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Effect of fish meal substitution with dried bovine hemoglobin on the growth, blood hematology, antioxidant activity and related genes expression, and tissue histoarchitecture of Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

Our study was designed to highlight the efficacy of fish meal (FM) replacement with dried bovine hemoglobin (DBH) on the *Oreochromis niloticus* (*O. niloticus*) growth, health antioxidant activity, and antioxidant-related gene expression for 70 days. Fish (n = 225, average weight: 36.37 ± 0.05 g) were fed diets with different replacement percentage of FM with DBH: 0 % (DBH0), 2.5 % (DBH2.5), 5 % (DBH5), 7.5 % (DBH7.5) and 10 % (DBH10), where DBH0 was kept as control diet. At the end of the feeding trial, the fish were exposed to bacterial challenge with the pathogenic bacteria *Aeromonas veronii* (*A. veronii*). Compared to the DBH0 group, there was a notable increase in the growth parameters with a lower feed conversion ratio in all DBH groups, with DBH10 recording the highest growth. The DBH (0–5 %) diets did not alter the leukogram and erythrogram of *O. niloticus*, whist, the DBH (7.5–10 %) diets badly affected the leukogram and erythrogram of the fish. The DBH7.5 and DBH10 diets significantly increased ($p < 0.05$) the levels of hepato-renal function indicators. A significant increase ($p < 0.05$) in the hepatic antioxidant parameters and its related gene expression in the spleen with a lower level of malondialdehyde was detected in the DBH5 and DBH7.5 groups, followed by DBH10 then DBH2.5 compared to DBH0 group. Hepatic and renal tissue architecture showed normal histomorphological structure in DBH (0–10 %), except a mild interstitial lymphocytic infiltration and melano-macrophages proliferative activation could be observed in DBH7.5 to10 %. As a result, the DBH at a percentage up to 5 % could be used as a substitution for FM without affecting the fish growth, health, hematological parameters, the oxidant/antioxidant balance, and hepatic and renal tissue architecture, which serves the sustainable aquaculture industry.

1. Introduction

Aquaculture is considered a large producer of aquatic foods, which significantly supply food and global nutrition safety and is expected to grow even more in reply to the rising need from a growing population (Edwards et al., 2019; FAO, 2020). Nile tilapia (*O. niloticus*) is the main significant freshwater fish species in tropical and subtropical areas (Amal and Zamri-Saad, 2011). Nile tilapia is an omnivorous fish

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characterized by high tolerance to different environmental circumstances; it's a valuable economic species because of its ability to accept natural and artificial feeds (Adeyemi et al., 2009; Canonico et al., 2005). The food of freshwater fishes is an object of continued research because it provides a base for improving a sustainable rearing program on fish production (Shalloof and Khalifa, 2009).

Food accounts for about 45 % of a fish farm's variable expenses, hence why the fish farming feeding study aims to reduce feeding costs by improving feeding strategies, either by lowering the price of diets through price reductions or by developing new feeding strategies through the use of less expensive protein sources as substitutes for FM (Martínez-Llorens et al., 2008). Nutritionists regard FM as an easily digestible and high-quality feed constituent preferred for use in the diets of agricultural animals, particularly shrimp and fish. FM has a high-calorie density per unit weight and is considered a valuable source of protein, lipids, vitamins, and minerals; it contains relatively little carbohydrate (Miles and Chapman, 2006). The accelerated growth of the aquaculture industry produced a surge in both cost and need for FM, making it the most expensive protein source in aquaculture feed (FAO, 2018). Searching for new replacers for a FM with high-quality proprieties in aquatic feeds is currently being conducted with enhanced vigilance (Amer et al., 2020; Amer et al., 2019).

Hemoglobin is a fraction from plasma extraction, manufactured via a spray method; it has a low isoleucine concentration despite having a high protein content and being notably high in lysine and valine (Martínez-Llorens et al., 2008). Hemoglobin contains a lot of digestible protein, lysine, and leucine, and it has a good pellet binding capability (Hertrampf and Piedad-Pascual, 2000b). Hemoglobin powder can be used in fish aquafeeds as an alternate protein source. Porcine hemoglobin powder has been replaced partially FM in the diets of Japanese eel (*Anguilla japonica*) (Lee and Bai, 1997a), Nile Tilapia (*O. niloticus*) (Lee and Bai, 1997b) and hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*) (Yao et al., 2018) diets. Past research has studied the inclusion of blood meal in cuneate drum diets (*Nibea miichthioides*) (Wang et al., 2006) or rainbow trout (*Oncorhynchus mykiss*) (El-Haroun and Bureau, 2007), and black carp fingerlings (*Mylopharyngodon piceus*) (Twahirwa et al., 2021).

Fish farms are always at risk of disease; one of the ways to control them is nutrition management (Ramesh et al., 2019; Herdyastuti et al., 2021; Forouharmehr et al., 2022). The use of animal and plant-based supplements has increased during the last decade to improve growth, immunity, and resistance to diseases (Durgawale et al., 2019; Datkhile et al., 2020).

Aeromonas species infection is an opportunistic pathogen that lives in water (Cai et al., 2004). *A. veronii* is one of the bacteria which causes Aeromonas infection in aquaculture (Abd-El-Malek, 2017)—infecting various fish species, among them freshwater goldfish, *Carassius auratus* (Shameena et al., 2020), Nile tilapia, *O. niloticus* (Abd El Latif et al., 2019; Reda et al., 2022), Chinese long snout catfish, *Leiocassis longirostris günther* (Cai et al., 2012), as well as catfish, *Ictalurus punctatus* (Hoai et al., 2019). The infection resulted in skin ulcers and hemorrhagic internal organs (Wang et al., 2021), in addition to alterations in the histology of the vital organ as a result of its virulence genes expression (Song et al., 2018; Zepeda-Velázquez et al., 2017).

To develop sustainable aquaculture development, it becomes necessary to provide an economic aquaculture industry by finding other alternatives of high-quality proteins to substitute high-cost FM. To date, few studies have evaluated the utilization of dried bovine hemoglobin in Nile tilapia meals. So, this research aims to investigate the effect of partial substitution of FM with DBH powder on the growth, health status, antioxidant and biochemical markers, as well as tissue histo-architecture of *O. niloticus*.

2. Material and methods

2.1. Rearing condition and experimental design

All experimental steps were carried out on animals in line with Egyptian laws on animal experimentation. With the approval of the Egyptian Veterinary Authority, Ethical Committee for Animal Experiments.

A total number of 225 *O. niloticus* fingerlings (36.37 ± 0.05 g) were purchased from Abbassa Fish Hatchery, Sharkia, Egypt. The fish had no previous disease outbreaks without any clinical abnormalities. The fish had been inspected to be sure they were apparently healthy (CCoA, 2005) and were kept for two weeks for acclimation to the laboratory settings before the experiment began, with a 12-hour light-dark cycle supported by fluorescent tubes as a light source. Fish were randomly assigned at a rate of 15 fish/ 100-L aquarium supported with compressed air through the aquarium's air pump (12 cm diameter, 20 mm length). The fish were fed a basal diet until satiated twice daily (9:00 a.m. and 2:00 p.m.). Every day, the fish waste and three-quarters of the aquarium's water were drained and refilled with fresh water.

Various water quality indices were monitored and measured weekly from each aquarium as water dissolved oxygen and temperature (portable oxygen meter, Jenway, London, UK), pH (Digital Mini-pH Meter, Fisher Scientific, Denver, USA), and unionized ammonia (Multi-parameters Ion Analyzer, HANNA Instruments, Rhode Island, USA). Dissolved oxygen ranged between (6.00–6.6 mg L^{-1}), temperature (26.1–26.8 °C), pH (6.5–6.9), and unionized ammonia (0.024–0.041 mg L^{-1}), which is considered within the permissible limits for rearing condition (Boyd and Tucker, 2012). The water quality parameters were measured according to APHA (1992) with no significant variations owing to DBH substitution.

After acclimatization, fish were randomly distributed into five groups in triplicates (N = 45 fish/ group, 15 fish/replicate). They were fed on five isoenergetic and isonitrogenous diets (20.97± 0.02 MJ/kg, 36.13±0.05 %, respectively) with five percentages of DBH powder for 70 days (0, 2.5, 5, 7.5, and 10 %), DBH0, DBH2.5, DBH5, DBH7.5, and DBH10, respectively, where the DBH0 was kept as a control diet. The diets were formulated to fulfill the nutrient needs of *O. niloticus* (NRC, 2011). The diet ingredients were mechanically mixed and then pelleted with a 1.5 mm diameter using a meat mincer. Afterward, air-dried at 25 °C for 24 hours with regular turning and kept in a refrigerator at 4 °C until further usage. The proximate chemical analysis of the basal diet was performed according to AOAC (2000) (Table 1).

2.2. Growth performance indices and survivability

Fish from each replicate were individually weighed at the beginning of the experiment to establish the initial body weight (IBW) and at the completion of the feeding trials (70 days) to demonstrate the final body weight (FBW). The growth and feed utilization parameters and survival rate were established according to the following formulas (Amer et al., 2019):

Total weight gain (TWG) (g fish–1) = (WT − WI), where WT is the final weight of fish in grams and WI is the initial weight of fish in grams.

Average daily gain (ABWG) (g $fish^{-1}$ day^{-1}) = total gain/experimental days.

Specific growth rate (SGR) (% day^{-1}) = 100 × (ln WT − ln WI)/duration/day.

Feed conversion ratio (FCR) = total feed intake (g)/total gain (g).

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

Survival percentage = (number of fish in each group remaining after the 70 days feeding period/initial number of fish) × 100.

Table 1
Proximate chemical composition of the experimental diets (g kg^{-1} on a dry weight basis).

Ingredients	DBH0	DBH2.5	DBH5	DBH7.5	DBH10
Fish meal 70.7 % CP	200	175	150	125	100
Yellow corn	220	220	225	234.5	244
Soybean meal 49 % CP	260	260	244.5	230	215
DBH*	0	25	50	75	100
Corn gluten 67 % CP	70	70	70	70	70
Wheat flour	100	100	100	100	100
Wheat bran	50	50	50	50	50
Fish oil	70	70	75	75	75
Methionine	0	0	0.5	0.5	1
Dicalcium phosphate	0	0	5	10	15
Vitamins and minerals mixture	30	30	30	30	30
Proximate composition (g kg^{-1})					
CP	358.6	363.9	362.3	361.3	360.6
Fat	109.2	106.8	109.3	107.1	104.8
NFE[1]	431.3	432	436.7	445.3	453.9
Crude fiber	34.7	34.6	33.6	32.8	31.9
Ash	66.0	62.4	57.8	53.4	48.9
Lysine	20.7	21.7	22.2	22.8	23.4
Methionine	7.5	7.2	7.3	6.9	7.0
GE MJ/kg[2]	20.85	20.90	21.02	21.04	21.06

[1] Nitrogen free extract, determined by difference = 100- (protein %+ fat %+ crude fiber %+ ash %).
[2] Gross energy (GE) was calculated according to NRC (2011) as 23.6 KJ/g protein, 39.5 KJ/g lipid and 17.0 KJ/g NFE. CP: crude protein.
* Spray-dried bovine hemoglobin powder, ACTIPRO® 95 BHS, Zwevezele, Belgium. Chemical composition of DBH (% on dry matter basis): Crude protein 92 %, ash 4 %, fat 0.4 %, Crude fiber0.5 %, Calcium 0.02 %, Phosphrus 0.3 %, Nitrogen free extract 3.1 %.

2.3. Sampling

Nine fish/groups were selected randomly to obtain blood samples from the caudal vessels at the end of the trial (70 days); a dose of 100 mg L^{-1} benzocaine solution was used for sedation of the fish, according to Neiffer and Stamper (2009). The blood samples were drained for serum separation using a 1 mL plastic syringe without anticoagulant; afterward, they were centrifuged for 10 minutes at 4°C at 3000 rpm, and serum was stored at -20°C until biochemical assays. Another blood samples were drained using a 1 mL heparinized syringe for determination of the leukogram and erythrogram. Samples of the fish's liver were collected for hepatic oxidant/antioxidant assays. The head spleen tissues were obtained and washed in ice-cold phosphate-buffered saline, then stored at −80°C for gene expression analyses. Liver and kidney tissues were collected and preserved in 10 % buffered formalin for histopathological examination.

2.4. Determination of erythrogram and leukogram

Total erythrocytes (RBCs) and leukocytes (WBCs) were counted by an automated hematology analyzer (Hospitex Diagnostics, Sesto Fiorentino, Italy) following the protocol of Feldman et al. (2000). The hemoglobin (Hb) concentration was measured immediately after blood sampling using the colorimetric diagnostic kits of spectrum-bioscience (Egyptian Company for Biotechnology, Cairo, Egypt).

2.5. Determination of biochemical parameters

We measured the serum levels of the following blood indices spectrophotometrically according to the stander protocols of their specific pamphlets using spectrophotometer (Lambda EZ201; Perkin Elmer, Waltham, USA); the serum activities of alanine aminotransferase (ALT, REF; 20764957322), aspartate aminotransferase (AST, REF; 20764949322) (Roche Cobas Co., Indianapolis, USA), lactate dehydrogenase (LDH, catalog No.; TK41214,Spinreact Co., Santa Coloma, Spain)., serum levels of total bilirubin (Catalog No.;202141, Diamond, Cairo, Egypt), total protein (Catalog No.; SB-0250-500), albumin (Catalog No.; SB- 028-500) (Stanbio Laboratory, USA), urea (catalog No.; URE118200,BioMed Co., Cairo, Egypt), creatinine (catalog No.; 10051,Human Co., Germany), uric acid (catalog No.; MD41001, Spinreact Co., Santa Coloma, Spain).

2.6. Determination of Oxidant/antioxidant indices

Total antioxidant capacity (TAC), glutathione peroxidase (GPX), superoxide dismutase (SOD), as well as malondialdehyde (MDA) were assayed in hepatic tissues (three fish/replicates). The liver samples were homogenized in buffer at pH 7.5 and centrifuged for 15 minutes at 10,000 rpm at 4°C to get the supernatant. The supernatant was then centrifuged for 1 hour to recover the final supernatant, and the pellet was collected, washed, and stored in the buffer (pH 7.5). The hepatic levels of malondialdehyde (MDA, catalog No. MD 25 29), GPX (catalog No. GP 2524), superoxide dismutase (SOD, catalog No. SD 25 21), and serum total antioxidant capacity (TAC, catalog No. TA 25 13) were measured spectrophotometrically using commercial kits (Bio diagnostics company, Cairo, Egypt).

2.7. The total RNA extraction and transcriptional assay

Total RNA was extracted from the spleen tissues of *O. niloticus* (N=9) following the manufacturer's technique using Quiazol (Qiagen, Germany). The extracted RNA was exposed to agarose gel electrophoresis and a spectrophotometer (BioRad, CA, USA) to determine its purity and concentration. The extracted RNA was processed with DNase (Takara, Shiga, Japan), and the reverse transcriptase kit (Applied Biosystem, California, United States) was used to create complementary DNA (cDNA) according to the manufacturer's approach. Real-time quantitative PCR (RT-qPCR) analyses of glutathione peroxidase (*GPX*), superoxide dismutase (*SOD*), and glutathione synthetase (GSS) were performed with specified primers (Sangon Biotech, Beijing, China) using beta-actin (β-actin) as a reference gene as listed in Table 2. The initial denaturation temperature was 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 15 seconds, with a dissociation analysis step in between; all were the conditions of RT-qPCR. Melting curve analysis was carried out after amplification to ensure that each amplicon was accurate. After ensuring that the primers were nearly 100 % efficient, the findings of gene expression were evaluated using the $2^{-\Delta\Delta}$CT method. The expression of these genes was normalized against β-actin and represented relative to the control (Schmittgen and Livak, 2008).

2.8. Histopathological investigation

Liver and kidney samples (3 samples/ group) were obtained and then fixed in formalin 10 %, dehydrated in gradual ascending ethanol (from

Table 2
O. niloticus primers of antioxidant related genes for the real-time quantitative PCR amplification.

Target gene	Primer sequence	Accession number
β-Actin	CCACCCAAAGTTCAGCCATG ACGATGGAGGGGAAGACAG	XM_003443127.5
GPX	GTGCCCTGCAATCAGTTTGG CGAGGAGCTGGAACTTTGGT	NM_001279711.1
SOD	TCACAGCAAGCACCATGCTA GCAACCTGTGTTGTCACGTC	XM_003449940.5
GSS	TAGCAAGCTAAAATGCGCGG AGAGCCGAGTTCATCAGCAC	XM_025901610.1

β-actin, beta actin; GPX, glutathione peroxidase; SOD, superoxide dismutase; GSS, Glutathione synthetase.

70 to 100 %), afterward, cleared in xylene, and embedded in paraffin. A microtome was used to slice five-micron thick paraffin slices (Leica RM 2155, England). Finally, the sections were stained with hematoxylin and eosin (H&E) (Bradford, 1976) and examined using the AmScope digital camera-attached Ceti England microscope for histopathological examination (Suvarna et al., 2013).

2.9. Bacterial preparation for the challenge testing

A pathogenic *A. veronii* strain was isolated in 2019 at the Fish Diseases and Management Department, Faculty of Veterinary Medicine, Zagazig University, Egypt, isolated from diseased *Cyprinus carpio* (*C. carpio*). Traditional biochemical tests and the VITEK® 2 system (BioM' erieux Inc., NC, USA) were used to identify the isolate, which was also confirmed as pathogenic to *C. carpio*. (Unpublished data). *A. veronii* was cultivated for 24 h at 27 ^{o}C on tryptic soy agar, and a single colony was chosen to incubate in tryptic soy broth for 24 h at 27 ^{o}C. The pellet was extracted and suspended in sterile phosphate-buffered saline solution after centrifuging the *A. veronii* cultured broth for 10 min at 3000 g at 4 ^{o}C.

The lethal dose (LD_{50}) of *A. veronii* was determined, and the fish were injected intraperitoneally (IP) with 0.1 ml with different doses of 24 h live bacteria. The mortality of inoculated fish was recorded seven days after injection and LD_{50} was evaluated as 3×10^7 CFU mL^{-1}. A sub-lethal dose of 1.5×10^7 CFU mL^{-1} was used in the bacterial challenge test using McFarland standard tubes and A drop plate assay Challenge test was used to validate the results.

8 randomly selected fish/replicate, 24 fish/group were selected randomly at the end of the feeding trial (70 days) to evaluate fish resistance against bacterial infection. *A. veronii* (1.5×10^7) CFU was injected IP into fish at a dose of 0.1 mL. The rest of the fish in each group was IP injected with 0.1 mL of saline solution as a control. The challenged fish was observed twice a day to record any aberrant clinical signs and mortalities. The bacterium was re-isolated from the fish organs to ensure that the mortalities were induced by *A. veronii.*

2.10. Data analysis

Normality and homogeneity tests were performed on the data, followed by an ANOVA test based on polynomial orthogonal contrasts. To determine the effect of replacing fish meal with DBH in the diet of *Oreochromis niloticus* on growth, health, antioxidant activity, and gene expression in fish challenged with *A. veronii*, linear and quadratic regression equations were calculated using SPSS Version 17 for Windows (SPSS Inc., Chicago, IL, USA). When $p < 0.05$, the regressions were judged significant. To evaluate differences between means, a post-hoc Tukey's test was performed. Unless otherwise noted, statistical significance is predicated on a $p < 0.05$. The data was reported as a mean with standard deviation.

3. Results

3.1. Effect of dietary DBH on fish survival and growth performance

The growth performance of *O. niloticus* fed diets substituted with DBH at various percentages is shown in Table 3. All experimental groups fed DBH diets with a 100 % survival rate along the 70-days feeding period. DBH substitution did not significantly affect the feed intake (FI) when compared to DBH0 ($p < 0.05$). DBH substitution linearly increased the FBW, WG, SGR %, PER, and ABWG and decreased the FCR by increasing the substitution percentage ($p < 0.05$).

3.2. Effect of dietary DBH on leukogram

Fish fed the DBH10 followed by DBH7.5 diets exhibited a quadratic increase ($p < 0.05$) in the count of WBCs compared to fish fed the DBH0, 2.5, and 5 diets (Table 4). The DBH10 diet quadratically increased the count of lymphocytes compared to all experimental diets; meanwhile, the fish fed the DBH10 and DBH7.5 diets showed a quadratic increase ($p < 0.05$) in the level of heterophils when compared to other DBH diets. The monocytes count was not significantly different ($p > 0.05$) between the experimental groups. The count of eosinophils and basophils quadratically increased ($p < 0.05$) in fish fed the DBH10 diet compared to other experimental groups. The DBH2.5 and DBH5 diets did not significantly affect the leukogram of *O.niloticus*.

3.3. Effect of dietary DBH on erythrogram

Table 5 shows the erythrogram of *O. niloticus* fed dietary DBH. The count of RBCs, MCH, and MCHC was not significantly different ($p > 0.05$) between the experimental groups. There was no significant difference in the concentration of Hb and PCV between DBH 0, 2.5, and 5 diets, while they were linearly and quadratically decreased ($p < 0.05$) in fish fed DBH7.5 and DBH10 diets compared to the control diet (DBH0). The MCV was linearly decreased ($p < 0.05$) in fish fed the DBH7.5, and DBH10 compared to fish fed the DBH0 diet. The DBH2.5 and DBH5 diets did not significantly affect the erythrogram of *O. niloticus*.

3.4. Effect of dietary DBH on biochemical parameters

The DBH diets did not significantly affect ($p > 0.05$) the levels of ALT, urea, uric acid, total protein, albumin, and globulin (Table 6). The fish fed the DBH10 followed by DBH7.5 diets had a linear and quadratic increase ($p < 0.05$) in the levels of AST, total bilirubin, LDH, and creatinine (Table 6).

Table 3
Growth performance of *Oreochromis niloticus* fed diet contained dried bovine hemoglobin (DBH).

	Experimental groups					Regression analysis	
Parameters	DBH0	DBH2.5	DBH5	DBH7.5	DBH10	Linear	Quadratic
IBW (g)	36.59±0.06	36.38±0.09	36.35±0.09	36.26±0.02	36.28±0.24	0.12	0.44
FBW (g)	88.35±0.35^{d}	91.25±1.25^{c}	93.18±1.81^{b}	94.31±1.45^{b}	99.00±1.23^{a}	0.00	0.58
TWG (g)	51.76±0.41^{d}	54.86±1.15^{c}	56.82±1.90^{bc}	58.04±1.43^{b}	62.71±1.24^{a}	0.00	0.64
SGR (%)	1.25±0.08^{c}	1.31±0.01^{c}	1.34±0.01^{b}	1.36±0.02^{b}	1.43±0.02^{a}	0.00	0.82
FI (g $fish^{-1}$)	68.50±2.30	68.78±1.21	72.72±3.50	65.09±0.87	67.20±2.79	0.43	0.41
FCR	1.32±0.05^{a}	1.25±0.04^{b}	1.27±0.01^{b}	1.12±0.01^{c}	1.07±0.06^{c}	0.00	0.39
PER	2.35±0.09^{c}	2.44±0.08^{b}	2.40±0.02^{b}	2.73±0.03^{a}	2.87±0.17^{a}	0.00	0.22
ABWG	0.73±0.05^{c}	0.78±0.01^{c}	0.81±0.02^{b}	0.82±0.01^{b}	0.89±0.03^{a}	0.00	0.64
SGR (%)	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	0.52	0.23

IBW, initial body weight; FBW, final body weight; TWG, total weight gain; SGR, specific Growth rate; FI, feed intake; FCR, feed conversion ratio; PER, protein efficacy ratio; ABWG, average body weight gain; SR (%), survival rate %. Values are mean ±SE. Values are not sharing same superscript letter in the same row are significantly different at $p < 0.05$. DBH0, DBH2.5, DBH5, DBH7.5, and DBH10: replacement of fishmeal with dried bovine hemoglobin (DBH) by 0, 2.5, 5, 7.5, and 10 %, respectively.

Table 4
Leukogram of *Oreochromis niloticus* fed dietary dried bovine hemoglobin (DBH).

Parameters	Experimental groups					Regression analysis	
	DBH0	DBH2.5	DBH5	DBH7.5	DBH10	Linear	Quadratic
WBCs $\times 10^{3}$/cmm	7.27±0.21^{c}	6.12±0.42^{c}	6.73±0.52^{c}	8.76±0.25^{b}	9.87±0.65^{a}	0.91	0.01
Lymphocytes	5.80±0.12^{b}	4.64±0.35^{b}	4.78±0.23^{b}	5.80±0.32^{b}	6.32±0.82^{a}	0.84	0.01
Heterophils	0.75±0.03^{b}	0.76±0.02^{b}	1.21±0.018^{b}	2.15±0.15^{a}	2.56±0.05^{a}	0.92	0.01
Monocytes	0.42±0.02	0.41±0.01	0.46±0.01	0.53±0.02	0.50±0.02	0.79	0.08
Eosinophils	0.18±0.01^{b}	0.19±0.01^{b}	0.19±0.04^{b}	0.18±0.01^{b}	0.30±0.01^{a}	0.33	0.01
Basophils	0.12±0.01^{b}	0.12±0.01^{b}	0.09±0.01^{b}	0.10±0.01^{b}	0.19±0.02^{a}	0.48	0.03

WBCs, white blood cell count. The regressions were considered significant at $P < 0.05$. Values are mean ± SE. Values are not sharing same superscript letter in the same row are significantly different at $p < 0.05$. DBH0, DBH2.5, DBH5, DBH7.5, and DBH10: replacement of fishmeal with dried bovine hemoglobin (DBH) by 0, 2.5, 5, 7.5, and 10 %, respectively.

Table 5
Erythrogram of *Oreochromis niloticus* fed dietary dried bovine hemoglobin (DBH).

Parameters	Experimental groups					Regression analysis	
	DBH0	DBH2.5	DBH5	DBH7.5	DBH10	Linear	Quadratic
RBCs $\times 10^{6}$/cmm	2.42±0.45	2.44±0.23	2.21±0.20	1.95±0.32	1.89±0.21	0.21	0.14
Hb (g/dl)	9.22±0.12^{a}	9.11±1.21^{a}	8.98±0.89^{a}	8.04±2.21^{b}	7.79±1.12^{b}	0.03	0.01
PCV (%)	28.75±3.21^{a}	29.25±12.00^{a}	27.92±0.1.2^{a}	21.75±1.23^{b}	20.34±2.51^{b}	0.01	0.01
MCV (fl)	118.80±6.54^{a}	119.87±8.2^{a}	115.85±13.20^{a}	111.53±15.10^{c}	107.61±11.2^{c}0.01		0.15
MCH (pg)	42.01±2.87	42.82±10.21	40.20±9.85	35.60±11.20	38.50±2.50	0.55	0.32
MCHC (g/dl)	34.02±3.24	37.80±12.10	35.89±8.21	32.90±12.00	36.82±3.5	0.14	0.68

RBCs, Red blood cells count; Hb, hemoglobin; PCV, packed cell volume; MCV, Mean corpuscular volume; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration. The regressions were considered significant at $P < 0.05$. Values are mean ± SE. Values are not sharing same superscript letter in the same row are significantly different at $p < 0.05$. DBH0, DBH2.5, DBH5, DBH7.5, and DBH10: replacement of fishmeal with dried bovine hemoglobin (DBH) by 0, 2.5, 5, 7.5, and 10 %, respectively

Table 6
Serum biochemical parameters of *Oreochromis niloticus* fed dietary dried hemoglobin meal (DHM).

Parameters	Experimental groups					Regression analysis	
	DBH0	DBH2.5	DBH5	DBH7.5	DBH10	Linear	Quadratic
ALT (U/L)	9.70±1.86	9.71±3.73	6.97±0.53	10.04±1.62	14.09±1.20	0.19	0.10
AST (U/L)	90.79±4.03^{c}	88.85±3.00^{c}	91.15±3.49^{c}	137.75±2.77^{b}	158.40±1.29^{a}	0.00	0.00
Total bilirubin (mg/dL)	0.49 ±0.00 ^{c}	0.52±0.01 ^{c}	0.63 ±0.01 ^{c}	0.77 ±0.02 ^{b}	0.87±0.13^{a}	0.02	0.01
LDH (U/L)	99.33±1.24 ^{c}	104.36 ±4.23 ^{c}	106.21±3.15^{c}	122.22±6.02 ^{b}	135.45±8.26^{a}	0.04	0.01
Urea (mg/dL)	10.37 ±0.18	10.79 ±0.27	11.92 ±1.14	12.75 ±1.23	12.44±1.85	0.21	0.33
Creatinine (mg/dL)	0.36±0.20^{c}	0.33±0.12^{c}	0.34±0.01^{c}	0.45±0.08^{b}	0.75±0.03^{a}	0.00	0.00
Uric acid (mg/dL)	7.25 ±0.22	7.45 ±0.20	8.62 ±0.07	8.24 ±0.15	8.75±0.26	0.40	0.29
Total protein (g dL^{-1})	3.98±0.066	3.99±0.04	4.39±0.02	4.01±0.08	4.39±0.0	0.20	0.12
Globulin (g dL^{-1})	2.08±0.72	2.27±0.01	2.89±0.72	2.03±0.13	2.27±0.01	0.20	0.80
Albumin (g dL^{-1})	1.90±0.02	1.72±0.08	2.22±0.09	1.98±0.05	1.72±0.01	0.13	0.41

ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase. The regressions were considered significant at $P < 0.05$. Values are mean ±SE. Values are not sharing same superscript letter in the same row are significantly different at $p < 0.05$. DHM0, DBH0, DBH2.5, DBH5, DBH7.5, and DBH10: replacement of fishmeal with dried bovine hemoglobin (DBH) by 0, 2.5, 5, 7.5, and 10 %, respectively.

Table 7
Oxidant/antioxidant parameters of *Oreochromis niloticus* fed dietary dried bovine hemoglobin (DBH).

Parameters	Experimental groups					Regression analysis	
	DBH0	DBH2.5	DBH5	DBH7.5	DBH10	Linear	Quadratic
TAC (ng mg^{-1})	0.20±0.01^{d}	0.37±0.02^{c}	0.92±0.04^{a}	0.95±0.04^{a}	0.57±0.05^{b}	0.00	0.01
GPX (U mg^{-1})	4.23±0.10^{d}	6.00±0.21^{c}	14.19±25^{a}	15±0.32^{a}	12±0.86^{b}	0.00	0.00
SOD (U mg^{-1})	2.88±0.21^{d}	5.19±0.29^{c}	11.13±0.70^{a}	12.97±0.77^{a}	8.07±0.73^{b}	0.01	0.03
MDA (mmol g^{-1})	7.92±0.75^{a}	6.10±0.72^{b}	3.19±0.09^{c}	2.13±0.63^{c}	5.5±1.05^{b}	0.00	0.01

TAC, total antioxidant capacity; SOD, superoxide dismutase; GPX, glutathione peroxidase; MDA, malondialdehyde. The regressions were considered significant at $P < 0.05$. Values are mean ± SE. Values are not sharing same superscript letter in the same row are significantly different at $p < 0.05$. DBH0, DBH2.5, DBH5, DBH7.5, and DBH10: replacement of fishmeal with dried bovine hemoglobin (DBH) by 0, 2.5, 5, 7.5, and 10 %, respectively.

3.5. Effect of dietary DBH on hepatic oxidant/antioxidant parameters

As illustrated in Table 7, The hepatic TAC, GPX, and SOD values were linearly and quadratically increased in fish that received DBH diets, where the highest significant values ($p < 0.05$) in fish given DBH5 and DBH7.5 followed by DBH10 then DBH2.5 compared to DBH0 group. While the hepatic MDA was diminished in the same manner ($p < 0.05$).

3.6. Effect of dietary DBH on the antioxidant gene expression

Different expression patterns of antioxidant-related genes were measured in the spleen of *O. niloticus,* as shown in Fig. 1. The expression of antioxidant genes (*SOD*, *GPX*, and *GSS*) (Fig. 1A, B, and C, respectively) was markedly boosted linearly and quadratically in the DBH5 (6.35-, 5.34-, and 4.95-fold) and DBH7.5 (4.68-, 4.00-, and 2.94 -fold) followed by DBH2.5 (3.29-, 2.76-, and 2.00- fold) and DBH10 (2.93-, 2.22-, and 1.99- fold), respectively, with no significant difference between DBH2.5 and BBH10.

3.7. Effect of dietary DBH on tissue architecture

Examined sections from the liver of control fish showed normal lobular arrangement, central venules, and portal area structures with close similarity to the mammalian hepatic portal structures but with the presence of intra-hepatic pancreases enclosing a branch from the portal vein. The hepatocytes, sinusoids, and reticulio-endothelial system are well-formed and histologically normal; occasionally, a few melano-macrophages are seen interepithelial, interacinar, in the vicinity of the vascular structures or among the interstitial tissue. Liver sections of treatment groups pointed out comparatively a normally active and healthy hepatic parenchyma, hepato-portal pancreases, and normal population of melano- macrophages. It is difficult to make a clear demarcation to distinguish which group is better histologically than the other it may require enzyme assays besides histochemical and immune-histochemical investigations. A proportional hepatocellular cytoplasmic basophilia (higher anabolic ribosomal contents) and active acinar arrangement with the presence of a large number of zymogenic granules were seen in DBH5 and DBH7.5 groups. (Fig.2A and B).

Kidneys of the control group (DBH0) revealed typical renal glomerular, tubular, and interstitial structures with preserved marine type Bowman's capsular histo-morphology, glomerular capillary morphology, and tubular epithelial length and widths with centrally located nuclei, normal brush borders, and granular cytoplasm. Kidneys of treatment groups (DBH2.5 to 10 %) exhibited approximately normal renal tissue with a standard histo-morphology. A mild to moderate interstitial lymphocytic infiltration was recorded, and more melano-macrophages proliferative activation could be observed in DBH7.5 and DBH10 (Fig. 3).

3.8. Effect of dietary DBH on A. veronii resistance

As seen in Fig. 4, the DBH0 group (control) had the highest mortality (78 ± 5 %). This group exhibited symptoms like skin and fin hemorrhages, fin rot, excessive mucus secretion, and erratic swimming with skin ulcerations. While the lowest mortality was noticed in the DBH5 group (25 ± 3 %), followed by the DBH7.5 and DBH10 groups (29 ± 4 and 32 ± 2 %, respectively), then the DBH2.5 group (59 ± 3 %) without any observable signs except only slight fin rot and hemorrhages

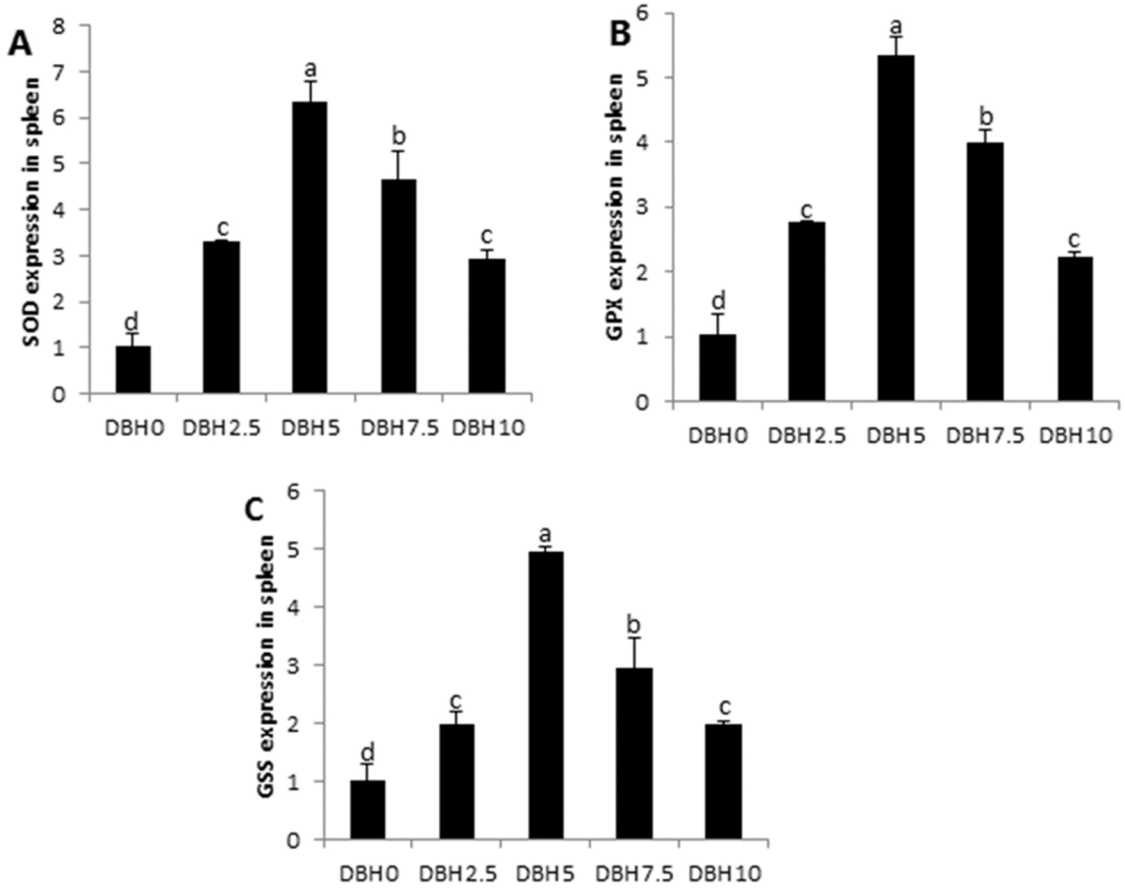


Fig. 1. Antioxidant-related gene expression in spleen of *Oreochromis niloticus* fed dietary dried bovine hemoglobin (DBH) for 70 days. [a, b, c, d.] The bars with different lowercase letters are significantly different ($P < 0.05$). DBH0, DBH2.5, DBH5, DBH7.5, and DBH10: replacement of fishmeal with dried bovine hemoglobin (DBH) by 0, 2.5, 5, 7.5, and 10 %, respectively.

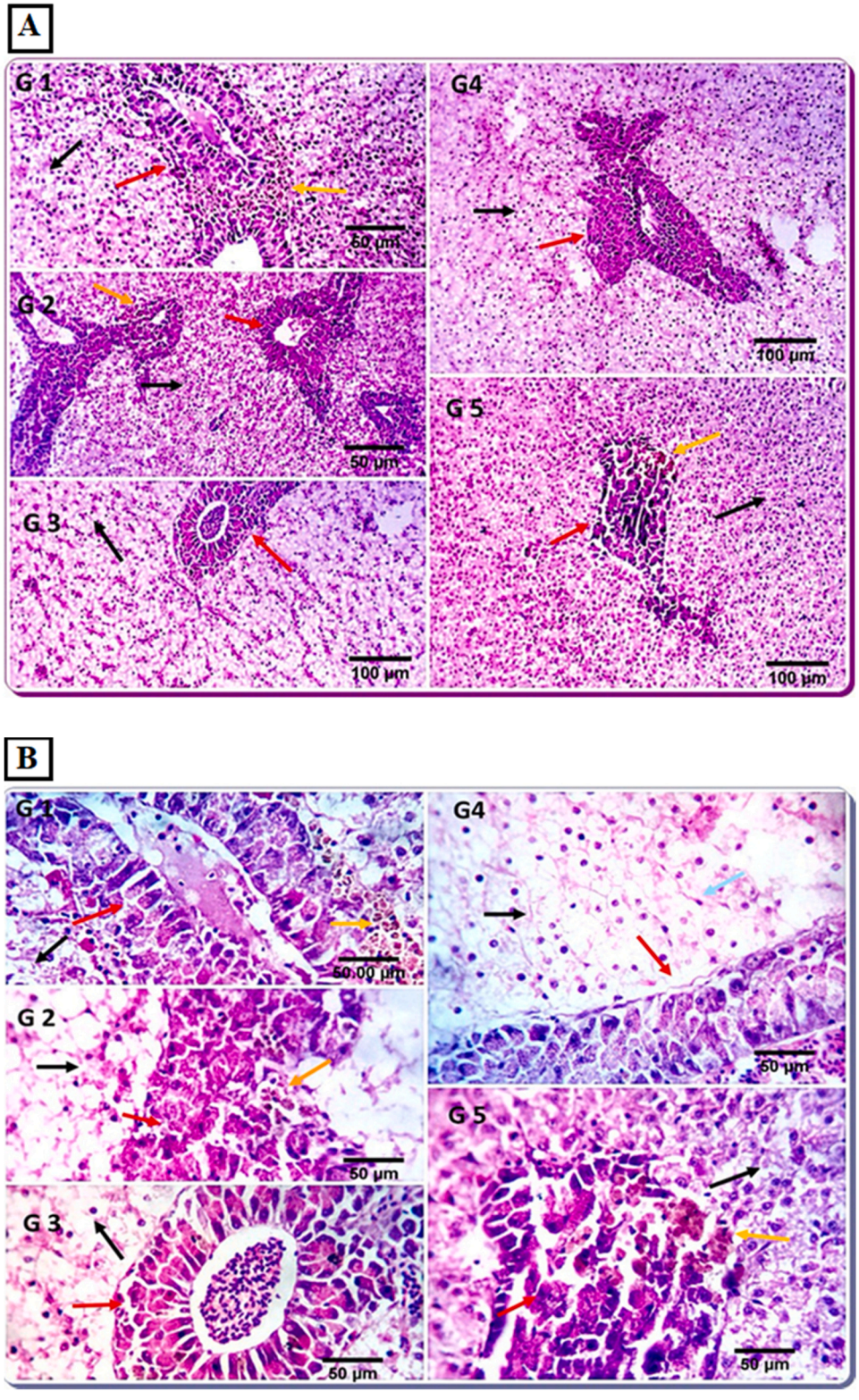


Fig. 2. (A-B) Photomicrographs demonstrate the histo-morphological structures in the control fish (G1) and in the liver of treatment groups (2-5). Apparently, normal lobular arrangement, central venules (black arrows), portal area structures, and intra-hepatic pancreases enclosing a branch from the portal vein (red arrows) and few inter and periacinar melan-macrophages (orange arrows) are seen in all groups. Liver sections of treatment groups (2-5) show a proportional hepatocellular cytoplasmic basophilia (higher anabolic ribosomal contents) and active acinar arrangement with the presence of a large number of zymogenic granules, particularly in G3 and G4. Groups (1 to 5) represent DBH0, DBH2.5, DBH5, DBH7.5, and DBH10, respectively. H&E X 100, 200, 400.

especially in the DBH2.5 group.

4. Discussion

Sustainable development in aquaculture has become an absolute necessity to meet the world's needs for animal proteins, especially in developing countries. The cost of aquafeed is a great challenge for the aquaculture industry. FM is an expensive source of energy and nutrients in fish diets. Finding alternative replacers for a high-cost FM with low-cost replacers becomes necessary to decrease the cost of aquafeed and consequently develop the industry. There is little available research on using DBH in fish diets; accordingly, the study was performed to clarify the growth and antioxidant indices, tissue histoarchitecture, blood hematology, as well as *A. veronii* challenge in *O. niloticus* in response to

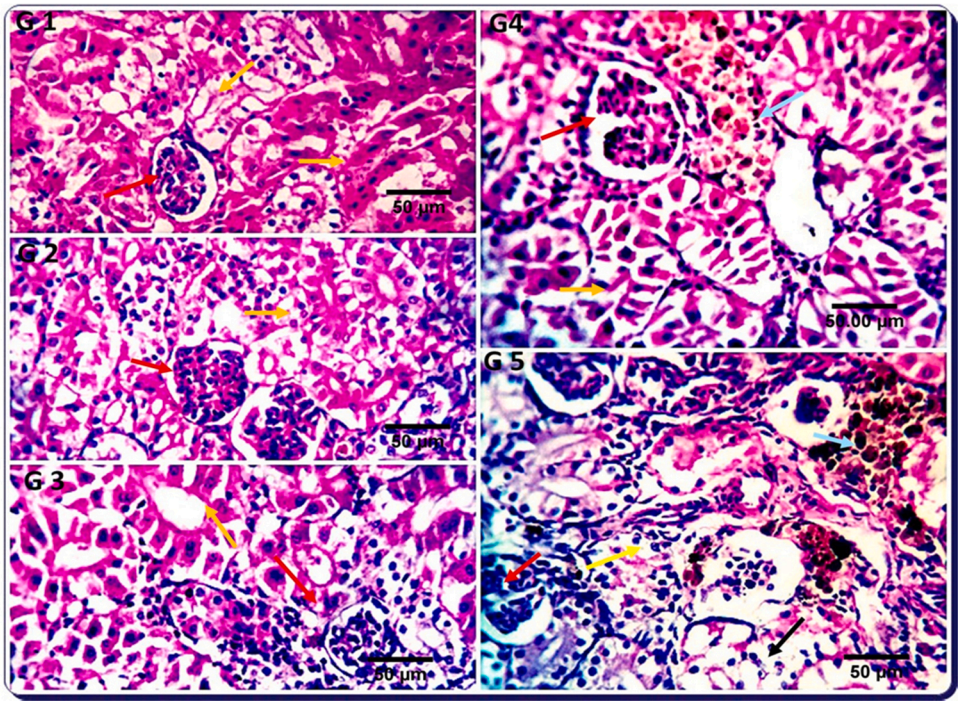


Fig. 3. Photomicrographs demonstrate the histo-morphological structures in the kidney of control fish (1) and treatment groups' kidneys (2-5). Normal renal glomerular, tubular, and interstitial structures with preserved marine type Bowman's capsular histo-morphology, glomerular capillary morphology (red arrows), tubular epithelial length, and widths with centrally located nuclei (orange arrows). Mild to moderate interstitial lymphocytic infiltration (yellow arrows) and melano-macrophages proliferative activation (blue arrows) are seen in groups 4 and 5. Groups (1 to 5) represent DBH0, DBH2.5, DBH5, DBH7.5, and DBH10, respectively. H&E X 50.

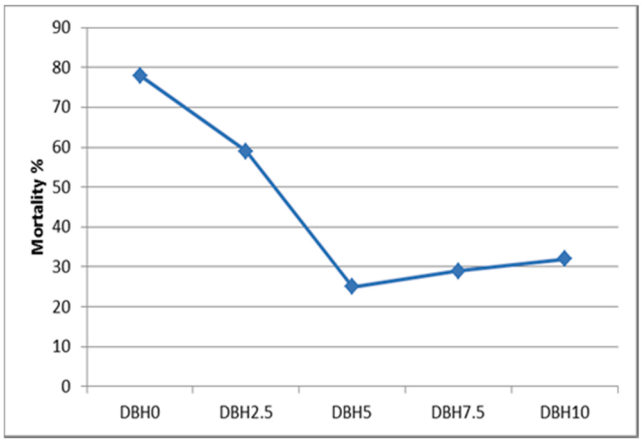


Fig. 4. Effect of partial replacement of fish meal with DBH on mortality % of *Oreochromis niloticus* fed dietary dried bovine hemoglobin (DBH) for 70 days and challenged with *Aeromonas veronii*. [a, b, c, d.] The bars with different lowercase letters are significantly different ($P < 0.05$). DBH0, DBH2.5, DBH5, DBH7.5, and DBH10: replacement of fishmeal with dried bovine hemoglobin (DBH) by 0, 2.5, 5, 7.5, and 10 %, respectively.

different FM replacement percentages with DBH.

In this study, the growth metrics were increased and FCR was decreased with increasing the percentage of DBH with 100 % survival rate in all experimental groups. This may be attributed to the high digestible protein content in hemoglobin powder; as a result, it's been recommended as an excellent alternative protein source in fish diets (Hertrampf and Piedad-Pascual, 2000a). Similar results were obtained in Nile tilapia (Lee and Bai, 1997a), Japanese eel (*Anguilla japonica*) (Lee and Bai, 1997b), gilthead sea bream (*Sparus aurata*) (Martínez-Llorens et al., 2008), and hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*) (Yao et al., 2018b). Ding et al. (2020) reported that 9.80 % of FM might be replaced by chicken hemoglobin powder (CHP) for largemouth bass. They also noted that a higher replacement percentage of CHP significantly reduces the growth, feed utilization, and health of the fish. In addition, Yao et al. (2018a) reported that 17.3 %–23.3 % FM could be substituted by hemoglobin powder in hybrid grouper diets without negatively impacting fish growth. Due to its high protein content, strong binding properties, and digestibility, hemoglobin powder can be utilized as an alternate protein source for FM (Chookird et al., 2010)

Blood analysis is an essential index for evaluating the physiological state of cultured fishes and the impact of diets and other stressors on fish health (Fagbenro et al., 2010). Environmental conditions, sex, age, food, and feeding regime can influence the results of hematological tests (Teixeira et al., 2000). The results of this study indicate that low dietary substitution of DBH (DBH2.5 and DBH5) did not affect the leukogram and erythrogram of *O.niloticus*. Similar results were obtained in largemouth bass (*Micropterus salmoides*) (Ding et al., 2020). In addition, increasing the WBCs count and decreasing the hematological parameters in the DBH7.5 and DBH10 groups may be attributed to progressive anemia in high substitution levels, which indicate organ dysfunction like liver and kidney (Olukunle et al., 2002).

ALT and AST enzyme activities are considered the most vital aminotransferases in the fish liver, which are related to hepatotoxicity (Bálint et al., 1997). The link between liver enzyme activities and diets shows that the enzymes under investigation respond swiftly to dietary alteration and can be utilized as markers of nutritional circumstances and cultured fish growth (Metón et al., 2007). In this study, high inclusion levels (DBH7.5 and DBH10), significantly increased the liver and kidney function indicators (AST, total bilirubin, LDH, creatinine), whist low inclusion levels (DBH2.5 and DBH5) did not alter the hepatic and kidney function indicators. This may be attributed to hemoglobin powder having a high protein content that may affect the hepatic and kidney indicators with DBH7.5 and DBH10 diets. In addition, the increased enzyme level may be attributed to liver and kidney tissue pathology. Along with the biochemical hepato-renal function indicators, the histopathological finding in liver and kidney tissues were assessed. The hepatic and renal tissues of DBH from 0 to 10 % revealed normal histomorphological structure; however, mild to moderate interstitial lymphocytic infiltration and melano-macrophages proliferative activation was observed in the renal tissue of DBH 7.5 and DBH10. Metón et al. (2007) reported that fish fed high protein or low-calorie diets had the

most increased AST enzyme activity. Another study reported that 5 % inclusion of blood meal to replace a FM is recommended for optimum growth, feed utilization, and un-impairment of hepatic enzymes in hybrid catfish (*Clarias gariepinus male x Heterobranchus longifilis* female) (Olukunle et al., 2002).

The activity of antioxidant enzymes is an important indicator of oxidative stress in fish (Iheanacho and Odo, 2020). Shifting in the enzyme activity within the antioxidant system is frequently indicative of oxidative stress, consequently cellular injury, and predisposes to various diseases (Ighodaro and Akinloye, 2018). Furthermore, fish require many antioxidants, among them SOD and GPX, to protect their immune system from reactive oxygen species (ROS) by eliminating superoxide anion radicals (O2) and hydrogen peroxide (H_2O_2) (Amer et al., 2022; El-Araby et al., 2022; Twahirwa et al., 2021). Antioxidants are plentiful in the tissues of fish (El-Houseiny et al., 2021; Ibrahim et al., 2021). MDA is a byproduct of lipid peroxidation caused by excess free radicals in the cell, consequently leading to toxic stress, deactivation of natural antioxidant enzymes, and impairment of cell membrane functions (Ahmed et al., 2021; Ibrahim et al., 2019; Liu et al., 2011; Mohamed et al., 2019).

In this study, hepatic antioxidant indicators (SOD, GPX, and TAC) were significantly higher in *O. niloticus* fed all DBH diets compared to the control diet (DBH0). However, higher values of MDA were significantly observed in the DBH0 group than in other experimental groups. Twahirwa et al. (2021) reported that the diets contained few blood meals increased the antioxidant capacities and decreased MDA levels in black carp (*Mylopharyngodon piceus*), while high blood meals may induce oxidative stress. Along with biochemical antioxidant activity, splenic antioxidant gene expression was investigated. Gene expression analysis is helpful for determining the antioxidant state of fish in response to dietary intervention (Ahmadifar et al., 2021). Glutathione and glutathione-related enzymes are key to the body's oxidative stress defenses (Stephensen et al., 2002). DBH substitution pointed out an up-regulation of the splenic antioxidant gene expression (*SOD, GPX,* and *GSS*), with the highest expression level detected in the DBH5 group. These results were in synergism with the results of hepatic antioxidant enzymes, which indicated that dietary DBH boosted the oxidative activity of *O. niloticus* up to a 5 % substitution level.

To investigate the protective effect of DBH in *O. niloticus*, a challenge test was used. In this study, dietary DBH exhibited a protective effect against *A. veronii* infection in fish which was proved by the decline in the mortality % in all DBH groups, especially the DBH5 group. DBH had good effects on fish resistance to bacterial infection, which can be attributed to the that bovine hemoglobin generates an antibacterial activity fragment (Froidevaux et al., 2001).

5. Conclusion

Our research suggests that dietary DBH substitution (2.5 to 10 %) improves fish growth and tolerance to *A. veronii* by reducing mortality. The 2.5 to 5 % DBH substitution level enhanced the antioxidant capacity and its related gene expression without destructive impact on hematology, health as well as hepatic and renal tissue architecture in *O. niloticus*. As a result, DBH may be appropriate for use as a dietary replacement for FM in *O. niloticus* diets, and the recommended percentage is 5 %.

CRediT authorship contribution statement

Conceptualization: Rowida E. Ibrahim and Shimaa A. Amer. **Methodology:** Rowida E. Ibrahim, Shimaa A. Amer, Shimaa A. Shahin, Mahmoud I. M. Darwish, Sarah Albogami, Abdelwahab A. Abdelwarith, Elsayed M. Younis, Maram H. Abduljabbar, Simon J Davies, and Ghadeer A. Attia. **Software and data curation:** Rowida E. Ibrahim. **Writing-Original draft preparation:** Rowida E. Ibrahim. **Writing-Reviewing and Editing:** Rowida E. Ibrahim and Shimaa A. Amer

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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