RESEARCH

Implications of ammonia stress for the pathogenicity of *Shewanella* spp. in *Oreochromis niloticus*: effects on hematological, biochemical, immunological, and histopathological parameters

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Abstract

Environmental stressors (such as ammonia) in aquaculture could increase the risk of pathogenicity, posing a more severe threat to farmed fish. The aim of this study was to investigate the effects of ammonia stress on the pathogenicity of *Shewanella* spp. in *Oreochromis niloticus*. First, a 96-hour static test was used to determine the median lethal concentration (LC_{50}) of unionized ammonia to Nile tilapia. After 96 h of exposure, the Un-ionized ammonia (UIA) LC_{50} was estimated to be 4.26 mg/L. Second, an experiment was conducted to test the effect of unionized ammonia stress on the pathogenicity of *Shewanella* spp. in *O. niloticus* for 30 days. A study involved 180 fish divided into six groups, with the first group serving as a control. The second group (AMN1/10) and the third group (AMN1/20) were not challenged and were exposed to 1/10 (0.42 mg/L) and 1/20 (0.21 mg/L) of the 96-hour LC_{50} of UIA, respectively. Then 0.2 mL (0.14×10^5) of *Shewanella* spp. was intraperitoneally injected into the fourth (SH), fifth (SH+AMN1/10), and sixth (SH+AMN1/20) groups, which were subjected to 0, 1/10 (0.42 mg/L), and 1/20 (0.21 mg/L) of the 96-hour LC_{50} of UIA, respectively. The survival rate, hematological indices, immunological parameters, and antioxidant activity of the fish significantly decreased when they were exposed to ammonia and *Shewanella* infection separately or together. Histopathological changes were also observed in the kidney and liver. Furthermore, both individual and combined exposures significantly altered renal and hepatic function, with notable increases in glucose and cortisol levels, as well as in the expression of proinflammatory cytokine genes (*TNF-α* and *IL-1ß*). However, the detrimental effects of co-exposure to ammonia stress and *Shewanella* infection were greater than those of separate exposures. As a result, we may say that increased ammonia concentrations enhance the

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infection of *Shewanella* spp. These findings could contribute to a better understanding of *Shewanella* infection in Nile tilapia.

Keywords *Shewanella*, Nile tilapia, Ammonia stress, Cortisol, Immunity

Introduction

In a world where environmental issues and growing populations are major concerns, the pursuit of sustainable development is more important than ever. In this effort, aquaculture is seen as a potent tool that provides a wide range of answers to complex problems that Sustainable Development Goals (SDGs) aim to solve [1, 2]. SDG 2, "Zero Hunger," aims to end hunger and malnutrition by 2030. Aquaculture stands as a potent weapon in this fight. It provides a reliable and nutritious source of protein that is essential for healthy growth and development, particularly in vulnerable populations [1, 3, 4]. Compared to terrestrial livestock, aquaculture offers higher protein conversion rates, requiring less feed and land per unit of protein produced [5, 6]. This makes it a particularly efficient option for food production in resource-constrained environments.

Despite its remarkable potential, aquaculture faces challenges, such as disease outbreaks and environmental stressors, that need to be addressed for its sustainable development. Ammonia, a byproduct of fish metabolism, is a natural component of aquatic environments. However, at high concentrations, it becomes highly toxic to aquatic organisms. In aquaculture, where stocking densities are often high, ammonia levels can easily rise above tolerable limits due to factors such as overfeeding, poor water quality management, and inadequate biofiltration [7–9]. This results in a cascade of negative consequences for farmed fish species [10–12]. One of the most concerning consequences of ammonia stress is its immunosuppressive effect. Elevated ammonia levels impair the immune function of fish and shellfish, reducing their ability to fight infections and resist disease. This weakens their defense against opportunistic pathogens, paving the way for disease outbreaks, especially through opportunistic pathogens [13–16]. In addition, disruptions in the microbiome can occur, allowing previously harmless or suppressed microbes to proliferate or introduce completely new microbes from the environment [14, 17, 18].

Shewanella is a genus of rod-shaped, gram-negative bacteria belonging to the Shewanellaceae family of the order Alteromonadales [19]. While often overshadowed by other aquatic pathogens, *Shewanella* bacteria can emerge as opportunistic threats in aquaculture settings [20]. These adaptable microorganisms, commonly found in diverse aquatic environments, pose a risk to farmed fish when stressed or weakened by factors such as ammonia stress [14, 18, 21]. *Shewanella* infection manifests in various ways depending on the species and strain involved. Skin lesions, hemorrhages in internal organs, and large-scale mortality were recorded in different cases, leading to severe economic losses [22–24].

The rise of *Shewanella* in aquaculture poses a new challenge to the industry. Previously, this bacterium has not been a major cause for concern compared to other pathogenic bacteria such as *Aeromonas*, *Streptococcus*, and *Edwardsiella*. However, recent reports suggest an increase in *Shewanella*-related infections and mortality rates within fish farms [22, 24–26]. To the best of our knowledge, no study has looked at how *Shewanella* spp. infections change with various concentrations of ammonia in *Oreochromis niloticus* (*O. niloticus*). Therefore, the goal of this study was to investigate how ammonia stress, which is considered a natural stressor in aquaculture systems, affects the pathogenicity of *Shewanella* spp. in *O. niloticus*.

Materials and methods

Fish

Three hundred and sixty *O. niloticus*, with an initial average weight of 26.13±0.07 g, were acquired from the Fish Research Unit of the Faculty of Veterinary Medicine at Zagazig University in Egypt. A total of 180 fish were used to determine the median 96-hour lethal concentration (96-h LC_{50}) of unionized ammonia (UIA), and the remaining 180 fish were used for the experimental trial. Fish were carefully transported to the Department of Aquatic Animal Medicine lab located on the same faculty. To help the fish adapt to laboratory conditions, they were placed in 100-liter aquariums with constant aeration and dechlorinated tap water for a period of 14 days. Fish were inspected for health upon arrival before being used in the experiment [27, 28]. According to APHA [29], the water parameters were adjusted during both the acclimatization and experimental phases. The water parameters were measured as follows: pH 6.60±0.50, temperature 22.5±0.50 ℃, ammonia 0.02±0.001 mg/L, nitrite 0.02±0.012 mg/L, nitrate 0.15±0.02 mg/L, dissolved oxygen 5.50±0.40 mg/L, and 12-hour photoperiod: 12-hour darkness. During the acclimatization and experimental period, The amount of food provided in each aquarium was calculated to equal 3% of the total live fish weight. Fish were fed a control diet (Table 1) [30] designed to fulfill the standard nutritional needs of tilapia. Fish were fed a pelleted diet (1.5 mm) at 9:00 a.m. and 3:00 p.m. Pellets were evenly distributed across the water surface of the aquarium. Feeding continued until fish showed signs of satiety, such as spitting out pellets,

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Table 1 Composition of basal diet (%)

Ingredients	Percentage of ingredient
Fish meal (65.4% CP)	40
Soybean meal (44%)	20
Yellow corn	13
Wheat flour	15
Wheat Bran	2
Fish oil	7
Monocalcium phosphate	2
[1] Vitamin mixture	0.45
[2] Mineral mixture	0.55
Chemical analyses (% DM)	
Crud Protein	38.90
Crude fat	10.50
Ash	5.84

[1]**Vitamin mix (IU or mg kg diet)**: vitamin A, 16,000 IU; vitamin D, 8000 IU; vitamin K, 14.72; thiamin, 17.8; riboflavin, 48; pyridoxine, 29.52; cynocobalamine, 0.24, tocopherols acetate, 160; ascorbic acid (35%), 800; niacinamide, 79.2; calcium-D- pantothenate,73.6; folic acid, 6.4; biotin, 0.64 L-carnitine, 100

[2]**Mineral mix (mg kg diet)**: Cu ($CuSO_4$), 2.0; Zn ($ZnSO_4$), 34.4; Mn ($MnSO_4$), 6.2; Fe ($FeSO_4$), 21.1; I (Ca (IO_3)2), 1.63; Se (Na_2SeO_3), 0.18; Co ($CoCl_2$), 0.24; Mg ($MgSO_4.H_2O$), 52.7

or until no more pellets were accepted by fish [31, 32]. Every day, the old water was replaced with fresh, dechlorinated water.

Ammonium chloride

The source of ammonia in this study was ammonium chloride (NH_4Cl), which was purchased from El-Gomhouria Co., Egypt. Daily, an NH_4Cl solution was prepared by the combination of NH_4Cl and dechlorinated, filtered potable water.

Shewanella spp. isolate

The *Shewanella* spp. (GenBank accession number: OP942237) used in this study were previously isolated from *O. niloticus* that were moribund. *Shewanella* spp. identification, characterization, and determination of their median lethal dose (LD_{50}) were determined as mentioned in Reda, El-Murr [33]. The *Shewanella* spp. isolate was re-cultured for 24–48 h at 22 °C on tryptic soy agar medium (Oxoid, England). The bacterial isolate concentration was adjusted to 0.14×10^5 CFU/mL [33] using McFarland Standard No. 0.5 for the experimental challenge.

Determination of the median 96-hour lethal concentration (96-h LC_{50}) of unionized ammonia (UIA)

One hundred eighty fish were divided into six groups. Each group had three replicates (10 fish per replicate, 30 fish per group). The groups from one to six were exposed to 0, 2, 4, 6, 8, or 10 mg/L of UIA. The fish were subjected to the previously specified UIA concentrations in a semistatic condition, with daily UIA measurements and water renewals (if necessary) to maintain a constant ammonia concentration [34, 35]. During the 96-hour exposure period, the fish were not fed in accordance with OECD recommendations [36]. Fish mortality was recorded daily and removed immediately. The 96-h LC_{50} value was calculated using probit analysis with IBM® SPSS® Statistics 23 [37]. After 96 h of exposure, the UIA LC_{50} was estimated to be 4.26 mg/L (Fig. 1).

Experimental plan

For 30 days, 180 fish were divided into six groups in triplicate (30 fish per group; 10 fish per replicate). The first group served as the control group (CONT), the second
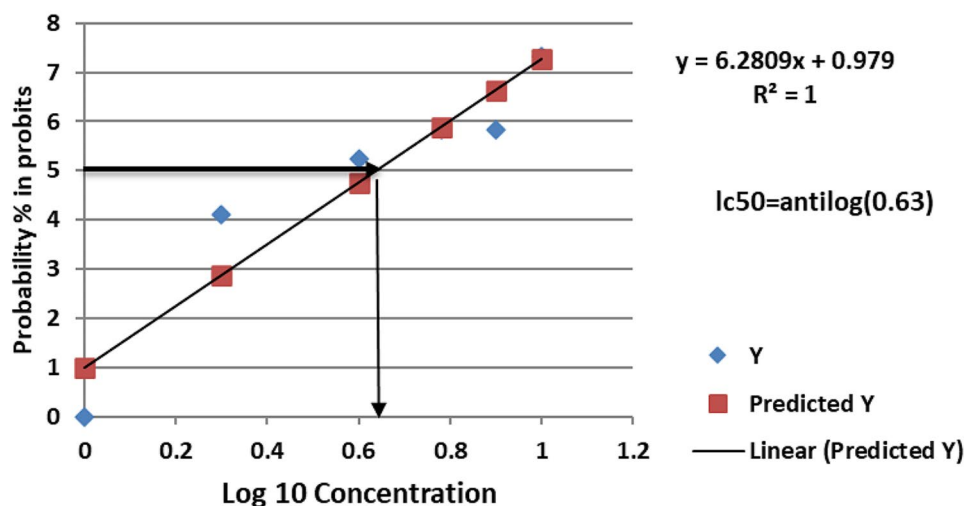

Fig. 1 Probit analysis graph showing LC_{50} of unionized ammonia in *Oreochromis niloticus*

group (AMN1/10), and the third group (AMN1/20) were exposed to 1/10 (0.42 mg/L) and 1/20 (0.21 mg/L) of the 96-hour LC_{50} of UIA, respectively, without being challenged. The fourth (SH), fifth (SH+AMN1/10), and sixth (SH+AMN1/20) groups were anesthetized with a 100 mg/L benzocaine solution [38] and then intra-peritoneal (IP) injected with 0.2 mL (0.14×10^5) of *Shewanella* spp. and exposed to 0, 1/10 (0.42 mg/L), or 1/20 (0.21 mg/L) of the 96-h LC_{50} of UIA, respectively. Water was completely changed daily with suction to uneaten food and waste materials and replaced with dechlorinated tap water with adjustment of the UIA concentration for the UIA-exposed group. The actual UIA concentration was measured twice a day for 24 h using the HI-715 (HANNA, Milan, Italy) before and after water exchange and the addition of a freshly prepared ammonium chloride solution to maintain UIA at the levels that were wanted [39]. UIA levels before and after water exchange were 0.50 ± 0.51 and 0.42 ± 0.30 mg/L, respectively ($n=9$/treatment), for AMN1/10 and AMN1/10+SH, and 0.32 ± 0.30 and 0.21 ± 0.25 mg/L, respectively ($n=9$/treatment) for AMN1/20 and AMN1/20+SH. Throughout the experiment, the clinical signs and mortality of the fish were documented daily.

Sampling

At the end of the experiment (after 30 days), nine fish per group were chosen at random and anesthetized with a 100 mg/L benzocaine solution [38]. Two sets of blood samples were collected from caudal vessels. To measure phagocytic activity (PA) and hematological indices, one set was taken using syringes that had been heparinized to hold one milliliter of blood. To separate the serum, another set of blood samples was drawn without the use of an anticoagulant and centrifuged for 10 min at 4 °C and 3000 rpm. Until the biochemical and immune parameters were assessed, the serum was kept at -20℃. Liver samples (9 fish/group) from the same fish that were killed by an overdose of benzocaine solution (400 mg/L) were obtained to calculate the oxidant/antioxidant indices [40].

Analytical parameters
Hematological indices
A hemocytometer (Hospitex Diagnostics, Sesto Fiorentino, Italy) was used to count the total number of white blood cells (WBCs) and red blood cells (RBCs). The Jain [41] method was used to measure the packed cell volume (PCV%) and hemoglobin (Hb) content.

Biochemical indices
The serum alanine aminotransferase (ALT, Catalog No.; MBS038444), aspartate aminotransferase (AST, Catalogue No.; MBS1601734), and urea (Catalog No.;

MBS8305389) levels were determined in accordance with the manufacturer's guidelines (MyBioSource, Inc., San Diego, CA 92,195−3308: USA). The method of Fossati, Prencipe [42] was used to measure the creatinine concentration. Calorimetric analysis was used to measure the serum cortisol and glucose levels using the methods of Saliu, Oluberu [43] and Trinder [44], respectively.

Oxidant/antioxidant assays
Using a spectrophotometric method, the oxidant/antioxidant indices in the hepatic homogenate were determined. The protocol described by Reda, Helmy [45] was followed for preparation of the hepatic homogenate. Spectrophotometry was used to estimate the total antioxidant capacity (TAC) (Catalog no. MBS2540515), catalase (CAT) (Catalog no. MBS038818), and malondialdehyde (MDA) (Catalog no. MBS007853) (MyBioSource, Inc., San Diego, CA 92,195−3308: USA). Colorimetric measurement of reduced glutathione (GSH) was conducted using the Beutler, Duron [46] method.

Immune indices assay
Using heat-inactivated *Candida albicans*, the phagocytic index (PI) and activity (PA%) were calculated using the following formulas [47]:

PI = total no. of phagocytized yeast/no. of phagocytic cells phagocytizing yeast

PA% = (No. of phagocytic cells phagocytizing yeast/ Total no. of phagocytic cells counted) × 100

Using spectrophotometry based on the lysis of freeze-dried *Micrococcus lysodeikticus* particles, serum lysozyme (LYZ) activity was determined [48]. Using the method described by Montgomery and Dymock [49], nitric oxide (NO) was measured. Interleukin-1β (IL-1β) (Catalog no. MBS283380) and tumor necrosis factor-α (TNF-α) (Catalog no. MBS704369) were estimated in accordance with the manufacturer's instructions (MyBioSource, Inc., San Diego, CA 92,195−3308: USA).

Histopathological study
Liver and kidney specimens (9 fish/group) were sampled and immersed in a 10% neutral buffered formalin fixative for 48 h. The fixed specimens were manually processed by the paraffin technique, sectioned at 4 μm thickness where two Sect. (50 μm apart) per organ per fish were obtained, and the tissue samples were routinely stained with hematoxylin and eosin as described previously by Suvarna, Layton [50]. Next, a multiparametric numeric assessment of the histological condition of the hepatic and renal tissues was performed to determine the organ histological indices was carried out as established by Bernet, Schmidt [51] using an AmScope CMOS C-Mount microscope digital camera (United Scope LLC., CA.,

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USA) attached to a Nikon light microscope (Nikon Inc., NY, USA).

Statistical analysis
To ensure that the gathered data were regularly distributed, Shapiro-Wilk's test was used. One-way analysis of variance (ANOVA), was used to identify noteworthy differences between the treatments. To identify differences between means, Tukey multiple range tests were used. Kaplan Meier analysis revealed noteworthy variations in the survival curve. SPSS Version 23 for Windows was used to conduct the statistical analysis (SPSS Inc., Chicago, IL, USA). The 96-h LC_{50} of UIA was determined using probit analysis with IBM® SPSS® Statistics 23. The means±standard errors (SE) are displayed for the data.

Results
Clinical symptoms
There were no abnormal symptoms recorded in the control group. The symptoms observed in both the AMN1/10 and AMN1/20 groups were almost identical, but they were more severe in the AMN1/10 group. The symptoms presented as darkness of the skin coloration and fin destruction (Fig. 2A). Some of the fish were aggregated near the water surface with lethargy movement and then died with open-mouthed. In the SH group severe fin rot, hemorrhage in the lower jaw was observed in some cases, and hemorrhage at the gill cover and ascites were observed in others (Fig. 2B, C, and D). The symptoms observed in the SH+AMN1/10 and SH+AMN1/20 groups were nearly the same, but they were more severe in the SH+AMN1/10 group (Fig. 2E, F, and H). The fish exhibited symptoms such as fin rot and scale loss, heightened mucus secretion, the presence of ulcers in certain cases, an expanded and exposed gill cover, and an open mouth. According to the Kaplan Meier curve data, 100% of the fish in the control group survived, while 83.3%, 93.3%, 80.0%, 70.0%, and 60.0% of the fish in the AMN1/10, AMN1/20, SH, SH+AMN1/20, and SH+AMN1/10 groups, respectively survived (Fig. 3). Internally, the main lesions recorded in the AMN1/10, and AMN1/20 groups were at the gills. The fish gills exhibited excessive mucus secretion and swelling in the gill filaments. In the AMN1/10 group, some fish displayed severe congestion in the gills, while others had pale gills. The same lesions were nearly the same in the SH, SH+AMN1/10, and SH+AMN1/20 groups, in addition to congestion in the liver, spleen, and kidney, as well as in the ascitic fluid in some fish (Fig. 4).

Hematological indices
As shown in Table 2, there were no significant differences in the RBC, Hb, PCV%, and WBC counts between the control, AMN1/10, or AMN1/20 groups. The RBC,

Hb, PCV%, and WBC counts were significantly lower (P=0.002, P=0.001, P=0.003, *and* P=0.0001, respectively) in the SH group than in the control group, followed by the SH+AMN1/10 group and the SH+AMN1/20 group.

Biochemical indices
ALT and AST levels were significantly elevated (P=0.007) in the SH+AMN1/10, SH+AMN1/20, SH, AMN1/10, and AMN1/20 groups compared to those in the control group (Fig. 5A and B). Compared to those in the control group, the creatinine levels in all groups were significantly different (P=0.033), following the pattern of SH+AMN1/20>SH+AMN1/10>SH>AMN1/10>AMN1/20 (Fig. 5C). Urea levels were significantly greater (P=0.003) in the SH, SH+AMN1/10, and SH+AMN1/20 groups than in the other groups, but there were no significant differences between them (Fig. 5D).

Similarly, the cortisol concentrations were significantly different (P=0.04) between the groups, following the pattern of the SH+AMN1/10>SH+AMN1/20>SH>AMN1/10>AMN1/20 compared to those in the control group (Fig. 6A). Glucose levels were significantly elevated (P=0.0001) in the SH+AMN1/10, SH+AMN1/20, and SH groups (without differences between them), followed by the AMN1/10 group, and the AMN1/20 group compared to those in the control group (Fig. 6B).

Oxidant/antioxidant indices
As shown in Fig. 7, compared with those in the control group, the antioxidant parameters (TAC, CAT, and GSH) were lower (P=0.0001) and the MDA level (P=0.0001) was greater noticed in the following pattern: SH+AMN1/10<SH<SH+AMN1/20<AMN1/10<AMN1/20.

Immune indices
Figure 8 shows that compared with those in the control group, the PA%, PI, and LYZ activities in the treatment groups were significantly lower (P=0.000, P=0.04, and P=0.01, respectively) in the order SH+AMN1/10<SH+AMN1/20<SH. AMN1/10 and AMN1/20 were also significantly lower in PA% and PI than in the control group, whereas the LYZ activity in AMN1/10 was significantly lower than that in AMN1/20. The NO levels were significantly lower (P=0.001) in the SH, SH+AMN1/10, SH+AMN1/20, AMN/10, and AMN1/20groups compared to those in the control group.

The IL-β and TNF-α levels were significantly elevated (P=0.0001) in the SH+AMN1/10, SH+AMN1/20, SH, AMN/10, and AMN1/20 groups compared to those in the control group (Fig. 9).
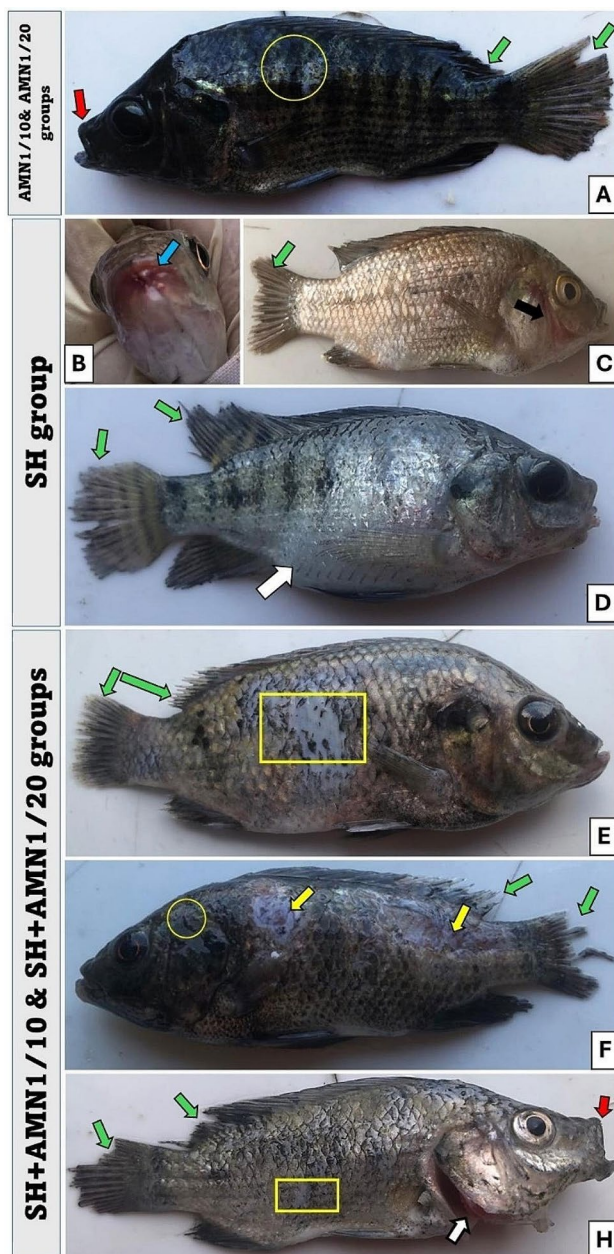
Fig. 2 Clinical signs of Nile tilapia challenged with *Shewanella spp.* and reared under different ammonia levels. (**A**) The symptoms in the AMN1/10 and AMN1/20 groups are nearly identical, with an increase in intensity in the AMN1/10 group. The symptoms manifest as darkness in skin coloration (yellow circle), fin rot (green arrows), and an open mouth (red arrow). (**B**, **C**), and (**D**) The SH group manifested hemorrhage in the lower jaw (blue arrow), hemorrhage at the gill cover (black arrow), severe fin rot (green arrow), and ascites in some cases (white arrow). (**E**, **F**), and (**H**) The symptoms in the SH+AMN1/10 and SH+AMN1/20 groups are almost identical, with an increase in intensity in the SH+AMN1/10 group. Fish manifested fin rot (green arrows) and scale loss (yellow rectangular), increased mucus secretion (yellow circle), ulcers in some cases (yellow arrow), an enlarged and opened gill cover (white arrow), and an open mouth (red arrow). AMN1/10, and AMN1/20 groups were non-challenged groups and exposed to 0.42, and 0.21 mg/L of unionized ammonia, respectively. SH, SH+AMN1/10, and SH+AMN1/20 groups were intraperitoneal challenged with Shewanella spp. and exposed to 0, 0.42, and 0.21 mg /L of unionized ammonia, respectively
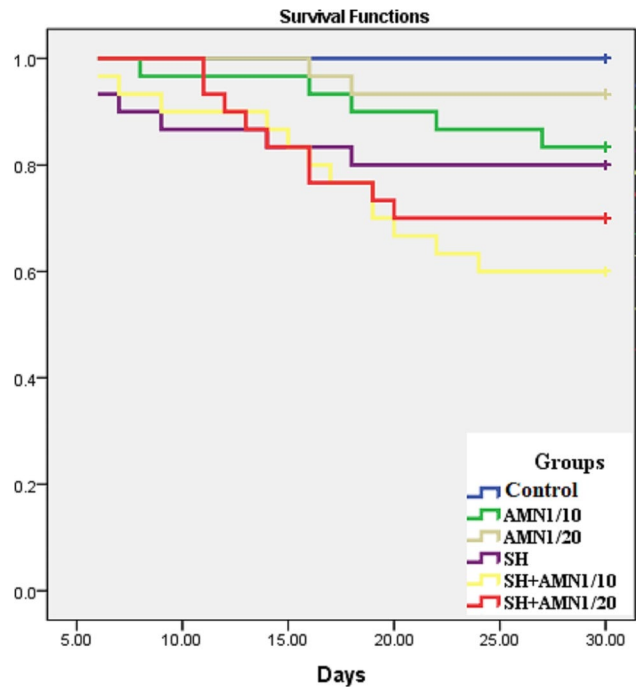

Fig. 3 Survival Kaplan Meier curves for Nile tilapia challenged with Shewanella spp. and reared under different ammonia levels. Control, AMN1/10, and AMN1/20 groups were non-challenged groups and exposed to 0, 0.42, and 0.21 mg/L of unionized ammonia, respectively. SH, SH+AMN1/10, and SH+AMN1/20 groups were intraperitoneal challenged with Shewanella spp. and exposed to 0, 0.42, and 0.21 mg/L of ammonium chloride, respectively

Histological findings

The microscopic examination revealed normal histoarchitectures of the hepatopancreas with no histological alterations in the control group (Fig. 10A). Conversely, exposure to high ammonia concentrations incited an array of morphological alterations, including notable cellular swelling of a lipoidal nature (clear cytoplasm with pushing of the nuclei to the periphery of the cells), congestions of the blood vessels, and sinusoidal capillaries, single cell necrosis, and lytic necrotic foci usually infiltrated by extravasated erythrocytes. These alterations were more severe in the AMN1/10 group (Fig. 10B) (liver index=15.90±1.64) than in the AMN1/20 group (Fig. 10C) (liver index=8.90±2.06). In the SH group numerous hepatopathic changes including necrosis of the melanomacrophage centers (MMCs), vascular congestion, inflammatory cell infiltration primarily with mononuclear cells, and lytic necrotic foci usually infiltrated with inflammatory cells (Fig. 10D), (liver index=16±0.55). Co-exposure to *Shewanella* spp. infection and high levels of ammonia caused significant hepatic damage, as indicated by notable single-cell necrosis, multifocal lytic necrotic foci, large areas of lytic necrosis, and obvious inflammatory cell infiltrates including eosinophilic granule cells. These alterations
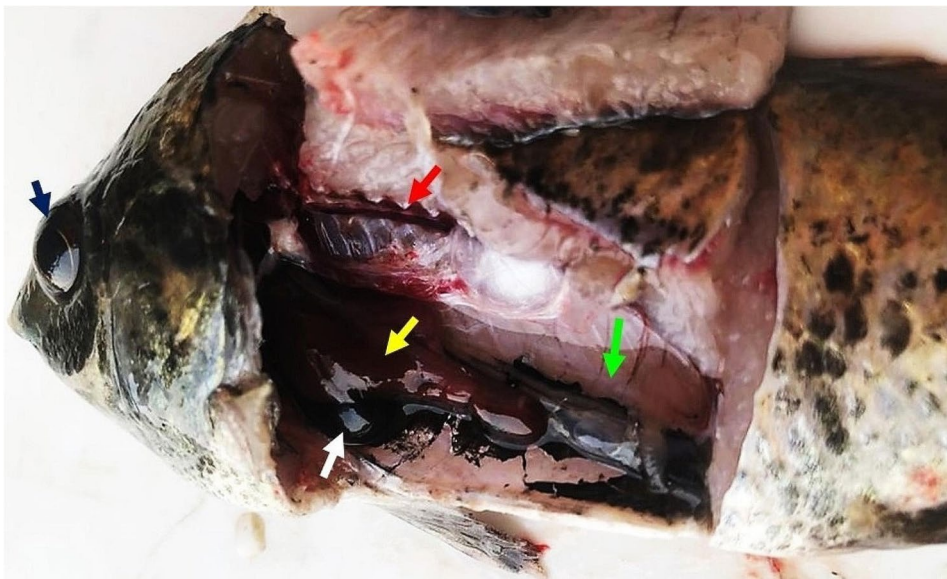
Fig. 4 Postmortem findings of Nile tilapia challenged with Shewanella spp. and reared under different ammonia levels showing congested liver (yellow arrow) and kidney (red arrow), enlarged gall bladder (white arrow) with ascitic fluid (green arrow), and slightly protruded eye (blue arrow)

Table 2 Hematological parameters of *O. niloticus* infected with *Shewanella spp* and exposed to ammonia

Groups	RBCs (10^6/cmm)	Hb (g/dL)	PCV (%)	WBCs ($\times 10^3$/cmm)
CONT	2.11 ± 0.13^a	4.35 ± 0.33^a	15.83 ± 1.03^a	32.63 ± 1.10^a
AMN1/10	2.15 ± 0.06^a	4.46 ± 0.23^a	15.64 ± 0.59^a	30.50 ± 0.88^a
AMN1/20	2.22 ± 0.09^a	4.33 ± 0.21^a	16.10 ± 0.55^a	31.33 ± 0.69^a
SH	1.26 ± 0.33^d	3.05 ± 0.81^c	9.18 ± 2.07^c	18.00 ± 0.70^d
SH+AMN1/10	1.37 ± 0.14^c	3.32 ± 0.41^b	11.03 ± 1.25^b	21.33 ± 1.44^c
SH+AMN1/20	1.50 ± 0.16^b	3.52 ± 0.37^b	11.16 ± 1.02^b	27.10 ± 1.74^b
P-value	<0.01	<0.01	<0.01	<0.001

RBCs, total counts of erythrocytes; WBCs, leukocytes; Hb, Hemoglobin, PCV %, Packed Cell Volume. Values (mean ± *SE*) that don't share superscripts in the same row significantly differ at *P* < 0.05 (One-way ANOVA; Duncan's post hoc test) (*n*=9/group). CONT, AMN1/10, and AMN1/20 groups were non-challenged groups and exposed to 0, 0.42, and 0.21 mg L^{-1} of ammonium chloride, respectively. SH, SH+AMN1/10, and SH+AMN1/20 groups were intraperitoneal challenged with *Shewanella spp* and exposed to 0, 0.42, and 0.21 mg L^{-1} of ammonium chloride, respectively

were more severe in the AMN1/10+SH group (Fig. 10E), (liver index=23.70±1.13) than in the AMN1/20+SH group (Fig. 10F), (liver index=20.30±1.59). There were no neoplastic, or preneoplastic alterations in the hepatocytes. Additionally, the scattered pancreatic tissue and biliary system showed no significant histological alterations in any of the groups. The details of the hepatopathic alterations and liver indices of all the groups are summarized in Table 3.

Normal histology with no histological alterations was evident in the renal tissue specimens of the control group (Fig. 11A). Kidneys from the AMN1/10 group exhibited a vast array of morphological changes, most importantly, notable vacuolation and necrosis of the tubular epithelium, and glomerular necrosis (Fig. 11B), (kidney index=21.40±0.96). However, the kidneys of the AMN1/20 group exhibited some nephropathic alterations such as vascular congestion, vacuolation of the tubular epithelium, and necrosis of the MMCs (Fig. 11C), (kidney index=15.30±2.01). Kidneys from the SH group exhibited a few nephropathic alterations represented by vascular congestion, and inflammatory cell infiltrates of a mononuclear nature (Fig. 11D), (kidney index=18.40±1.52). Infection with *Shewanella* spp. in combination with high levels of ammonia in water exacerbated kidney cell injury in the AMN1/10+SH and AMN1/20+SH groups. In both groups, tubular vacuolations, necrosis, glomerular lobulations, necrosis, interstitial inflammatory cell infiltration, vascular congestion, endothelial hypertrophy, and hyperplasia are accompanied by hyalinization and vacuolations of the tunica media. Kidney cell injury was more severe in the AMN1/10+SH group (Fig. 11E), (kidney index=31.80±1.29) than in the AMN1/20+SH group (Fig. 11F), (kidney index=26.70±1.22). The details of the nephropathic alterations and kidney indices of all the groups are summarized in Table 3.

Discussion

Understanding and maintaining ammonia levels are crucial in aquaculture systems. Fish excretion, uneaten feed decomposition, and high stocking density in aquaculture systems all contribute to ammonia [52]. Most ammonia exists in a harmless form (ionized ammonia, NH_4). However, a portion of ammonia exists as unionized ammonia (NH_3), which is highly toxic to fish [53, 54]. When fish are stressed due to factors such as unionized ammonia,

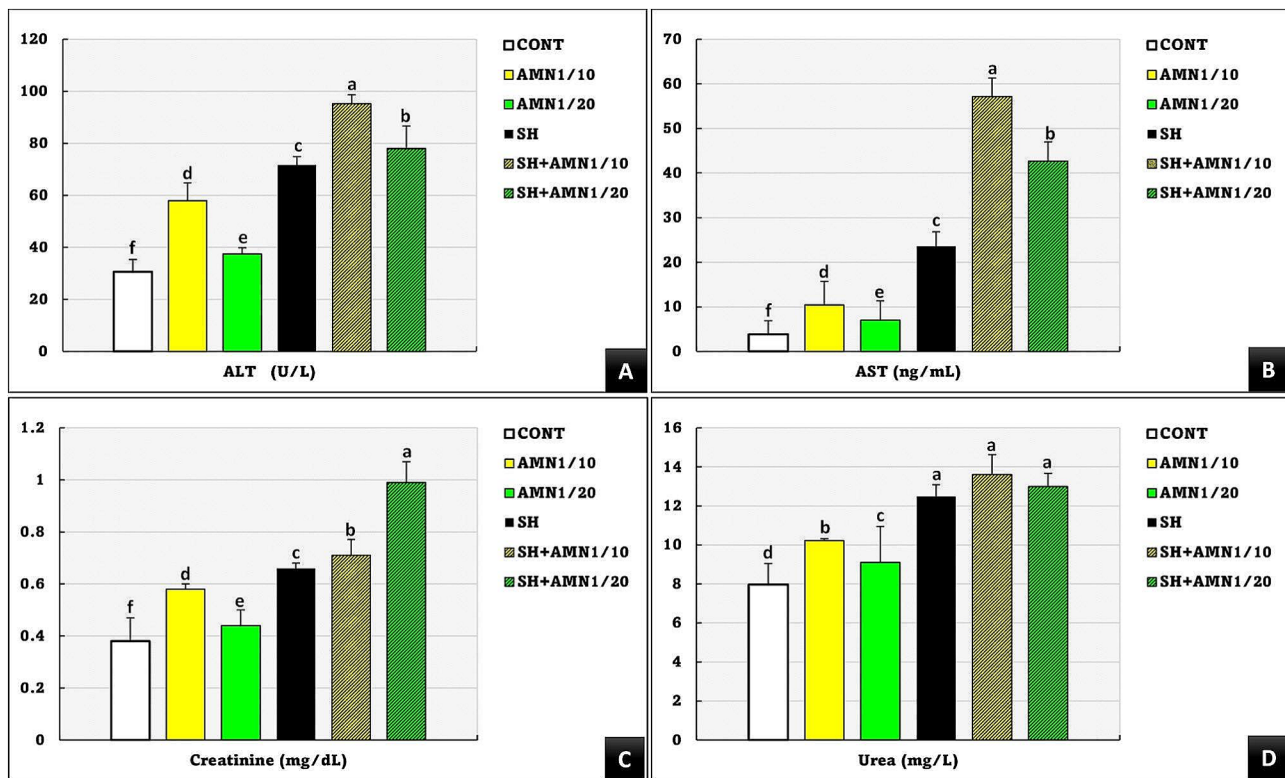Reda *et al. BMC Veterinary Research*   (2024) 20:324

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Fig. 5 Alanine aminotransferase (ALT, **A**), aspartate transaminase (AST, **B**), creatinine (**C**), and urea (**D**) for Nile tilapia challenged with Shewanella spp. and reared under different ammonia levels. Values are presented as the mean ± SE ($n = 9$/group). The bars with different superscripts (**a**, **b**, **c**, **d**, **e**, and **f**) are significantly different ($P < 0.05$, one-way ANOVA). Control, AMN1/10, and AMN1/20 groups were non-challenged groups and exposed to 0, 0.42, and 0.21 mg/L of unionized ammonia, respectively. SH, SH + AMN1/10, and SH + AMN1/20 groups were intraperitoneal challenged with Shewanella spp. and exposed to 0, 0.42, and 0.21 mg/L of ammonium chloride, respectively

their immune system becomes less effective. This creates an opportunity for opportunistic bacteria present in the water to invade the fish and cause disease. Stress can also alter the internal environment of fish, increasing susceptibility to bacterial growth and potentially even increasing the virulence (harmfulness) of certain bacteria [10, 15, 55–57]. Some studies have linked the outbreak of some diseases in aquatic systems to the quality of water parameters [14, 58–61]. A meta-analysis by Paredes-Trujillo and Mendoza-Carranza [59] revealed a significant association between ammonia (NH_3) and infectious diseases in 41% of the investigated publications.

This study revealed similar symptoms in the AMN1/10 and AMN1/20 groups, but more severe symptoms in the AMN1/10 group. Symptoms included skin color change to dark coloration, fin destruction; lethargy, open mouths, excessive mucus secretion, swelling, and congestion in the gills. The detrimental effects of ammonia on muscle membranes and metabolism, which can result in lethargy, could explain the recorded symptoms. Additionally, the damaging impact of ammonia on gill filaments can result in excessive mucus secretion, which can cause respiratory distress [11, 12, 53]. In another study, *O. niloticus* newly hatched larvae exposed

to 0.5–0.6 mg/l UIA exhibited signs of evidence of losing equilibrium, swimming sideways, and attempting to breathe oxygen. Some were pale and had excess mucus in their skin and gills [62]. Furthermore, hyperactivity and rapid operculation were recorded in O. niloticus exposed to 0.5 mg/L UIA after 120 h of exposure [55]. Variations in fish response and severity of signs of ammonia toxicity across studies can stem from differences in ammonia concentration, exposure duration, fish species and their life stages, and water quality parameters. In the present study, the SH group exhibited severe fin rot, lower jaw hemorrhage, and ascites in some cases. The virulence factors of *Shewanella* species, including their capacity to release cytotoxins, adhere to host cells, and produce siderophores, may be attributed to the appearance of these symptoms [20]. However, the SH + AMN1/10 group in this study showed more severe symptoms, including scale loss, mucus secretion, ulcers, expanded gill cover, and fish death with an open mouth with congestion in the internal organs. In a similar study, Abdel-Latif, Shukry [56] reported that exposure of *O. niloticus* to both ammonia and *Aeromonas hydrophila* simultaneously worsened the severity of this disease. Research on the impact of bacterial infection in the presence of
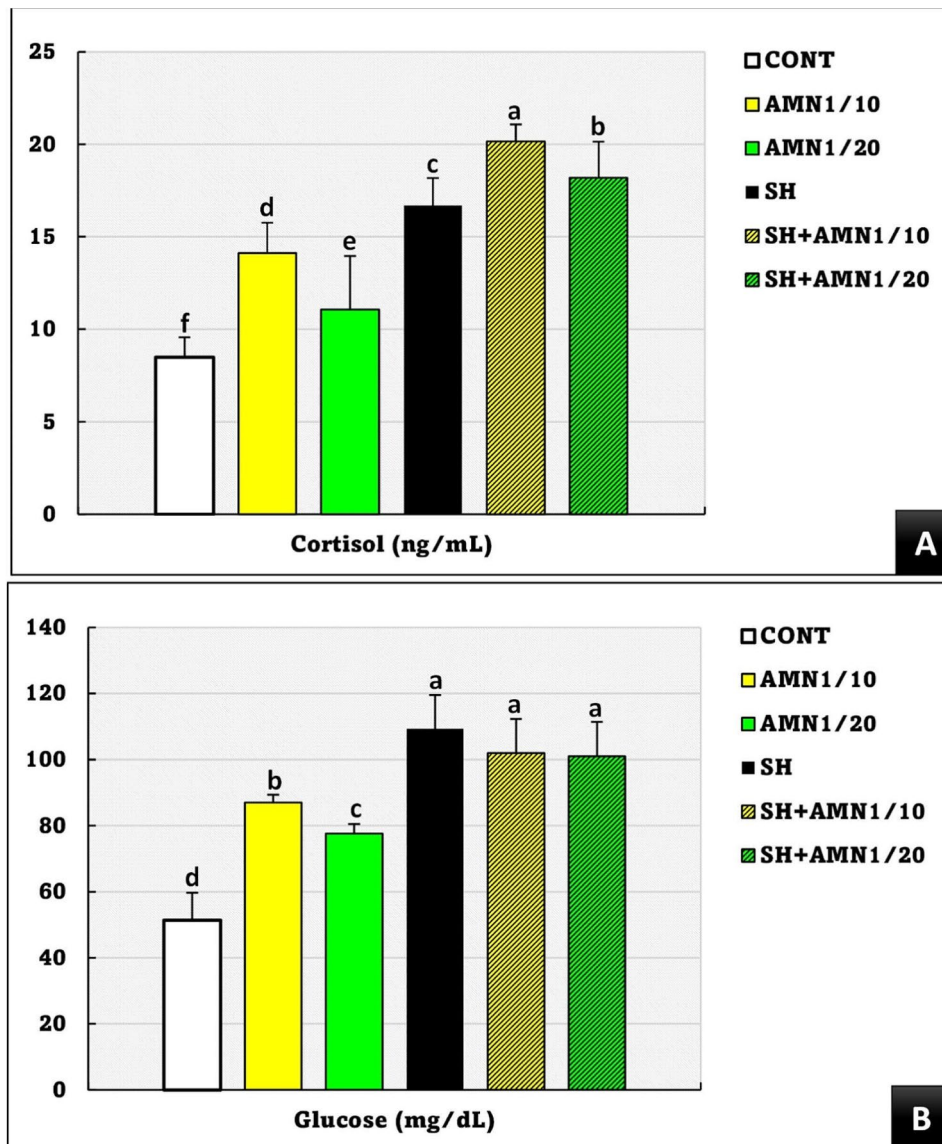
Fig. 6 Cortisol (**A**), and glucose (**B**) levels for Nile tilapia challenged with Shewanella spp. and reared under different ammonia levels. Values are presented as the mean ± SE ($n = 9$/group). The bars with different superscripts (**a**, **b**, **c**, **d**, **e**, and **f**) are significantly different ($P < 0.05$, one-way ANOVA). Control, AMN1/10, and AMN1/20 groups were non-challenged groups and exposed to 0, 0.42, and 0.21 mg/L of unionized ammonia, respectively. SH, SH + AMN1/10, and SH + AMN1/20 groups were intraperitoneal challenged with Shewanella spp. and exposed to 0, 0.42, and 0.21 mg/L of ammonium chloride, respectively

ammonia stress on fish survival has been inconsistent. Several studies agree with us that bacterial infection in the presence of ammonia stress has a negative effect on the survival rates of fish [14, 18, 56]. Abreu, Magalhães [63] reported that this might be because certain bacteria, such as those in the Aeromonadaceae family, use ammonia as a growth factor, which promotes their proliferation and virulence. On the contrary, Farmer, Mitchell [64] reported that immersing fish in NH_4Cl at a concentration of 46.3 mg/L decreased the mortality rate of *Flavobacterium columnare* infected fish. The authors of this study have proposed several hypotheses, the most important of

which is that ammonia hampers the ability of bacteria to attach to the fish body.

Hematological examination is crucial in assessing fish health following exposure to environmental stressors like ammonia [65]. Changes in red blood cell counts, hemoglobin content, and white blood cell counts can indicate anemia, infections, or immune system stress. These alterations can be linked to disease or exposure to pollutants in the water, making blood analysis a valuable tool for monitoring fish health and water quality [66, 67]. Therefore, examining these hematological parameters in *Shewanella* spp.-challenged *O. niloticus* exposed to ammonia
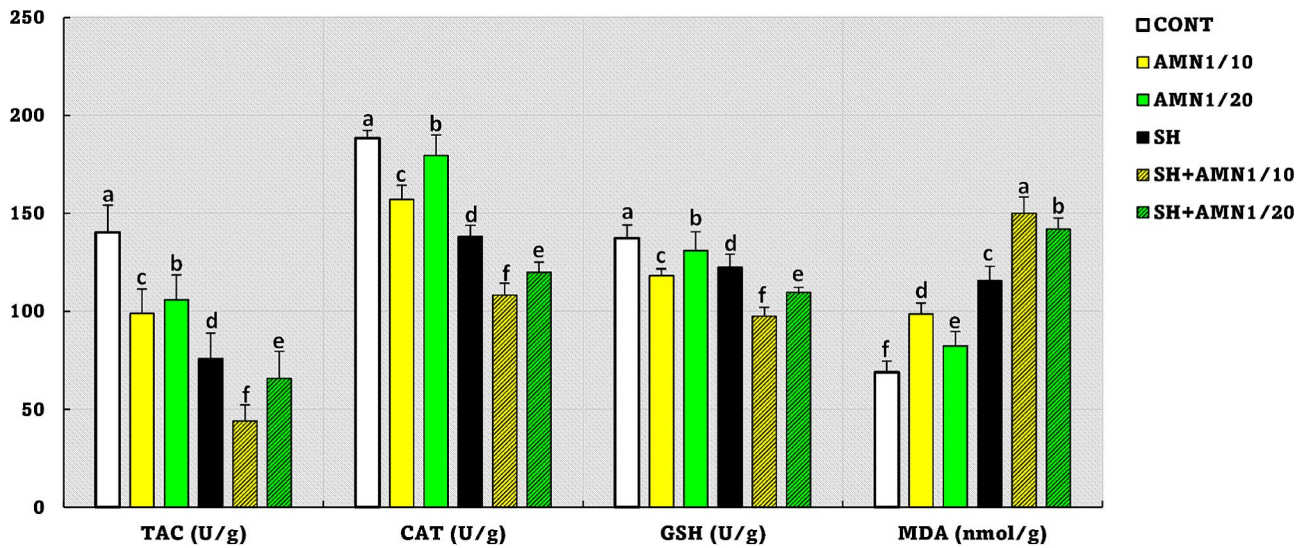
Fig. 7 Total antioxidant capacity (TAC), catalase (CAT), reduced glutathione (GSH), and malondialdehyde (MDA) levels for Nile tilapia challenged with She-wanella spp. and reared under different ammonia levels. Values are presented as the mean ± SE ($n = 9$/group). The bars with different superscripts (**a**, **b**, **c**, **d**, **e**, and **f**) are significantly different ($P < 0.05$, one-way ANOVA). Control, AMN1/10, and AMN1/20 groups were non-challenged groups and exposed to 0, 0.42, and 0.21 mg/L of unionized ammonia, respectively. SH, SH + AMN1/10, and SH + AMN1/20 groups were intraperitoneal challenged with Shewanella spp. and exposed to 0, 0.42, and 0.21 mg/L of ammonium chloride, respectively
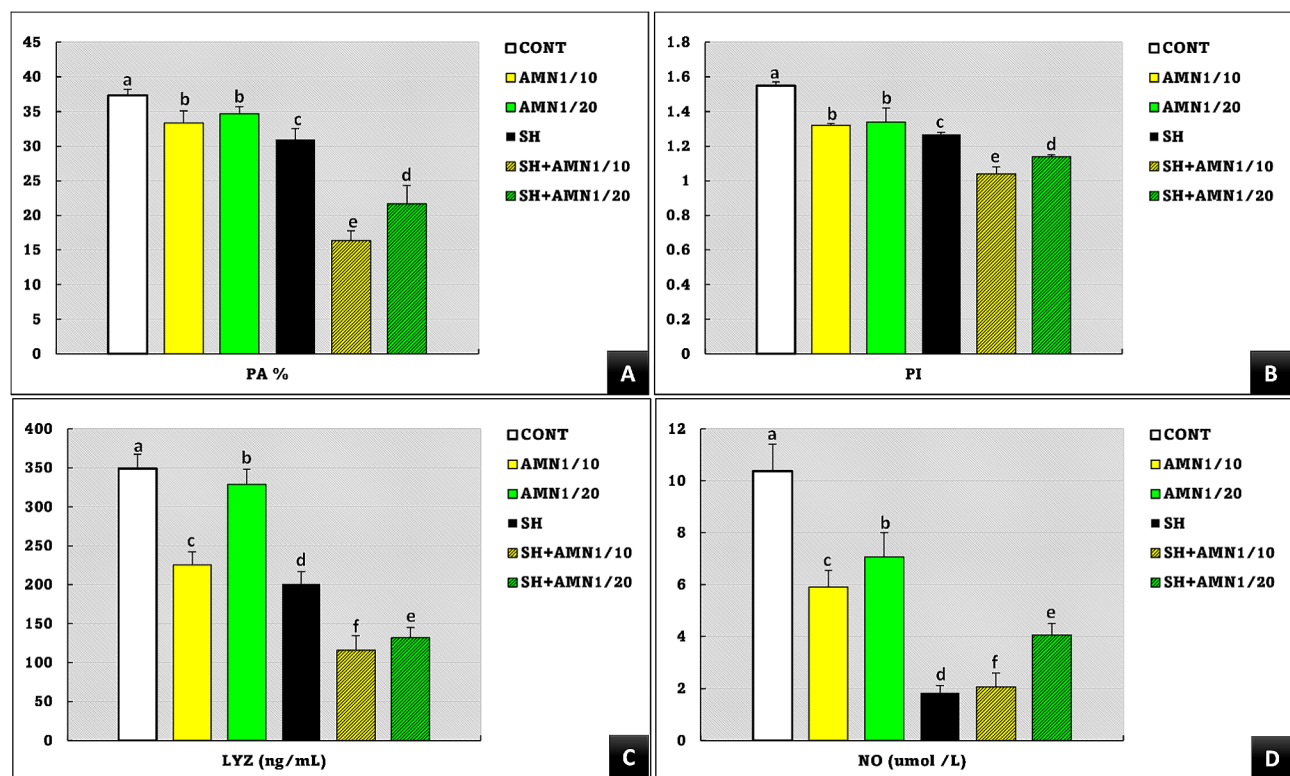
Fig. 8 Phagocytic activity (PA%, **A**), phagocytic index (PI, **B**), lysozyme activity (LYZ, **C**), and nitric oxide (NO, **D**) levels for Nile tilapia challenged with She-wanella spp. and reared under different ammonia levels. Values are presented as the mean ± SE ($n = 9$/group). The bars with different superscripts (**a**, **b**, **c**, **d**, **e**, and **f**) are significantly different ($P < 0.05$, one-way ANOVA). Control, AMN1/10, and AMN1/20 groups were non-challenged groups and exposed to 0, 0.42, and 0.21 mg/L of unionized ammonia, respectively. SH, SH + AMN1/10, and SH + AMN1/20 groups were intraperitoneal challenged with Shewanella spp. and exposed to 0, 0.42, and 0.21 mg/L of ammonium chloride, respectively

Fig. 9 Interleukin-1β (IL-1β, A), and tumor necrosis factor-α (TNF-α, B) for Nile tilapia challenged with Shewanella spp. and reared under different ammonia levels. Values are presented as the mean ± SE ($n = 9$/group). The bars with different superscripts (**a**, **b**, **c**, **d**, **e**, and **f**) are significantly different ($P < 0.05$, one-way ANOVA). Control, AMN1/10, and AMN1/20 groups were non-challenged groups and exposed to 0, 0.42, and 0.21 mg/L of unionized ammonia, respectively. SH, SH + AMN1/10, and SH + AMN1/20 groups were intraperitoneal challenged with Shewanella spp. and exposed to 0, 0.42, and 0.21 mg/L of ammonium chloride, respectively
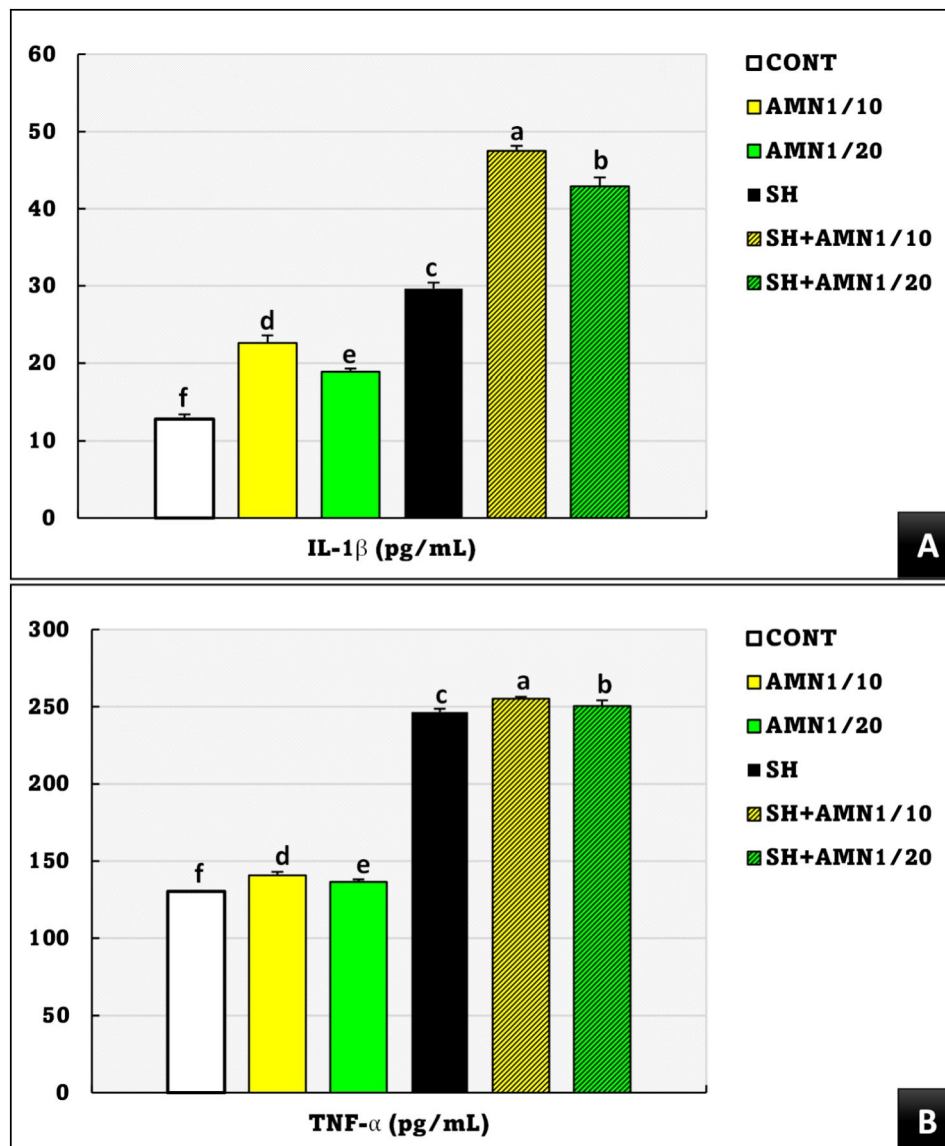
stress provides valuable insights into the combined effects of these stressors on fish health. Unexpectedly, the hematological indices measured in this study showed no differences among the control, AMN1/10, and AMN1/20 groups. The study by Handayani, Soegianto [68] stated that *O. niloticus* exposed to mercury (Hg) at concentrations of 0.1 and 1 mg/L showed considerably higher levels of RBC, WBC, and Hb; however, only for the 0.1 mg/L dosage did these values recover to control levels after 7 days of exposure. Exposure to mercury did not alter the levels of Ht. This finding aligns with the findings of *Salmo salar* blood Hct, RBC, and MCV, which were unaffected

by exposure to low ammonia concentrations (22 µg/L NH_3-N) for 14–15 days [69].Therefore, this result could be attributed to the low concentrations of ammonia to which the fish were exposed, and the fish may require longer-term stressor exposure. In addition, the fish compensating mechanisms [70] could be the reason for the reported lack of direct effects of NH_3 on hematological markers. This was corroborated by Witeska, Kondera [65] who mentioned that hematological variables may demonstrate either compensating or destructive consequences of toxicity exposure. Therefore, it is challenging to draw conclusions about the mechanisms underlying
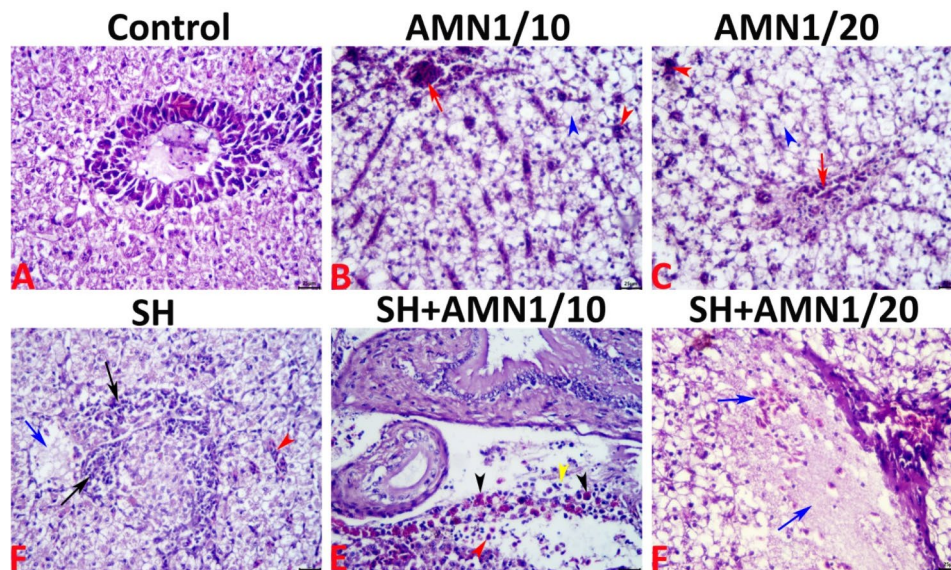
Fig. 10 Representative light micrographs of the H&E-stained hepatic tissue sections showing: a normal histological picture in the control group (**A**), acute cellular swelling with notable vacuolations (blue arrowheads), sinusoidal congestions (red arrowheads), and lytic necrotic foci infiltrated by extravasated erythrocytes (red arrows) in the AMN1/10 (**B**), and the AMN1/20 (**C**) groups, sinusoidal (red arrowhead), and vascular (blue arrow). Lytic necrotic foci infiltrated with inflammatory cells (black arrow) in the SH group (**D**), and vascular congestion (red arrowhead), and infiltration with lymphocytes (black arrowhead), and eosinophilic granular cells (black arrowheads) in the SH + AMN1/10 group (**E**) and lytic necrotic foci infiltrated with erythrocytes (blue arrows) in the SH + AMN1/20 group (**F**). The scale bar equals 25 µm

the hematological alterations that fish exposed to toxicity frequently exhibit since they are a general, nonspecific stress response. Moreover, in the present study, exposure to UIA at various concentrations and *Shewanella* spp. challenge had the lowest harmful effect on hematological parameters compared with exposure to *Shewanella* spp. challenge alone. This finding could be attributed to the fact that fish were exposed to *Shewanella* challenge simultaneously as they were exposed to ammonia, so the ammonia concentration did not negatively influence the hematological parameters. Farmer, Mitchell [64] made a similar attribution about the combined impact of ammonia exposure and the *F. columnare* challenge on channel catfish survival rate. Prior research has shown that infection by *Shewanella* spp. has harmful impacts on the blood parameters of fish [33]. This could be attributed to the secretion of protease and hemolysin enzymes by *Shewanella* spp., which can cause damage to hemopoietic organs [71, 72].

The liver and kidneys are vital organs in fish that play a crucial role in detoxification and waste removal. As a result, their function can serve as a valuable indicator of fish health. Changes in liver and kidney function can signal exposure to toxins or pollutants in the water, making them ideal biomarkers for aquatic toxicology [73, 74]. Additionally, altered liver and kidney function can be indicative of infectious diseases in fish [33]. In this study, there was a positive correlation between UIA concentration and the levels of ALT and AST in *Shewanella*-challenged groups, which presented the highest

ALT and AST levels compared to those in the control group. Furthermore, compared to the control group, the SH group and *Shewanella*-challenged groups exposed to UIA had greater creatinine and urea levels than the groups exposed to UIA alone without challenge. These results indicate that co-exposure to ammonia stress and *Shewanella* infection can have a synergistic effect on the liver and kidney functions of fish. Previous studies have proven that ammonia can damage the liver and kidneys, which are essential organs for detoxification and waste removal [10, 12, 53]. Additionally, other studies have shown alterations in liver and kidney function in *Shewanella*-infected fish, and some have attributed these changes to virulence factors and toxins [22, 25, 33]. Therefore, the combined effects of both ammonia stress and *Shewanella* infection on *O. niloticus* may be more severe than the effects of either stressor alone.

Cortisol and glucose levels serve as common indicators of stress in fish [75, 76]. An increase in cortisol and ammonia levels has been documented in previous research in response to individual exposure to ammonia stress [12, 77, 78] or *Shewanella* infection [33]. By examining cortisol and glucose levels; we can better understand the interplay between stress response, *Shewanella* spp. infection, and metabolic function in *Oreochromis niloticus* exposed to these stressors. The present results corroborate that co-exposure to ammonia stress and *Shewanella* infection (SH + AMN1/10 and SH + AMN1/20) had synergistic effects on the levels of cortisol and glucose, which were greater than those in the control group.

Table 3 The hepatic and renal histology of *O. niloticus* infected with Shewanella spp and exposed to ammonia

Organ	Reaction pattern	Alteration	W	Control F	Control Index	AMN1/10 F	AMN1/10 Index	AMN1/20 F	AMN1/20 Index	SH F	SH Index	SH+AMN1/10 F	SH+AMN1/10 Index	SH+AMN1/10 F	SH+AMN1/10 Index	P-value
Liver	Regressive pattern	- Acute cellular swelling	1	0	0.0±0.0	33	0.0±0.0	21	0.0±0.0	11	0.0±0.0	39	0.0±0.0	32	0.0±0.0	-
		- Single-cell necrosis	3	0	0.0±0.0^d	19	3.0±0.51^b	11	2.0±0.29^c	17	0.8±0.20	27	4.0±0.29^a	21	2.7±0.15^{bc}	0
		- Vacuolation foci	2	0	0.0±0.0^c	7	3.6±0.40^{ab}	4	3.0±0.00^b	3	3.3±0.30^{ab}	9	4.2±0.48^a	5	3.9±0.45^{ab}	0
		- Lytic necrotic foci	3	0	0.0±0.0^c	13	1.4±0.30^{ab}	7	0.8±0.32^{bc}	15	0.6±0.30^{bc}	21	1.8±0.20^a	19	1±0.30^{ab}	0
		- MMCs necrosis	3	0	0.0±0.0^c	6	3±0.00^{ab}	4	2.1±0.45^b	6	3.3±0.30^a	14	3.9±0.45^a	8	3.6±0.40^a	0
	Inflammatory pattern	- Leukocytic infiltration	2	0	0.0±0.0^d	7	1.8±0.48^{bc}	5	1.2±0.48^c	20	1.8±0.48^{bc}	27	3.0±0.00^a	25	2.4±0.40^{ab}	0
		- Eosinophilic granular cell infiltrate	2	0	0.0±0.0^d	0	1.4±0.30^c	0	1±0.33^{cd}	3	2.6±0.42^b	4	3.8±0.55^a	3	3±0.44^{ab}	0
	Circulatory pattern	-Vascular congestion	1	0	0.0±0.0^c	21	0.0±0.0^c	15	0.0±0.0^c	19	0.6±0.30^{ab}	31	0.8±0.32^a	29	0.6±0.30^{ab}	0.02
		- Sinusoidal congestion	1	0	0.0±0.0^c	21	2.60±0.58^{ab}	15	1.40±0.22^b	17	2.30±0.47^{ab}	29	3.10±0.52^a	25	2.70±0.55^{ab}	0
		- Hemorrhages	2	0	0.1±0.1^c	7	2.30±0.15^{ab}	6	2.00±0.25^b	4	1.30±0.15^b	13	3.10±0.52^a	11	3.10±0.60^a	0
	Progressive alterations	- Regenerated hepatocytes	2	0	0.20±0.20^d	3	1.50±0.34^{bc}	3	1.00±0.33^c	4	0.80±0.32^{cd}	5	2.50±0.16^a	5	2.10±0.10^{ab}	0
		- Hyperplastic cholangiocytes	2	0	0.0±0.0^b	0	0.60±0.30^{ab}	0	0.60±0.30^{ab}	0	0.80±0.32^{ab}	0	1.00±0.33^a	0	0.80±0.32^{ab}	0.02
		- Basophilic foci	1	0	0.0±0.0	0	0.0±0.0	0	0.0±0.0	0	0.0±0.0	1	0.0±0.0	0	0.0±0.0	-
		- MMCs hyperplasia	2	0	0.0±0.0	0	0.20±0.20	0	0.20±0.20	0	0.20±0.20	2	0.20±0.20	2	0.40±0.26	0.83
	Neoplastic alterations	- Epithelial neoplasms	3	0	0.0±0.0	0	0.0±0.0	0	0.0±0.0	0	0.0±0.0	0	0.40±0.26	0	0.40±0.26	0.12
		- Non-epithelial neoplasms	3	0	0.0±0.0	0	0.0±0.0	0	0.0±0.0	0	0.0±0.0	1	0.0±0.0	0	0.0±0.0	-
	Liver index				0.0±0.0^d		15.90±1.64^b		8.90±2.06^c		16±0.55^b		23.70±1.13^a		20.30±1.59^a	0
kidney	Regressive alterations	- Glomerular collapse	2	0	0.0±0.0^c	13	2.20±0.20^a	7	1.40±0.30^b	9	1.80±0.20^{ab}	15	2.40±0.26^a	14	2.00±0.00^{ab}	0
		- Glomerular necrosis	3	0	0.0±0.0^d	13	3.00±0.00^a	5	1.50±0.50^c	6	1.80±0.48^c	11	3.30±0.30^a	7	2.10±0.45^{bc}	0
		- Tubular vacuolation	1	0	0.0±0.0	39	2.30±0.36^b	26	1.00±0.00^c	21	1.00±0.00^c	41	3.10±0.52^a	32	1.40±0.22^c	0
		- Tubular necrosis	3	0	0.0±0.0^c	19	3.90±0.45^a	8	2.40±0.40^b	11	3.00±0.00^{ab}	21	3.60±0.40^a	17	3.30±0.30^{ab}	0
		- Tubular dilatation	2	0	0.0±0.0^c	5	1.00±0.33^{ab}	4	0.80±0.32^{abc}	2	0.40±0.26^{bc}	7	1.40±0.30^a	6	1.20±0.32^{ab}	0.01
		- Cast formation	2	0	0.0±0.0^d	7	1.40±0.30^{bc}	3	0.80±0.32^c	9	1.80±0.20^{ab}	15	2.40±0.26^a	11	2.00±0.00^{ab}	0
		- MMCs necrosis	3	0	0.0±0.0^c	6	1.80±0.48^a	2	0.60±0.40^{bc}	5	1.50±0.50^{ab}	9	2.70±0.30^a	7	2.10±0.45^a	0
		- Tunica media vacuolations	2	0	0.0±0.0^b	4	0.80±0.32^{ab}	1	0.20±0.20^{ab}	3	0.60±0.30^{ab}	5	1.00±0.33^a	5	1.00±0.33^a	0.05
	Inflammatory alterations	- Leukocytic infiltration	2	0	0.0±0.0^b	6	1.20±0.32^b	2	0.40±0.26^b	22	3.20±0.44^a	31	4.20±0.55^a	23	3.80±0.55^a	0
		- Eosinophilic granular cell infiltrate	2	0	0.0±0.0	0	0.0±0.0	0	0.0±0.0	0	0.0±0.0	0	0.0±0.0	0	0.0±0.0	-
	Circulatory alterations	- Vascular congestion	1	0	0.0±0.0^d	17	1.50±0.16^{bc}	11	1.00±0.00^c	21	2.30±0.15^a	32	2.40±0.47^a	24	2.00±0.33^{ab}	0
		- Interstitial hemorrhage	2	0	0.0±0.0^b	6	1.20±0.32^a	5	1.20±0.32^a	4	0.80±0.32^{ab}	7	1.40±0.30^a	7	1.40±0.30^a	0
		- Interstitial edema	2	0	0.0±0.0^c	4	0.80±0.32^b	2	0.60±0.30^{bc}	7	1.60±0.26^a	9	1.80±0.20^a	8	1.60±0.26^a	0.009
	Progressive alterations	- Regenerated tubular epithelium	2	0	0.0±0.0^c	7	1.40±0.30^a	2	0.40±0.26^{bc}	3	0.60±0.30^{abc}	5	1.00±0.33^{ab}	4	0.80±0.32^{abc}	0
		- Endothelial hypertrophy	1	0	0.0±0.0^c	9	0.90±0.10^a	3	0.30±0.15^b	2	0.20±0.13^b	14	1.00±0.00^a	7	0.70±0.15^a	0.19
		- Endothelial hyperplasia	2	0	0.0±0.0	0	0.0±0.0	0	0.20±0.20	0	0.0±0.0	2	0.40±0.26	2	0.20±0.20	0
		- MMCs hyperplasia	2	0	0.0±0.0	0	0.0±0.0	0	0.0±0.0	0	0.0±0.0	2	0.0±0.0	1	0.0±0.0	0.39
	Neoplastic alterations	- Epithelial neoplasms	3	0	0.0±0.0	0	0.0±0.0	0	0.0±0.0	0	0.0±0.0	0	0.0±0.0	0	0.0±0.0	0.2
		- Non-epithelial neoplasms	3	0	0.0±0.0	0	0.0±0.0	0	0.0±0.0	0	0.0±0.0	0	0.0±0.0	0	0.0±0.0	-
	Kidney index				0.0±0.0^e		21.40±0.96^c		15.30±2.01^d		18.40±1.52^{cd}		31.80±1.29^a		26.70±1.22^b	0

F, alteration frequency; W, importance factor; LI, liver Index; KI, Kidney Index. The values are shown in means ± SE. The means within the same row carrying different superscripts are significant at $P<0.05$
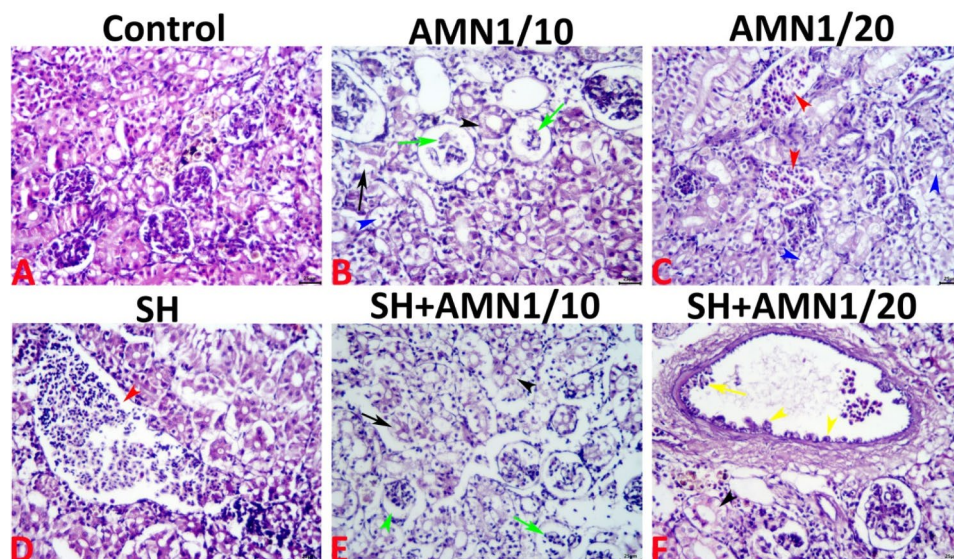
Fig. 11 Representative light micrographs of the H&E-stained renal tissue sections showing normal histological picture in the control group (**A**), glomerular necrosis (green arrows), single-cell necrosis (blue arrowhead), and complete tubular necrosis (black arrow) in the AMN1/10 group (**B**), necrotic MMCs (black ellipses), vacuolated tubular epithelium (blue arrowheads), and vascular congestions (red arrowheads) in the AMN1/20 group (**C**). Vascular congestion (red arrowhead), and inflammatory cell infiltrate (red ellipse) in the SH group (**D**). Glomerular necrosis (green arrow), and collapse with widen Bowman's space (green arrowhead), tubular single-cell necrosis (black arrowhead), and complete tubular necrosis (black arrow) in the SH+AMN1/10 group (**E**). Glomerular necrosis (green arrowhead), tubular single-cell necrosis (black arrowhead), and vascular congestion associated with endothelial hypertrophy (yellow arrowhead), and hyperplasia (yellow arrow), hyalinization (red arrowhead), and vacuolations (blue arrowheads) of the tunica media in the SH+AMN1/20 group (**F**). The scale bar equals 20 µm

Evans, Pasnik [55] reported that *O. niloticus* in both the UIA-exposed and control groups exhibited physiologic responses to *Streptococcus agalactiae* challenge via increased glucose levels. The researchers attributed this result to either handling stress or infection. On the other hand, Benli and Yildiza [79] suggested that the glucose stress response in Nile tilapia infected with *Edwardsiella tarda* was due to poor water quality conditions, not infection.

Fish exposed to both *Shewanella* infection and ammonia stress face dual stress. Bacterial infections ramp up the production of harmful molecules called reactive oxygen species (ROS) within fish cells [80, 81]. Ammonia exposure also disrupts cellular balance and causes ROS production to overdrive [82–84]. Furthermore, the increase in stress factors (cortisol and glucose) in this study plays a major role in the production of ROS by activating an enzyme called NADPH oxidase that generates ROS directly [85, 86]. To combat this oxidative stress, fish activate antioxidant defenses, including enzymes such as TAC, CAT, and GSH. TAC is one of the most crucial metrics because it provides a holistic evaluation of the total antioxidant status of fish samples by providing a thorough assessment of the cumulative effect of all antioxidants, both enzymatic and non-enzymatic [87]. Catalase plays a critical role in the breakdown of hydrogen peroxide, a common reactive oxygen species (ROS), into water and oxygen [88]. Glutathione, a non-enzymatic

antioxidant, is essential for cellular redox equilibrium because it scavenges free radicals and serves as a substrate for antioxidant enzymes [89]. These antioxidants work to neutralize ROS and protect cells from damage. However, the combined factors, ammonia stress and *Shewanella* infection, can disrupt the antioxidant system, as demonstrated in the present study. The antioxidant indices (TAC, CAT, and GSH) decreased significantly with increasing oxidant concentrations (MDA) in either group only exposed to the UIA or simultaneously infected and exposed to UIA (SH+AMN1/10 and SH+AMN1/20). These findings demonstrate that fish exposed to dual stress (ammonia and *Shewanella* infection) experience oxidative stress. This finding supports the explanations for the altered liver and kidney functions in these study groups. In contrast, the expression of SOD genes in Nile tilapia increased under ammonia stress and *A. hydrophila* infection, while glutathione-S-transferase (GST) gene expression decreased in hepatic tissues exposed to single or dual stressors [56]. Several variables can impact the results, including variations in experimental technique, type of fish and bacteria used, level of ammonia, duration of exposure, organs analyzed, and timing of sample collection among different investigations [81].

Phagocytosis is the mechanism by which a cell engulfs various particle targets. Lysozyme is crucial for breaking down bacterial cell walls [90]. On the other hand, NO is a signaling molecule with antimicrobial properties. It's

produced by immune cells to combat pathogens [91]. IL-1β and TNF-α are pro-inflammatory cytokines that orchestrate the immune response [92]. By examining these parameters, we can establish a more comprehensive understanding of how ammonia stress influences the fish's immune response to *Shewanella* spp. infection. In this study, the fish groups exposed to the combined stress of ammonia and *Shewanella* infection (SH+AMN1/10, then SH+AMN1/20) had the lowest levels of immune parameters (PI, PA%, and LYZ) and the highest levels of proinflammatory cytokines (IL-1β and TNF-α). Cortisol, an important regulator of the neuro-immunoendocrine system, is associated with immune suppression in fish because it affects the hypothalamic-pituitary-interrenal axis [93]. Our results can be explained by the evidence that shows a correlation between elevated levels of cortisol in the bloodstream and a decrease in immune parameters (PI, PA%, and LYZ) [94]. The degree of suppression can vary depending on the severity and duration of the stress and the specific fish species. Several studies suggest that chronic stress can lead to long-term impairments in fish immune function [95–97]. Abdel-Latif, Shukry [56] reported heightened levels of proinflammatory cytokines and CXC chemokines in the kidney tissues of *O. niloticus* subjected to ammonia stress or *A. hydrophila*. These findings suggest that the immune system of fish responds by increasing the levels of these substances to reduce inflammatory reactions.

The liver and kidneys are crucial organs for detoxification but are vulnerable to water pollutants such as ammonia, suggesting that they are targets for ammonia poisoning [10]. Numerous papers document histopathological changes in the liver and kidney of ammonia-exposed fish, the extent of which these changes vary based on the concentration and exposure period [10, 56, 98, 99]. Additionally, different kinds of fish exhibit histological changes in their internal organs, mostly in the liver and kidneys [24, 25, 33]. These alterations are mainly due to the enzyme activity of *Shewanella* infection, ability to adhere to cells, and the release of cytotoxins [20]. In the present study, high ammonia exposure caused morphological alterations, including lipoidal swelling, blood vessel congestion, single cell necrosis, and lytic necrotic foci. These alterations were more severe in the AMN1/10 and SH groups. Co-exposure to *Shewanella* spp. infection and high ammonia concentrations cause significant hepatic damage, with single-cell necrosis, multifocal lytic necrotic foci, and inflammatory cell infiltrates. Additionally, the kidneys of the AMN1/10 group exhibited many changes in shape, such as tubular epithelium vacuolations and necrosis, glomerular necrosis, and vascular congestion. The kidneys of AMN1/20 also exhibited nephropathic alterations. Infection with *Shewanella* spp. and high ammonia levels in the AMN1/10+SH and

AMN1/20+SH groups worsened kidney cell damage. Similarly, *O. niloticus* exposed to dual stress (ammonia and *A. hydrophila* infection) displayed vacuolation of hepatocytes, protuberant fatty degeneration, and peri-acinar necrosis in hepatopancreatic tissues. However, the posterior kidney of these fish displayed infiltration of inflammatory cells with severe necrosis and degeneration [56].

Conclusion

This study indicated that ammonia exposure synergistically affects *Shewanella* infection in Nile tilapia. In the groups co-exposed to ammonia and *Shewanella* infection, there was a significant increase in the mortality rate and stress markers (cortisol and glucose levels) and changes in liver and kidney function and structure. Conversely, significant decreases in hematological indices, antioxidant activity, and immunological parameters were observed. Accordingly, these results thoroughly grasp the *Shewanella* infection in Nile tilapia and the role that ammonia stress plays in those infections. The results also highlighted the need for water quality maintenance to protect the aquaculture sector against these opportunistic bacteria.

Author contributions
Rasha M. Reda, Abdelhakeem El-Murr, Nehal A. Abdel-Basset, Mohamed M. M. Metwally, Rowida E. Ibrahim were involved in conceptualization, data curation, formal analysis, investigation, methodology, resources, validation, visualization, roles/writing original draft, writing review and editing.

Data availability
All data generated or analyzed during this study are included in this published article.

Declarations

Ethical approval and consent to participate
The Institutional Animal Care and Use Committee of Zagazig University in Egypt was authorized all experimental techniques, and all appropriate institutional guidelines were followed when caring for and utilizing animals in this work (ZU-IACUC/2/F/142/2021).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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