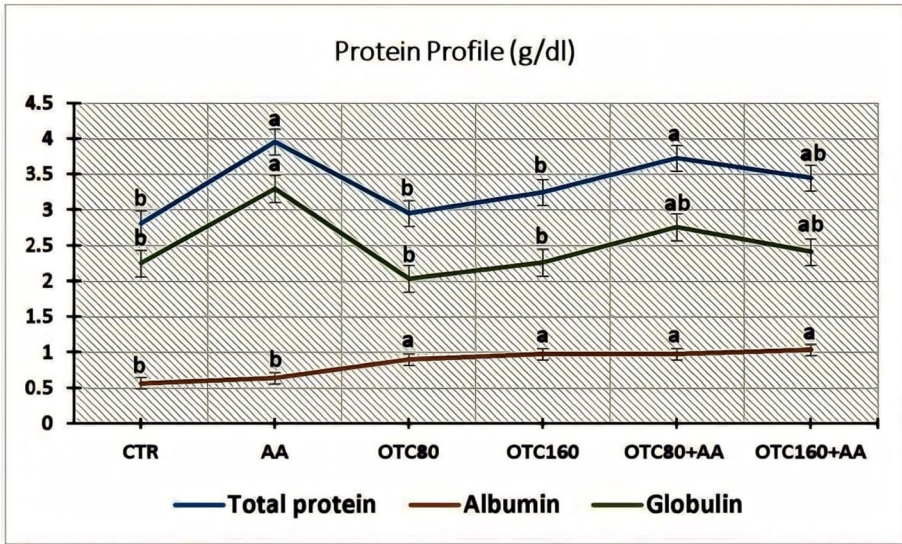


Fig. 2 Serum protein profile of experimental *O. niloticus* groups, values are means; the error bars represent SD ($n=9$ /group). Significant differences between treatments are indicated by different subtitles above the bars

Table 6 Protein electrophoresis of *O. niloticus* experimental groups

Parameter	CTR	AA	OTC80	OTC160	OTC80+AA	OTC160+AA	<i>p</i> -value
Albumin %	20.00 ± 5.8 ^b	16.23 ± 3.46 ^b	30.5 ± 9 ^a	30.13 ± 11.37 ^a	26.24 ± 4.05 ^b	30.7 ± 21.7 ^a	0.03
Alpha 1%	9.87 ± 0.12	8.4 ± 1.02	10.0 ± 2.00	11.7 ± 8.2	9.36 ± 1.53	7.7 ± 1.5	0.32
Alpha 2%	32.73 ± 0.15 ^a	31.18 ± 1.68 ^a	21.33 ± 12.6 ^a	19.77 ± 11.34 ^a	26.02 ± 1.55 ^b	25.7 ± 1 ^b	0.007
Beta 1%	8.2 ± 0.5 ^b	11.07 ± 2.53 ^a	13.8 ± 7.66 ^a	7.8 ± 0.5 ^b	5.67 ± 1.69 ^b	7.3 ± 3.1 ^b	0.05
Beta 2%	20.7 ± 2.8	21.9 ± 3.2	13.4 ± 11.8	19.7 ± 6.2	23.64 ± 4.05	19.3 ± 4.6	0.15
Gamma %	8.5 ± 2.65 ^b	12.56 ± 3.66 ^a	10.97 ± 1.8 ^b	10.9 ± 2.40 ^b	9.07 ± 0.25 ^b	9.3 ± 2.5 ^b	0.02

Values are expressed as means ± SD ($n=9$ /group). Different subtitles in the same row differ significantly at $p \leq 0.05$.

OTC80 group than in the OTC160 group, which was more severe. Including AA in the diets of OTC-treated groups significantly improves the picture of internal organs.

Histological changes in the hepatopancreas and posterior kidney were briefly tabulated (Table 7). They revealed normal hepatopancreatic histoarchitecture where central veins surrounded hepatocytes and pancreatic acini in the control group (Fig. 5a). Normal histoarchitecture was similarly detected in the hepatopancreas of the AA, OTC80, and OTC80 + AA groups (Fig. 5b, c, and e). However, in the OTC160 group, considerable tissue damage was seen in the form of marked necrosis of hepatic cells and pancreatic acini accompanied by mononuclear inflammatory cell infiltration and severe vacuolar degeneration of hepatic cells (Fig. 5d). This disordered tissue damage was somewhat restored in the OTC160 + AA group, which presented only vacuolation of hepatocytes and normal hepatopancreas (Fig. 5f).

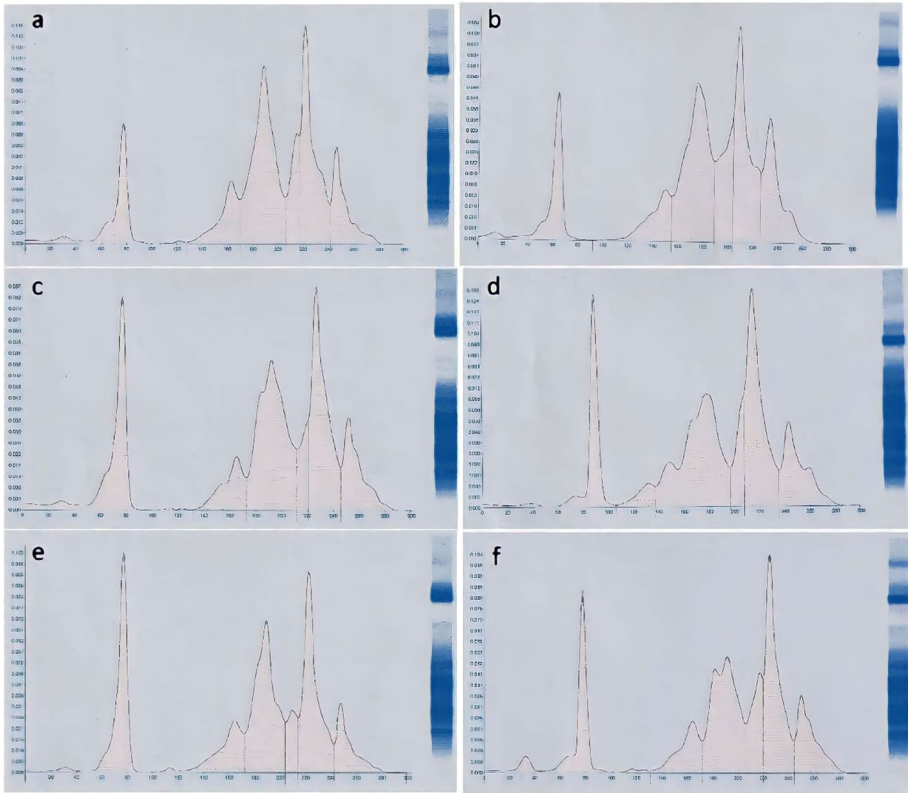


Fig. 3 Protein electrophoresis of *O. niloticus* experimental groups, **a** CTR; **b** AA showed a significant increase in beta 1 % and gamma %; **c** OTC80 showing a significant increase albumin % and beta 1 %; **d** OTC160 showing a significant increase albumin %; **e** OTC80+AA showing a substantial decrease in alpha 2 %; **f** OTC160+AA showing a significant increase in albumin % and a substantial decrease in alpha 2 %



Fig. 4 Internal examination of *O. niloticus* experimental groups. **a** OTC80 showing hemorrhagic spots on the liver, **b** OTC160 showing hemorrhagic abdomen, adhesion of internal organs, and hemorrhagic liver, **c** OTC80+AA showing normal internal organs except the enlarged spleen, **d** OTC160+AA showing pale hemorrhagic liver

Like hepatopancreas, the posterior kidney of CTR, AA, and OTC80 + AA groups had a normal renal histoarchitecture composed of renal tubules (Fig. 6a, b, and c). The posterior kidney of the OTC80 group displayed mild vacuolation of the renal tubular epithelium (Fig. 6d), and that of the OTC160 group showed a marked degree of renal tubular degeneration (Fig. 6e). Once more, the kidney of the OTC160 + AA group presented signs of improvement, with foci of degenerative changes within some renal tubules (Fig. 6f).

Comet assay and DNA damage

The comet assay results on DNA damage (Fig. 7) revealed that OTC160 displayed a significantly ($p \leq 0.05$) higher percent of DNA damage in hepatocytes, represented by decreased % DNA in the head, increased tail length, % DNA in the tail, and increased tail moment. This damage was partially restored by including AA in *O. niloticus* diets (OTC160 + AA). The OTC80 and OTC80 + AA groups showed damage in the form of an increase in tail length and an increase in the tail moment only in the OTC80 group. There were no significant differences between the AA and CTR groups (Fig. 7).

Experimental infection

The clinical examination of the infected fish (Fig. 8) with both strains (*A. hydrophila* and *V. parahaemolyticus*) revealed hemorrhagic spots and skin ulcers, tail erosion, exophthalmia, and ocular opacity. Post-mortem examination of challenged fish revealed congestion of internal organs, hemorrhagic liver, and enlarged spleen (Fig. 8). The frequency and severity of signs were less severe in treated groups than CTR and in *V. parahaemolyticus* infected groups than in *A. hydrophila* infected groups.

The morbidity and mortality percentages (Fig. 9) were significantly lower in the OTC80 + AA group, followed by the OTC160 + AA group and the OTC80 group than in the CTR group challenged with both examined strains. The dietary AA achieved RPS of 50% in the case of *A. hydrophila* infection and 33.33% in the case of *V. parahaemolyticus*, which was increased to 100% when combined with OTC80 treatment (OTC80 + AA),

Table 7 Histological lesions in the hepatopancreas and posterior kidney of *O. niloticus* experimental groups

Fish group	Hepatopancreas	Posterior kidney
CTR	NC	NC
AA	NC	NC
OTC80	NC	Mild vacuolation of renal tubular epithelium
OTC160	Marked necrosis of hepatic cells and pancreatic acini mononuclear inflammatory cell infiltration severe vacuolar degeneration of hepatic cells	Marked degree of renal tubular degeneration
OTC80 + AA	NC	NC
OTC160 + AA	Vacuolation of hepatocytes and normal hepatopancreas	foci of degenerative changes within some renal tubules

NC no change.

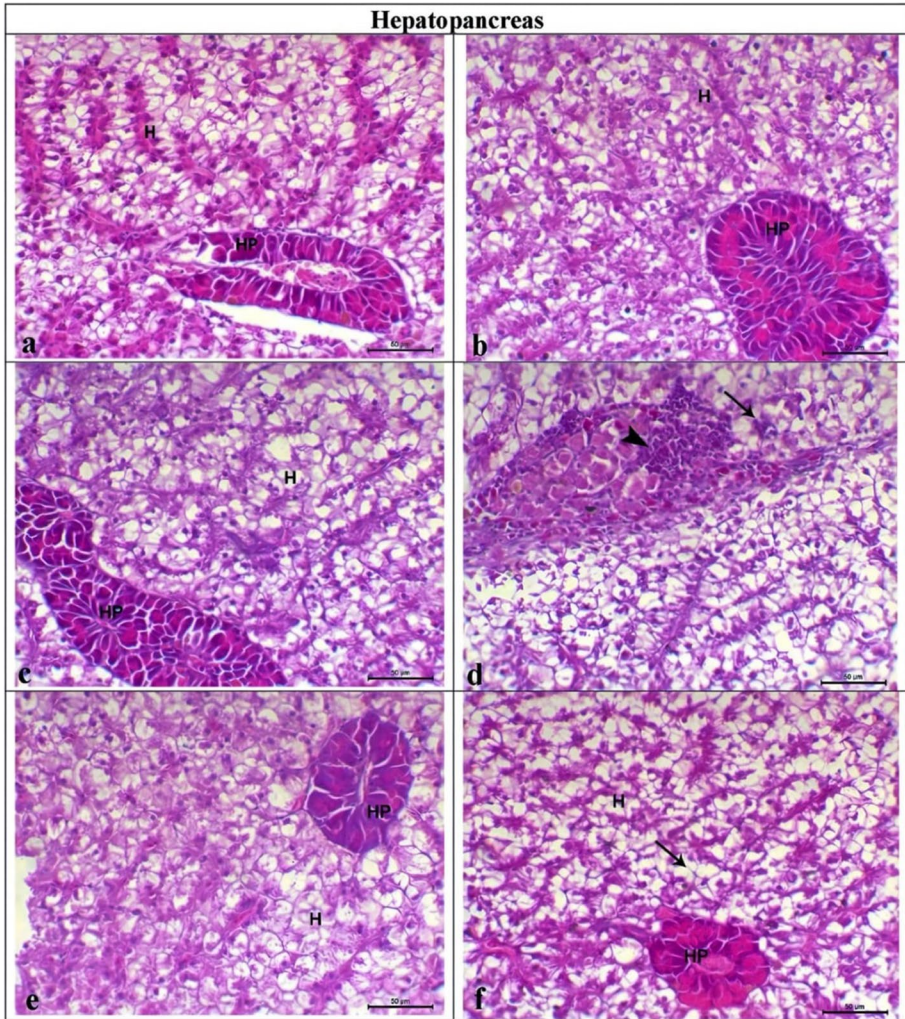


Fig. 5 Representative photomicrograph of H&E-stained hepatopancreas (H indicates hepatocytes and HP indicates hepatopancreas). Scale bar = 50 µm. **a** Hepatopancreas of CTR group showing normal hepatopancreatic histoarchitecture where hepatocytes cords (H) and pancreatic acini surround central vein, **b** hepatopancreas of AA group showing normal hepatopancreatic histoarchitecture, **c** hepatopancreas of OTC80 showing normal hepatopancreatic histoarchitecture, **d** hepatopancreas of OTC160 group showing marked necrosis of pancreatic acini accompanied with mononuclear inflammatory cells infiltration (arrowhead) and severe vacuolar degeneration of hepatic cells (arrow), **e** hepatopancreas of OTC80+AA group showing normal hepatopancreatic histoarchitecture, **f** hepatopancreas of OTC160+AA group showing vacuolation of hepatocytes (arrow) and normal hepatopancreas

while OTC80 treatment alone had RPS of 70% and 83.33% in the case of *A. hydrophila* infection and *V. parahaemolyticus*, respectively, and OTC160 treatment achieved a low RPS of 30% in the case of *A. hydrophila* infection and 0% in the case of *V. parahaemolyticus*, which was raised to 80% and 83.33% when combined with AA (OTC160+AA).

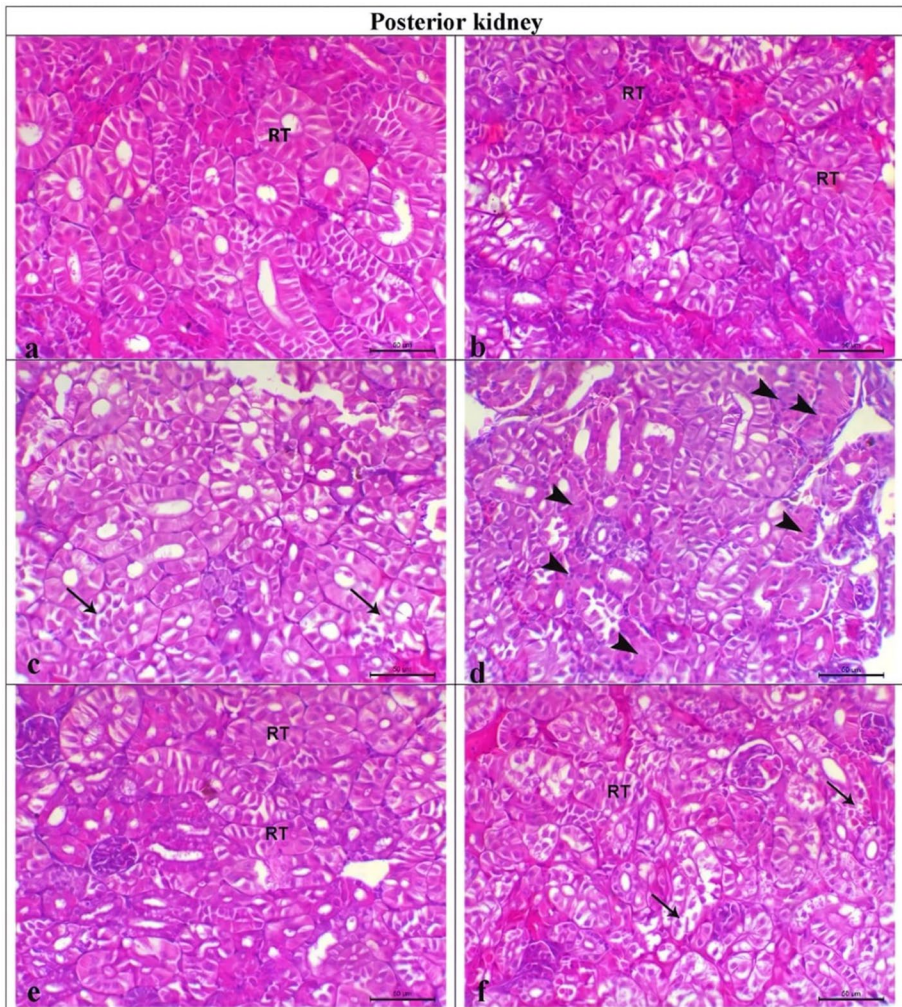


Fig. 6 Representative photomicrograph of H&E-stained posterior kidney (RT shows renal tubules). Scale bar=50 μ m. **a** The posterior kidney of the CTR group showing normal renal histoarchitecture where composed of renal tubules, **b** posterior kidney of the AA group showing normal renal histoarchitecture, **c** posterior kidney of OTC80 group showing mild vacuolation of the renal tubular epithelium (arrows), **d** posterior kidney of OTC160 group showing a marked degree of renal tubular degeneration (arrowheads), **e** posterior kidney of OTC80+AA group showing normal renal histoarchitecture, **f** posterior kidney of OTC160+AA group showing foci of degenerative changes within some renal tubules (arrows)

Discussion

Because of its broad-spectrum antibacterial properties, OTC is usually added to fish feeds in countries where it is not prohibited as a growth promoter. This prolonged therapy, however, causes OTC deposits in fish tissue, which has serious consequences for both fish and consumers (Reda et al. 2013). OTC has been shown to cause oxidative stress and liver dysfunction. OTC has the potential to promote the production of ROS, which promotes

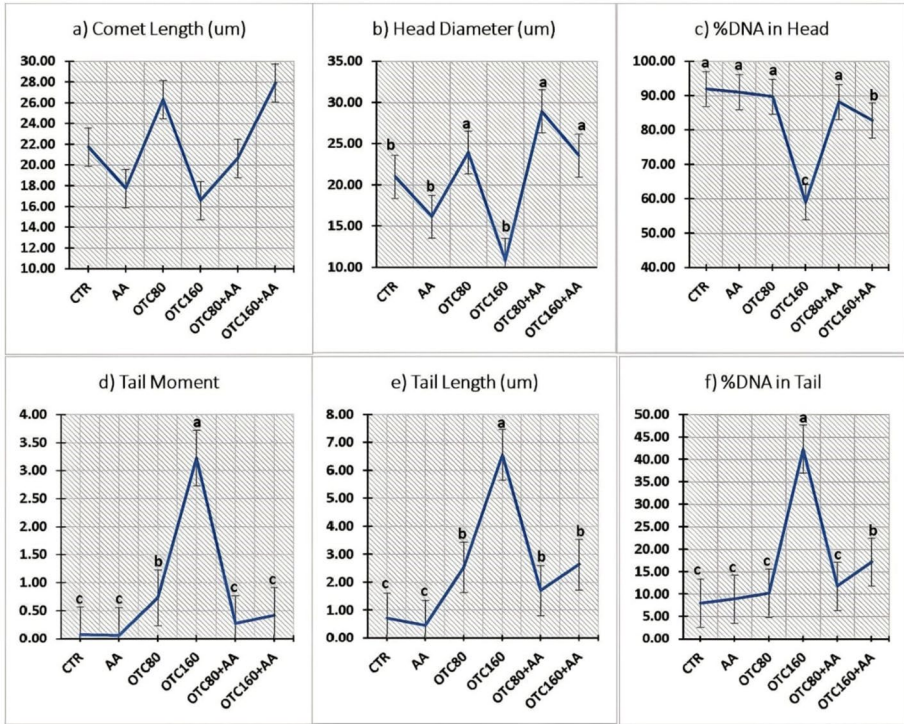


Fig. 7 Comet assay screening DNA damage in hepatocytes of *O. niloticus* experimental groups. a Comet length (um); b head diameter (um); c % DNA in head; d tail moment; e tail length (um); f % DNA in tail; values are means; the error bars represent SD ($n = 9/\text{group}$). Significant differences between treatments are indicated by different subtitles above the bars ($p \leq 0.05$)

lipid peroxidation in the hepatocyte membranes, leading to further outflow of intracellular enzymes into the bloodstream (Nakano et al. 2018). Furthermore, OTC induced adverse effects on immune response, nephrotoxicity, genotoxicity, histological alterations, and impaired antioxidant capacity and hematological parameters in fish species (Reda et al. 2013). This drives us to seek appropriate neoadjuvants to act synergistically with OTC and diminish negative effects. AA is a micronutrient that is essential for fish growth and physiological processes. Dietary acidifiers are well-recognized to improve OTC absorption (Ng and Koh 2016). We investigated whether AA might improve OTC’s antibacterial potential and, as a result, the fish’s immunity and assessed the potential role of dietary AA in reducing OTC-induced stress in *O. niloticus*.

Our findings demonstrated that the AA and OTC+AA groups significantly increased RBCs, Hb, PCV%, and PLT counts more than the OTC and control groups. The enhancement might be attributed to acidic pH levels produced by acidifiers, which effectively liberate essential minerals like potassium, calcium, ferrous, and copper from feed (Khajepour and Hosseini 2012). Also, this could be owing to AA’s antioxidative capabilities, which extend the life span of RBCs through its effective ROS scavenging ability (Nayak et al. 2007). Our results corroborate the findings of Affonso et al. (2007) that feeding *Brycon amazonicus* diets enriched with AA substantially increased RBCs and PCV values. An upsurge in RBC, PCV, and Hb is related to decreased hemolysis or increased

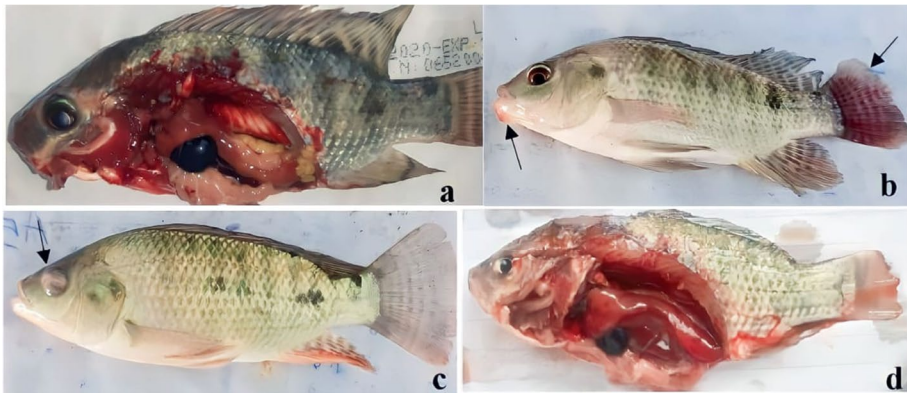


Fig. 8 Clinical and internal examination of experimentally challenged *O. niloticus* groups. **a** CTR infected with *A. hydrophila* showing congested internal organs, hemorrhagic liver, distended gall bladder, and enlarged spleen; **b** OTC80+AA infected with *A. hydrophila* showing hemorrhage and erosion of the oral region and tail; **c** OTC160 infected with *A. hydrophila* showing ocular opacity and hemorrhage; **d** CTR infected with *V. parahaemolyticus* showing hemorrhagic liver, distended gall bladder, enlarged spleen, and tail erosion

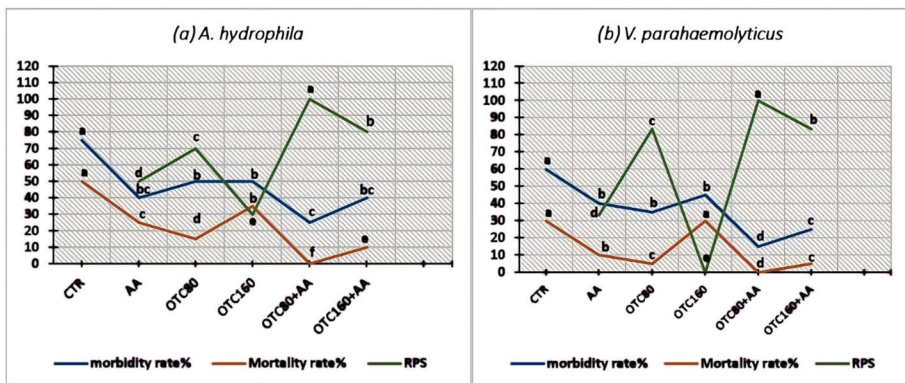


Fig. 9 Morbidity, mortality rates, and RPS of experimentally challenged *O. niloticus* groups ($n = 20$ /sub-group). **a** *O. niloticus* subgroups challenged with *A. hydrophila*, **b** *O. niloticus* subgroups challenged with *V. parahaemolyticus*. Significant differences between treatments are indicated by different subscripts ($p \leq 0.05$)

hematopoiesis. Our findings showed that high doses of OTC (OTC160) caused the anemic condition in *O. niloticus*, possibly due to the inhibition of erythropoiesis and increased lysis of RBCs (Miş'e Yonar et al. 2020). Similarly, Omoregie and Oyebanji (2002) detected a substantial reduction in RBCs, PCV, and Hb in *O. niloticus* that fed OTC added to diets.

The numbers of TLC and DLC are useful tools for assessing fish immunity. Here, we conclude that AA upsurges the immune responses and disease resistance. This conclusion depends on our results that dietary AA can increase TLC, particularly lymphocytes, which are involved in antibody production, hence increasing the immunological response of fish against infection. A comparable upsurge in lymphocyte count was detected in poultry fed

an acidified diet (Wang et al. 2010). In the current trial, OTC treatment insignificantly affected the TLC and DLC values, with a significant increase in MID in the OTC160 group. In contrast to our results, Dobšíková et al. (2013) demonstrated that OTC reduces the TLC in *Cyprinus carpio*. These variations may be related to differences in exposure periods and fish species.

In the current study, the AA when added alone or combined with OTC improved *O. niloticus* immunity, as evidenced by high levels of total protein, globulin, beta 1%, gamma %, IgM, and NBT. In agreement with our findings, many studies revealed that dietary AA increased the fish's immunity (Hardie et al. 1991; Moradi et al. 2022). In this study, OTC160 significantly decreased NBT with no effect on IgM and this result confirms that high doses of OTC reduced phagocytosis. Furthermore, OTC80 was reported to increase IgM levels with an insignificant effect on NBT. Other studies have found that OTC down-regulates antibody production, TLC, and phagocytic activity in *Cyprinus carpio* and *O. niloticus* (Kasagala and Pathiratne 2008; Reda et al. 2013, 2016), respectively. Likewise, Siwicki et al. (1989) reported suppression of the NBT activity and phagocytosis of *O. mykiss* spleen phagocytes following I/P injection of OTC (10 mg/kg). In contrast to our results, oral administration of OTC to *Scophthalmus maximus* at 200 mg OTC kg/BW/day for 12 days did not influence the NBT or phagocytosis activity (Tafalla et al. 2002), while Lundén et al. (2002) discovered in vitro that commonly used antibiotics (oxolinic acid, OTC, florfenicol, and sulphadiazine combined with trimethoprim) markedly decreased NBT activity in *O. mykiss* at the highest drug dose tested (100 g/ml). These confused results could be attributed to the multiple conditions used in different trials, such as species of fish, dosage, route of antibiotic administration, and water temperature, all of which affect drug absorption.

Lundén et al. (2002) discovered that OTC is reserved in the pronephros, a major fish lymphoid organ; thus, OTC immune suppression may be attributed to OTC penetration and impairment of immune cell function. However, when OTC was combined with AA, the immunological response and resistance to infection were maximized. This was evident in decreased rates of morbidity and mortality in the AA+OTC group compared to fish fed each alone. These findings indicate that innate immunity in fish is among the targets of OTC. To our knowledge, it is the first study to evaluate the effect of OTC on fish IgE. Our results demonstrated a significant elevation in IgE levels in fish serum treated with OTC160, which indicated a type of sensitivity toward high doses of OTC.

Increased ROS levels are well known to cause oxidative stress, which can impact fish health and cause tissue damage. Some key antioxidant enzymes, like SOD, GPx, and CAT help prevent ROS production. Our results revealed a significant decrease in the activity of all measured antioxidant enzymes (SOD, GPx, and CAT) in the OTC160 group compared to the OTC80 group, which showed insignificant differences from the CTR. These observed changes in the activities of antioxidant enzymes could be due to an increase in ROS production, resulting in excessive usage of these enzymes during ROS removal. While Nazeri et al. (2017) similarly pointed to the antioxidant activity of tetracycline, Abraham et al. (2021) reported a non-significant decrease in SOD activity in the hepatic and muscular tissues affected by the therapeutic dose of OTC for 10 days. The reduced antioxidant activities as observed by the parameters measured may have hindered detoxification and increased OTC toxicity at high doses in *O. niloticus* at the cellular level. In contrast, feeding fish with AA significantly restored serum antioxidant enzyme activities, demonstrating the protective role of dietary AA against oxidative damage induced by OTC. It could be owing to AA's high antioxidative activity, allowing it to neutralize ROS and reduce oxidative stress. Through H⁺ donation, AA binds to ROS and recovers free radicals. AA was

also demonstrated to operate as a reducing agent, principally by inhibiting the transfer of metal ions like Fe and Cu ions, which interact with H₂O₂ to form hydroxyl radicals (Babior 2000). Many earlier studies have found that AA as a feed additive increases CAT, SOD, and GPX activity in *Acipenser baerii* (Xie et al. 2006) and *Pelteobagrus fulvidraco* (Liang et al. 2017). In contrast, Chen et al. (2015) reported that dietary AA would not affect the GPx activity of juvenile largemouth bass. Based on earlier comparable situations, species differences could explain this phenomenon.

Serum biochemical enzyme activity (AST, ALT, and LDH) can provide useful information about liver tissue health status, as changes in the activities of these enzymes reveal liver tissue damage. The biochemical outcomes demonstrated the histological findings of the hepatopancreas and posterior kidney. In the current study, high doses of OTC (OTC160/OTC160+AA) significantly increased liver enzyme activities compared to the CTR and these results suggest OTC is a probable bother in amino acid and carbohydrate metabolism in the liver, which is the main organ of detoxification. This increase could be attributed to oxidative damage induced by OTC, which affects hepatocyte permeability, causing the outflow of these enzymes into fish blood (Yonar 2012). In conformity with our findings, increased liver enzymes in OTC-treated fish have previously been detected in *Oncorhynchus kisutch* and *O. mykiss* (Nakano et al. 2018; Moradi et al. 2022). The influence of OTC on these biomarkers was more distinct in the OTC160 compared to the OTC80 in a dose-dependent manner. OTC exposure resulted in changes in the activities of enzymes involved in gluconeogenesis in other fish species. OTC altered the activity of LDH, one of the enzymes related to gluconeogenesis, in zebrafish (Oliveira et al. 2013) and rainbow trout (Rodrigues et al. 2018). Thus, OTC may increase gluconeogenesis, which causes excessive consumption of energy and may result in a decline in organ weight.

High serum creatinine and urea levels demonstrate renal dysfunction, a low glomerular filtration rate, and muscular atrophy. The oral dosing with high doses of OTC (OTC160) notably increased the serum creatinine and urea levels in *O. niloticus*, while OTC80 only significantly elevated the serum urea levels demonstrating the nephrotoxic potential of OTC. Dietary inclusion of AA (100 mg/kg diet) significantly reduced these parameters toward control value and the histological findings of the hepatopancreas and posterior kidney proved this protective role. In this report, liver and kidney biomarkers significantly decreased following the inclusion of AA in *O. niloticus* diets, suggesting the efficacy of this nutrient in protecting hepatocytes and renal cells from damage. This was consistent with findings from previous studies on thornfish, *Terapon jarbua* (Chien & Hwang 2001), juvenile cobia (Zhou et al. 2012), and *O. niloticus* (Ibrahim et al. 2020).

Serum protein electrophoresis is a laboratory method for identifying the levels of specific proteins in serum. This enables them to be monitored and analyzed separately. It entails exposing serum in a special gel to an electric charge. This causes the various protein types to relocate and band together. The pattern of serum protein electrophoresis outcomes is determined by the fractions of two main protein types (albumin and globulins). The largest peak, albumin, is located near the +ve electrode. The following 5 constituents (globulins) are identified as alpha 1, alpha 2, beta 1, beta 2, and gamma. The peaks observed for these constituents are oriented near the -ve electrode, with the gamma peak being the nearest to it. The increased levels of beta 1% and gamma % were reported in the AA group. These types of proteins support immunity. Antibodies or immunoglobulins are usually the only proteins found in the normal gamma region. OTC+AA groups showed a substantial decrease in alpha 2% which may be related to liver disease. Albumen level and percent increased in all OTC-treated groups except OTC80+AA which showed only an increase in albumen level with an insignificant increase in albumen percent.

Similar to our results, poor feed intake and growth were also observed in *Clarias gariepinus* juveniles (Lawal et al. 2012) and *O. niloticus* (Roy 2017) at higher doses of OTC. The current study's findings supported several previous studies that found that the general acceptance of medicated feeds reduced as drug doses increased (Robinson et al. 1990; Toften and Jobling 1997; Julinta et al. 2019). The reduced survival of *O. niloticus* with increasing OTC dosing which was recorded in the current study supported by Stojanova and Luque (2017), who stated that any drug or antibiotic at higher doses than its allowable level outcomes in a toxicity reaction and becomes toxic to the host organism.

Internally, fish fed high doses of OTC showed hemorrhagic abdomen, adhesion of internal organs, and hemorrhagic liver. These results were consistent with Julinta et al. (2019) who reported pale liver and kidney and liquefaction and discoloration of internal organs in *O. niloticus* fed the higher doses of OTC.

Histopathology is a well-established method for investigating the toxicological effects of drug treatment in fish because it can distinguish, identify, and evaluate histological changes in specific key organs (Rodrigues et al. 2017). Because the liver and kidney are the organs that respond most readily to toxicants in fish, they are regarded as the best targets for histopathological studies (Rodrigues et al. 2017). In this study, the histological changes were directly correlated to the OTC dose. The most severe pathological changes of the hepatopancreas observed in OTC160 were in the form of marked necrosis of hepatic cells and pancreatic acini, accompanied by mononuclear inflammatory cell infiltration and severe vacuolar degeneration of hepatic cells. The inclusion of AA in the fish diet partially restored the disordered tissue damage in the OTC160+AA group, which presented only vacuolation of hepatocytes and normal hepatopancreas. Hepatic vacuolization was a pronounced feature that was generally caused by extra fat deposition in the cytoplasm as a result of metabolic dysfunction caused by various toxicants (Velma and Tchounwou 2010). Various authors have reported hepatic vacuolation after exposure to tetracycline in various species, which is consistent with our findings (Nunes et al. 2015; Rodrigues et al. 2017). These hepatotoxic impacts must be regarded, particularly when the fish are exposed to dosages over the prescribed dose or for a longer period than recommended.

During histopathological examination, some necrotic areas were detected in hepatic tissue. The observed hepatocyte necrosis in organisms exposed to high doses of OTC might be a result of the inhibition of DNA synthesis, which is necessary for liver growth and development. Hepatic damage in *O. niloticus* exposed to 1- and 10-days OTC at 80 and 800 mg/kg/day was also reported. In line with our findings, OTC treatment caused hepatic damage and atrophy in *O. niloticus* (Julinta et al. 2019; Limbu et al. 2021). The most common inflammatory characteristic was mononuclear inflammatory cell infiltration, which was more common in OTC160, indicating the existence of an inflammatory reaction to the high dose of OTC.

The most obvious changes observed in the posterior kidney varied from mild vacuolation of the renal tubular epithelium (OTC80) to a marked degree of renal tubular degeneration (OTC160). Once more, the kidney of the OTC160+AA group presented signs of improvement, with foci of degenerative changes within some renal tubules. Previous trials on the OTC impact on renal histology in various fish species have yielded conflicting results, with observed lesions varying from no alterations to severe necrosis (Reda et al. 2013; Soler et al. 1996). In our earlier study, another antibiotic (florfenicol; 10 mg/kg BW/day/10 days) was demonstrated to cause mild hepatocyte vacuolization consistent with glycogen storage with no changes in the renal tissues of *O. niloticus* (Abu-Zahra et al. 2023). In the current study, histopathological changes were not detected in tissues of the hepatopancreas and posterior kidney affected by AA.

The comet assay, which can detect damaged DNA strands at the individual cell level in an alkaline medium, is one of the most widely used methods for assessing genotoxicity in fish (Singh et al. 1988). It permits the accessing of DNA damage at the level of single cell by determining movement of fragmented DNA in gel-implanted cells (Kousar et al. 2022) and allows investigation of the entire genome throughout the cell cycle, not just during mitosis (Bae et al. 2020). The majority of genotoxic substances have been found to produce ROS that interacts with the DNA and causes its disturbance. In our study, high dose of OTC was reported to cause oxidative stress which plays an important role in inducing genotoxicity and cytotoxicity in hepatocytes. Furthermore, DNA damage in liver cells may be linked to OTC160-induced genetic abnormalities, which may result in the modulation of deviant and physiologically nonfunctional proteins that cause mitochondrion malfunction and nuclear protein damage. The observed OTC genotoxicity also might be clarified by OTC-DNA binding. According to Khan et al. (2003), OTC and tetracyclines overall have a strong DNA affinity and subsequently resulted in the creation of a TC-DNA binary compound that causes changes in the secondary structure of the native DNA duplex. Secondly, ROS generation and toxic metabolites formed during OTC metabolism, which causes oxidative stress and disrupts enzymatic activity, maybe another toxicological mechanism for antibiotic toxicity (Liu et al. 2014). Also, OTC forms bonds with divalent ions for instance Ca^{2+} and Mg^{2+} leading to further disruption of several biological processes (Madhu Sharma et al. 2019). More damaged DNA portions move to the comet's tail section as DNA damage increases in the cell nucleus, causing the tail region to glow more intensely and the tail length to lengthen (Kousar et al. 2022) as detected in the current study. After 28 days of OTC exposure, the antioxidant activity was impaired, resulting in oxidative stress and genotoxicity, such as abnormalities in the RBCs nucleus (Rodrigues et al. 2018). So far, only a few researches on the mutagenic and genotoxic effects of antibiotics on fish have been performed. In another research, Itoh et al. (2006) revealed DNA changes in the comet assay caused by several quinolone antibiotics. Di Cerbo et al. (2016) and Gallo et al. (2017) demonstrated that OTC induces in vitro inflammatory response, genotoxic damage, and apoptosis. In the current study, there was a dose-dependent increase in DNA damage which is consistent with Ahmed et al. (2011) who found that DNA damage in the erythrocytes of *O. mossambicus* increased with increasing arsenic concentration. The inclusion of AA in *O. niloticus* significantly reduced hepatocyte DNA damage in OTC-treated groups. Consequently, the results of this study augment the fact that fish like *O. niloticus* are good in vivo models for tracking the mutagenic effect of drugs on aquatic systems.

Once again, fish-fed AA + OTC had the lowest morbidity and mortality rates after bacterial challenge, implying that AA and OTC have another type of synergism against infections. This can be clarified by the assembly of AA's antimicrobial activity with that of OTC. Most pathogenic bacteria in the intestine prefer a pH of 7 or slightly higher; nevertheless, beneficial microbes thrive in an acidic pH range (5.8–6.2). As a result, acidifying the gastrointestinal content of tilapia more frequently decreases pathogens (particularly Gram-negative) while increasing beneficial microbes (Ng et al. 2009). Notably, AA increased the RPS of all treatments after infection, corroborating the findings of a previous study on *Pelteobagrus fulvidraco* Richardson challenged with *A. hydrophila* (Liang et al. 2017). Furthermore, AA increased resistance to other bacteria, including *Escherichia coli* (Leal et al. 2017) and *Vibrio harveyi* (Zhou et al. 2012).

In our study, fish fed on AA were healthier than those fed a control diet, which was demonstrated by the high levels of antioxidants, immune parameters, safety, and survival rates in the challenge test.

Conclusion

To the best of our knowledge, it is the first study to investigate the effect of dietary AA on OTC-induced stress in *O. niloticus*. The present study revealed that the dietary administration of high doses of OTC in *O. niloticus* notably impacted the hematological parameters, immunity, and antioxidant activity of *O. niloticus*. Also, OTC160 induced DNA damage and histological changes in *O. niloticus*. AA supplementation was reported to reestablish the suppressed immunity, as indicated by increased activities of serum total protein, globulin, IgM, NBT, gamma, and beta protein % and augmented TLC and lymphocyte values. Furthermore, the serum activity of antioxidant enzymes (SOD, CAT, and GPx) increased and that of biochemical enzymes (ALT, AST, and LDH) decreased. Also, AA is capable of reducing DNA damage and histological changes induced by OTC. Thus, dietary supplementation of AA might be an effective approach to reduce the adverse impacts of OTC on *O. niloticus* and work synergistically to increase fish disease resistance and decrease morbidity and mortality rates. The results of this trial on OTC biosafety would enhance tropical fish safety and might aid in the development of effective fish health approaches. We anticipate that our study's findings will help better understand the potential environmental risks of antibiotics, and support the progress of management approaches to minimize the impacts of these substances in the ecosystem and the consequences for farmed fish.

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Author contribution NISAZ: methodology, formal analysis, writing—original draft, writing—review and editing, resources, supervision, investigation, visualization. SS: ideas, writing—original draft, formulation of overarching research goals and aims, writing—review. AAA: resources, investigation, visualization, validation, writing—review. MME: ideas, formulation of overarching research goals and aims, project administration, writing—review.

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Data availability The authors confirm that the data supporting the findings of this study are available within the manuscript, figures, and tables.

Declarations

Competing interests The authors declare no competing interests.

Animal welfare and ethics The Institutional Aquatic Animal Care and Use Committee, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University approved the experimental protocols for the care and maintenance of live fish (approval number: IAACUC-KSU-2022-0023).

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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