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Effects of dietary anthocyanin on innate immune parameters, gene expression responses, and ammonia resistance of Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

The present study investigated the effects of dietary anthocyanin on the growth performance, haematological, non-specific immune, and spleen gene expression responses of Nile tilapia, *Oreochromis niloticus*. Five experimental groups of fish with mean weights of 8.24 ± 0.64 g were used in the study; four of these were fed with diets incorporating anthocyanin (20 mg kg -^{1}, 40 mg kg^{-1}, 80 mg kg^{-1} and 160 mg kg^{-1}), while the fifth was a control group without dietary anthocyanin. Growth performance and haematological parameters of tilapia were not affected by anthocyanin-supplemented diets ($p > 0.05$). Dietary anthocyanin significantly increased respiratory burst activity, phagocytic activity, phagocytic index, lysozyme activity, myeloperoxidase activity, serum total superoxide dismutase (T.SOD) activity, and serum catalase (CAT) activity ($p < 0.05$). The total immunoglobulin level was highest in the 80 mg kg^{-1} group compared with the other groups ($p < 0.05$). In addition, with the anthocyanin-containing diets, the gene levels of interleukin 1, beta (*IL-1β*), interleukin 8 (*IL-8*), tumor necrosis factor (*TNF-α*), heat shock protein 70 (*HSP70*), and interferon gamma (*IFN-γ*) were increased in the fish spleen, and the gene levels of *CAT*, *GPx*, and *SOD* were also increased in fish liver ($p < 0.05$). At the end of the experiment, the fish were subjected to ammonia stress. The groups fed with 20 and 40 mg kg^{-1} anthocyanin exhibited higher survival rates than the other groups. In summary, feeding Nile tilapia with anthocyanin-containing diets caused increases in the innate immune parameters, gene expression responses, and the survival rate of the fish subjected to ammonia stress.

1. Introduction

Tilapia is a high-value species that is cultured worldwide and is one of the most rapidly growing forms of aquaculture, with a production of 5.6 million tons in 2015 [1]. High stock density of tilapia causes an increase in the level of ammonia in aquaculture pond water. For this reason, fish become stressed and the risks of developing diseases increase [2].

The continuous ammonia exposure results in reduction of fish growth, and causes gill hyperplasia, liver tissue degeneration, hyperexcitability, coma, convulsions, and fish mortality [3]. Previous studies reported that ammonia significantly restrained the antioxidant system and immune response in fish [4,5].

Recent research showed that the use of natural feed additives in aquaculture improved growth performance and disease resistance in fish and influenced blood parameters [6–8]. Moreover, previous studies found that the cumulative mortality rate of fish or shellfish exposed to ammonia toxicity was decreased by the use of feed additives [9,10].

Anthocyanins are glycosidic forms of anthocyanidins that give fruits and flowers bright and attractive colours, and they form a subgroup of flavonoids [11]. Fruits such as blueberries, blackberries, raspberries, strawberries, red grapes, and Saskatoon berries contain high levels of antioxidants [12]. To date, in nature, there are at least 653 anthocyanins involving six common aglycones and many types of glycosylations and acylations [13,14]. Anthocyanins have many effects on humans and animals, including antioxidant, anti-inflammatory, antimicrobial, and anticarcinogenic activities; and are used for treatment of hyperglycaemia and oxidative liver damage, cardiovascular disease prevention, obesity control, improvement of vision, and diabetes alleviation [15–17]. Anthocyanins also prevent atherosclerosis, inhibit free radical activity, and reduce inflammation and aging [18]. These properties show that anthocyanin can be considered as a proper feed additive. There are studies showing the effects of anthocyanin on various living organisms [19–23]; however, the effects of anthocyanin additives on fish have not yet been determined. Therefore, the effects of anthocyanin on growth performance, some blood parameters, and gene

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Abbreviations

T.SOD	total superoxide dismutase
CAT	catalase
IL-1β	interleukin 1, beta
IL-8	interleukin 8
TNF-α	tumor necrosis factor
HSP70	heat shock protein 70
IFN-γ	interferon gamma
GPx	glutathione peroxidase
SOD	superoxide dismutase
RGR	relative growth rate
SGR	specific growth rate
FCR	feed conversion ratio
RBC	red blood cell count
Hb	hemoglobin concentration
Hct	hematocrit ratio
CC1	CC-chemokine
IgM	immunoglobulin M
IFW	mean initial fish weight
FFW	mean final fish weight
MCV	mean corpuscular volume
MCH	mean corpuscular haemoglobin
MPO	myeloperoxidase
Ig	total immunoglobulin
ROS	reactive oxygen species
IFNs	interferons

expression in the spleen and liver of Nile tilapia, *Oreochromis niloticus*, were determined in this study.

2. Materials and methods

2.1. Experimental diet

Fresh blackberries were obtained from a bazaar in the Çanakkale district of north-western Turkey. After drying in air at 40 °C in a circulation drying oven, they were ground to a flour in a coffee grinder, and 50 g of blackberry flour was mixed with 100 mL of a 1:1 ethanol/acidified water (1% HCl) mixture. The amount of total anthocyanin was determined spectrophotometrically after different concentration processes and lyophilization [24]. The purity (≥91%) of the product was determined by HPLC analysis [25]. The HPLC chromatograms of the investigated sample revealed the presence of anthocyanin [Supplementary Fig. 1]. Anthocyanin powder was included in feed prepared in the laboratory at rates of 0 mg kg^{-1}, 20 mg kg^{-1}, 40 mg kg^{-1}, 80 mg kg^{-1}, and 160 mg kg^{-1} for diets named Antho0, Antho20, Antho40, Antho80, and Antho160, respectively. The ingredients (Table 1) were mixed in a laboratory blender, then compressed into 2-mm tablets in a pelleting machine and the pellets were dried in a drying cabinet (40 °C) until the moisture level fell to about 10%. They were then packaged and frozen in a deep freezer at −20 °C until used. The dietary concentrations were determined according to previous studies [26].

2.2. Proximate composition

Proximate analyses were conducted using standard methods. Moisture analyses were accomplished by drying the samples at 105 °C for 24 h in an oven to obtain a stable weight, crude protein was analysed by the Kjeldahl method, and crude ash was determined by incineration at 525 °C in a muffle furnace for 12 h [27]. The methanol chloroform extraction method was used to measure the crude fat content of the samples [28].

2.3. Fish and experimental design

The experiment was carried out in the Faculty of Marine Sciences and Technology, Çanakkale Onsekiz Mart University, Turkey. A total of 450 Nile tilapia (*Oreochromis niloticus*) (initial mean weight: 8.24 ± 0.64 g) were used. The US Environmental Protection Agency guidelines for qualitative evaluation of fish health were followed [29]. Each tank contained 140 L of aerated fresh water that was recirculated at a rate of 150 L/h. All fish were fed *ad libitum* twice a day (08:00 and 17:00 h) for 60 days. During the study, the photoperiod was set to a 12L:12D cycle and temperature was controlled using a chiller. Dissolved oxygen, conductivity, temperature, and pH were measured daily with a YSI Pro2030 multiparameter water analyser and a Hanna HI2221 pH-meter. Nitrite, total ammonia, and nitrate were measured weekly with an Optizen POP UV/VIS spectrophotometer and analysed by spectrophotometry using Merck test kits. Throughout the experiments, water quality parameters of temperature, pH, dissolved oxygen, total ammonia, nitrite, and nitrate were measured as 28.1 ± 0.2 °C, 7.3 ± 0.2, 7.5 ± 0.1 mg/L, 0.018 ± 0.0011 mg/L, 0.027 ± 0.003 mg/L, and 0.7 ± 0.1 mg/L, respectively. All fish experiments were conducted at Çanakkale Onsekiz Mart University in compliance with the guidelines for fish research approved by the animal ethics committee (Protocol Number: 2018/12–03).

2.4. Growth performance and biometric indices

Calculations for the relative growth rate (RGR, %), specific growth rate (SGR, % per day), and feed conversion ratio were as follows:

Table 1
Percentage and proximate composition of the experimental diets.

Ingredients (% dry matter)	Experimental Groups				
	Antho0	Antho20	Antho40	Antho80	Antho160
Fish meal (anchovy meal)[a]	305	305	305	305	305
Fish oil (anchovy oil)[a]	73	73	73	73	73
Soybean meal[b]	215	215	215	215	215
Wheat flour[c]	298	298	298	298	298
Wheat starch[d]	78.99	78.97	78.95	78.91	78.83
Anthocyanin powder	0	0.02	0.04	0.08	0.16
Vitamin mix[e]	10	10	10	10	10
Mineral mix[f]	20	20	20	20	20
BHT[g]	0.01	0.01	0.01	0.01	0.01
Total	1000	1000	1000	1000	1000
Chemical analyses (% DM)					
Protein	34.99	35.01	35.00	35.02	35.04
Fat	9.00	9.06	9.02	9.03	9.05
Ash	5.01	5.03	5.00	5.05	5.04
NFE[h]	41.00	40.90	40.98	40.90	40.87
Energy (kJ/g)[i]	18.78	18.79	18.79	18.78	18.79

[a] Anchovy fish meal, Anchovy fish oil, Karsusan A.Ş., Trabzon, Turkey.
[b] Soybean meal, Kırcı Soybean AŞ., Balıkesir, Turkey.
[c] Wheat flour, İpek Wheat AŞ., Nevşehir, Turkey.
[d] Wheat starch, Cargill, İstanbul, Turkey.
[e] Vitamin Mix (mg kg^{-1} feed, NRC 1977): vitamin A, 5500 IU; vitamin D3, 1000 IU; vitamin E, 50 IU; vitamin K, 10 mg; choline, 550 mg; niacin, 100 mg; riboflavin, 20 mg; pyridoxine, 20 mg; thiamin, 20 mg; biotin, 0.1 mg; folic acid, 5 mg; B12, 20 μg; inositol, 100 mg; choline chloride, 5000 mg.
[f] Mineral Mix (mg kg^{-1} feed, H440): NaCl, 1.0; $MgSO_4$, 7; NaH_2PO_4, 25; KIO_3, 0.0003; $ZnSO_4$, 0.353; $MnSO_4$, 0.162.
[g] BHT, Sigma-Aldrich, St. Louis, MO, USA.
[h] Nitrogen-free extracts (NFE) = dry matter - (crude lipid + crude ash + crude protein).
[i] Energy was calculated based upon 23.6 kJ/g protein, 39.5 kJ/g lipid and 17.0 kJ/g NF.

Relative growth rate (%) = 100 (final fish weight–initial fish weight)/initial fish weight.

Specific growth rate (SGR, % per day) = 100 (ln final fish weight) - (ln initial fish weight)/experimental days.

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

2.5. Blood sampling

Three fish from each tank (9 fish per group) were used for blood sampling. Fish were quickly removed from the tanks and anesthetized with 20 mg/L clove oil [30]. The area behind the anal fin was cleaned with alcohol in order to prevent the mixing of the mucous membrane into the blood, and then blood from the caudal vein was taken immediately with a 2.5-mL plastic syringe. Aliquots of the blood samples were transferred into tubes containing K_3EDTA (MiniCollect®, Austria) for analyses of haematological and some immune-related parameters (phagocytic index, phagocytic activity, and respiratory burst). The remainder of the blood was transferred into serum tubes (Z Serum Separator Tubes, MiniCollect®, Austria) and centrifuged at 5000 × *g* for 10 min, and available serum samples were stored at −80 °C prior to analysis [31]. After blood sampling, spleen and liver tissues were collected as soon as possible and placed in RNAlater solution (Lot No. #R0901; Sigma-Aldrich, St. Louis, MO) at 4 °C overnight and then stored at −20 °C until gene expression analysis [32].

2.6. Haematological parameters

Red blood cell count (RBC, 10^6 mm^{-3}), haemoglobin concentration (Hb, g dL^{-1}), and hematocrit ratio (Hct, %) were measured with an automatic blood count instrument (Mindray BC-3000Plus) [33].

2.7. Immune-related parameters

Myeloperoxidase activity and respiratory burst activity of phagocytes were determined by the methods reported in the literature [34]. Phagocytic activity was determined according to the microscopic counting method [35]. The method described by Nudo and Catap [36] was used to determine the activity of lysozyme. Total immunoglobulin concentration was measured following the method described by Siwicki and Anderson [35].

2.8. Serum antioxidant enzymes

The serum total superoxide dismutase (T.SOD, EC 1.15.1.1) levels were determined using a T. SOD Assay Kit (Product 19160; Sigma-Aldrich) according to the manufacturer's instructions. Catalase (CAT, EC 1.11.1.6) activity was measured spectrophotometrically using a published method [37].

2.9. RT-qPCR analyses of gene expression

RT-qPCR analysis of gene expression was conducted according to the method described by Yılmaz and Ergün [33]. The expression levels of the *SOD* (superoxide dismutase), *CAT* (catalase), *GPx* (glutathione peroxidase), *CC1* (CC-chemokine), *HSP70* (heat shock protein 70), *IFN-γ* (interferon gamma), *IL-8* (interleukin 8), *IL-1β* (interleukin 1), *TNF-α* (tumor necrosis factor), and *IgM* (immunoglobulin M) genes (Table 2) were determined with an Applied Biosystems 7500 Sequence Detection System (USA). β-Actin was used as the internal control. Gene expression levels were analysed using $2^{-\Delta\Delta Ct}$, and β-actin was used as a reference to normalize the RNA input [38].

2.10. Ammonia nitrogen stress experiment

At the end of the experiment, the fish (24 fish tanks, 72 fish groups) were exposed to 543 mg/L ammonium chloride for 96 h as this was the LC_{50} value previously calculated by probit analysis [39]. For this purpose, a stock solution was prepared and the ammonia concentration in the tanks was measured photometrically using a Spectroquant test kit (1.00683.0001; Darmstadt, Germany), and ammonium chloride was added to the tanks at 12 h intervals [40]. Fish deaths were recorded daily.

2.11. Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's multiple comparison tests were conducted using SPSS 19.0 (IBM Corp., Armonk, NY, USA) and the significance level was considered to be 0.05. In each ammonia stress treatment group, Kaplan-Meier analysis was implemented according to the method of Yilmaz [41].

Table 2
Primers used for relative quantitative real-time PCR.

Gene	FWD or REV	Sequence (5′–3′)	Product size (bp)	References
IL-1β	Forward	TGCTGAGCACAGAATTCCAG	60	Kayansamruaj et al. [68]
	Reverse	GCTGTGGAGAAGAACCAAGC		
IL-8	Forward	GCACTGCCGCTGCATTAAG	85	Ming et al. [69]
	Reverse	GCAGTGGGAGTTGGGAAGAA		
TNF-α	Forward	GAGGTCGGCGTGCCAAGA	119	Chen et al. [70]
	Reverse	TGGTTTCCGTCCACAGCGT		
IgM-heavy chain	Forward	AGGAGACAGGACTGGAATGCACAA	171	Pang et al. [71]
	Reverse	GGAGGCAGTATAGGTATCATCCTC		
IFN-γ	Forward	TGACCACATCGTTCAGAGCA	128	Chen et al. [70]
	Reverse	GGCGACCTTTAGCCTTTGT		
HSP70	Forward	TGGAGTCCTACGCCTTCAACA	238	Chen et al. [70]
	Reverse	CAGGTAGCACCAGTGGGCAT		
SOD	Forward	GACGTGACAACACAGGTTGC	20	Xie et al. [72]
	Reverse	TACAGCCACCGTAACAGCAG		
CAT	Forward	TCAGCACAGAAGACACAGACA	21	Xie et al. [72]
	Reverse	GACCATTCCTCCACTCCAGAT		
GPx	Forward	CCAAGAGAACTGCAAGAACGA	21	Xie et al. [72]
	Reverse	CAGGACACGTCATTCCTACAC		
CC-hemokine	Forward	ACAGAGCCGATCTTGGGTTACTTG		
	Reverse	ACAGAGCCGATCTTGGGTTACTTG		Abo-Al Ela et al. [73]

FWD, forward primer; REV, reverse primer; *IL-1β*, interleukin 1 beta; *IL-8*, interleukin 8; *TNF-α*, tumor necrosis factor; *IgM*, immunoglobulin M; *IFN-γ*, interferon gamma; *HSP70*, heat shock protein 70; *SOD*, superoxide dismutase; *CAT*, catalase; *GPx*, glutathione peroxidase; *CC1*, CC-chemokine.

Table 3
Influence of dietary anthocyanin (0, 20, 40, 80, or 160 mg kg^{-1} diet) on growth parameters of Nile tilapia fed with alternative experimental diets for 60 days.

	Antho0	Antho20	Antho40	Antho80	Antho160
Mean initial fish weight (g)	7.73 ± 0.32^{a}	8.10 ± 0.40^{a}	8.40 ± 0.78^{a}	8.36 ± 0.70^{a}	8.63 ± 1.04^{a}
Mean final fish weight (g)	23.50 ± 0.62^{a}	23.20 ± 1.70^{a}	25.63 ± 1.25^{a}	24.80 ± 1.04^{a}	24.53 ± 0.77^{a}
RGR (%)	204.67 ± 15.10^{a}	188.27 ± 35.09^{a}	205.22 ± 15.07^{a}	197.33 ± 19.63^{a}	187.09 ± 27.32^{a}
SGR (%/day)	1.24 ± 0.05^{a}	1.17 ± 0.13^{a}	1.24 ± 0.05^{a}	1.21 ± 0.07^{a}	1.17 ± 0.10^{a}
FCR	0.90 ± 0.04^{a}	0.95 ± 0.12^{a}	0.83 ± 0.01^{a}	0.87 ± 0.05^{a}	0.92 ± 0.05^{a}
Survival (%)	100	100	100	100	100

Table 4
Haematological values of Nile tilapia fed experimental diets with different concentrations (0, 20, 40, 80, or 160 mg kg^{-1} diet) of anthocyanin for 60 days.

	Antho0	Antho0	Antho20	Antho40	Antho80	Antho160
RBC (× 10^{6} mm^{-3})	Automatic	4.89 ± 0.30^{a}	5.05 ± 0.33^{a}	5.02 ± 0.33^{a}	5.01 ± 0.44^{a}	5.09 ± 0.41^{a}
	Manual	4.91 ± 0.45^{a}	5.09 ± 0.28^{a}	5.05 ± 0.30^{a}	5.03 ± 0.38^{a}	5.08 ± 0.36^{a}
Hgb (g dL $^{-1}$)	Automatic	9.90 ± 1.12^{a}	10.82 ± 0.94^{a}	10.88 ± 0.96^{a}	10.81 ± 0.68^{a}	9.83 ± 1.46^{a}
	Manual	9.95 ± 1.18^{a}	10.83 ± 1.02^{a}	10.87 ± 1.09^{a}	10.82 ± 0.87^{a}	9.85 ± 1.93^{a}
Hct (%)	Automatic	35.78 ± 1.58^{b}	36.92 ± 1.63^{ab}	37.02 ± 1.60^{ab}	37.24 ± 1.71^{ab}	38.22 ± 1.93^{a}
	Manual	35.79 ± 1.93^{b}	36.95 ± 2.01^{ab}	37.05 ± 1.99^{ab}	37.25 ± 2.04^{ab}	38.21 ± 2.10^{a}

Values (mean ± SEM, n = 9) with different superscript letters in the same line are significantly different between groups ($p < 0.05$). RBC: Red blood cell count, Hct: hematocrit, Hb: hemoglobin.

Table 5
Effects of dietary anthocyanin on immune-related parameters in Nile tilapia receiving different experimental diets for 60 days.

	Antho0	Antho20	Antho40	Antho80	Antho160
Respiratory burst activity (OD at 620 nm)	0.14 ± 0.020^{c}	0.25 ± 0.016^{b}	0.36 ± 0.065^{a}	0.31 ± 0.044^{ab}	0.18 ± 0.040^{c}
Phagocytic activity (%)	30.13 ± 1.34^{c}	31.27 ± 1.58^{c}	54.64 ± 4.03^{a}	36.72 ± 1.77^{b}	32.39 ± 2.11^{c}
Phagocytic index	3.49 ± 0.35^{b}	3.79 ± 0.41^{ab}	8.67 ± 1.01^{ab}	5.21 ± 0.46^{ab}	4.08 ± 0.55^{a}
Lysozyme activity (μg/mL)	9.75 ± 2.39^{b}	20.77 ± 4.84^{a}	20.64 ± 2.85^{a}	20.45 ± 3.17^{a}	14.10 ± 4.02^{b}
Myeloperoxidase activity (OD at 450 nm)	0.33 ± 0.11^{c}	0.63 ± 0.22^{b}	0.78 ± 0.13^{ab}	0.95 ± 0.17^{a}	0.36 ± 0.08^{c}
Total immunoglobulin (mg/mL)	13.23 ± 2.33^{b}	12.52 ± 1.76^{b}	13.33 ± 4.41^{b}	19.63 ± 4.54^{a}	6.98 ± 1.29^{c}

Values (mean ± SEM, n = 9) with the same superscript letters in the same line are not significantly different between groups ($p < 0.05$).

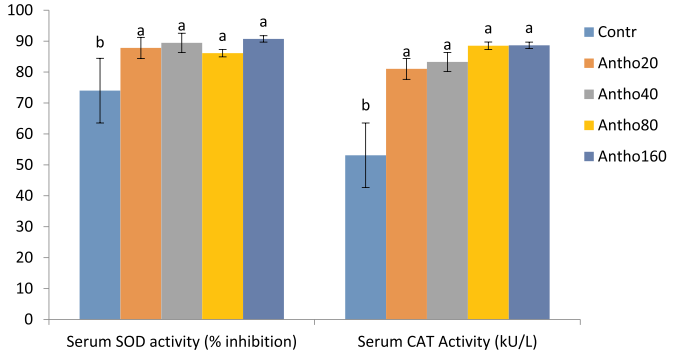

Fig. 1. Superoxide dismutase (SOD) and catalase (CAT) activities of Nile tilapia *Oreochromis niloticus* fed diets supplemented with different concentrations of anthocyanin (0, 20, 40, 80, and 160 mg kg^{-1} diet). Data are represented as mean ± SEM (n = 9 per group). Values with different superscript letters are significantly different ($p < 0.05$).

3. Results

3.1. Growth performance

The mean initial fish weight (IFW), mean final fish weight (FFW), relative growth rate (RGR), feed conversion ratio (FCR), and specific growth rate (SGR) of Nile tilapia measured at the end of the experiment are given in Table 3. There were no statistical differences between the growth performance of Nile tilapia fed with anthocyanin concentrations of 0 mg kg^{-1} (control), 20 mg kg^{-1}, 40 mg kg^{-1}, 80 mg kg^{-1}, and 160 mg kg^{-1} ($p > 0.05$).

3.2. Haematological variables

No statistical differences were observed between manual and automatic haematological results ($p > 0.05$). Haematological parameters of the tilapia are shown in Table 4. The RBCs and Hb concentrations in the treatment groups did not vary significantly from the values observed for the control group ($p > 0.05$). The Hct ratio was significantly higher in the Antho160 group compared with the other experimental groups ($p < 0.05$).

3.3. Immune-related parameters

Immunological variables are given in Table 5. The respiratory burst activity and phagocytic activity were significantly higher in the Antho40 group compared with the other experimental groups ($p < 0.05$). The phagocytic index of the Antho40 group was also significantly higher than those of the other experimental groups ($p < 0.05$). The lysozyme activity increased significantly in the Antho20, Antho40, and Antho80 groups compared with the Antho0 (control) and Antho160 groups. Significantly higher myeloperoxidase activity and total immunoglobulin were detected in the Antho80 group compared with all other experimental treatment groups ($p < 0.05$).

3.4. Serum antioxidant enzymes

Results for the serum antioxidant enzyme parameters are shown in Fig. 1. Significantly higher serum T. SOD and CAT activities were observed in the Antho20, Antho40, Antho80, and Antho160 groups compared with the Antho0 control group ($p < 0.05$).

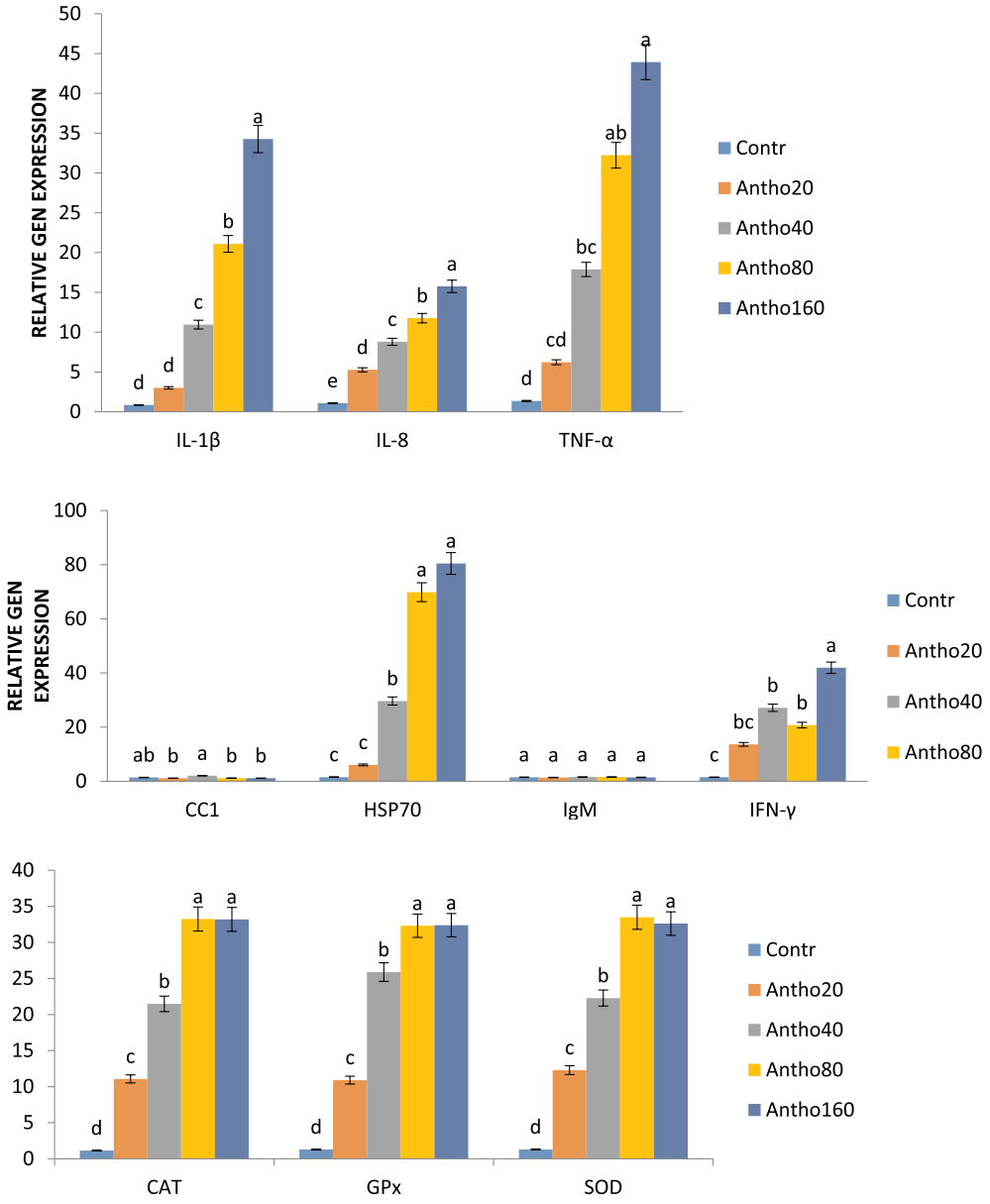

Fig. 2. Gene expression profiles of Nile tilapia fed diets supplemented with different concentrations of anthocyanin (0, 20, 40, 80, and 160 mg kg^{-1}) including interleukin 1 beta (IL-1β), interleukin 8 (IL-8), tumor necrosis factor (TNF-α), CC chemokine (CC1), heat shock protein 70 (HSP70), immunoglobulin M (IgM), and interferon gamma (IFN-γ), involved in the spleen, and glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD), involved in the liver. Data are represented as mean ± SEM (n = 9 per group). Values with different superscript letters are significantly different ($p < 0.05$).

3.5. Expression of immune and antioxidant genes in the spleen and liver of Nile tilapia

The expression profiles of seven immune-related genes in the spleen and three antioxidant genes in the liver of *O. niloticus*, after dietary anthocyanin treatments, are shown in Fig. 2. Significantly higher expression levels of the *IL-1β* gene were found in the Antho40, Antho80, and Antho160 groups compared with the Antho20 and Antho0 groups ($p < 0.05$). Significantly higher *IL-8* and *TNF-α* gene expression levels were seen in the Antho20, Antho40, Antho80, and Antho160 groups compared with the Antho0 control group ($p < 0.05$). The *CC1* gene expression level was higher in the Antho40 group than in the Antho20, Antho80, and Antho160 groups ($p < 0.05$). Higher *HSP70* and *IFN-γ* gene expression levels were observed in the Antho160 group compared with the other experimental groups ($p < 0.05$). The *IgM* level was similar in all the experimental groups ($p > 0.05$). The *CAT*, *GPx*, and *SOD* gene expression levels were higher in the Antho80 and Antho160 groups than in the other experimental groups ($p < 0.05$).

3.6. Ammonia nitrogen stress experiment

The effects of dietary anthocyanin supplementation on cumulative survival of Nile tilapia after ammonia nitrogen stress for 96 h are presented in Fig. 3. At the end of the experiment, there was no significant difference between the Antho80 and Antho160 groups ($p > 0.05$). However, the Antho20 and Antho40 groups showed significantly higher survival rates than the other groups ($p < 0.05$). The challenge test indicated that dietary anthocyanin supplementation could enhance the resistance of Nile tilapia to ammonia nitrogen stress.

4. Discussion

In the present study, the dietary incorporation of anthocyanin did not improve fish growth. Our results are consistent with earlier reports

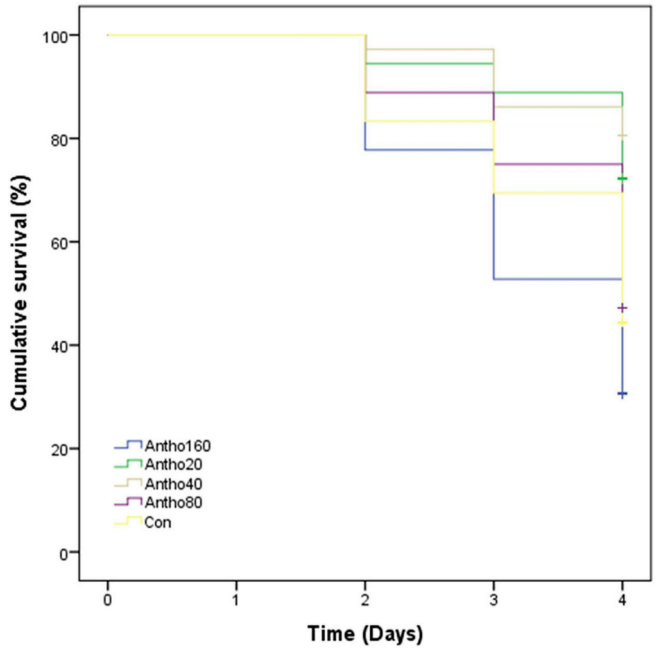

Fig. 3. Kaplan-Meier survival curves. Effects of ammonia nitrogen stress on the cumulative survival of Nile tilapia fed experimental diets at 96 h.

on broiler chickens [30,42] that found no correlation between dietary inclusion of anthocyanin and growth performance.

Haematological parameters are the most important blood parameters that indicate whether fish are under stress and give information about fish health [43–45]. We found that haematological parameters did not change (except in the Antho160 group) in Nile tilapia fed with anthocyanin-supplemented experimental diets compared to those in the control group. Similarly, feeds containing blackberry syrup (7.5, 15, 30 g/kg), which is high in anthocyanin, did not result in any changes in haematological parameters of tilapia [46]. Ströher et al. [47] fed hypercholesterolemic rats with blueberry extract containing anthocyanin and observed that there were no differences in the levels of Hb, Hct, MCV, MCH and erythrocytes ($p > 0.05$). In another study, increased RBCs and Hb concentrations were observed in female rabbits fed a diet including the ethanolic extract of *Hibiscus sabdariffa*, which contains a high proportion of anthocyanin, for 4 weeks [48]. The different haematological findings of the abovementioned studies may be the result of differences in the anthocyanin dose or the experiment duration.

We found that the immunological parameters of respiratory burst activity, phagocytic activity, phagocytic index, lysozyme activity, and myeloperoxidase activity all increased in the experimental groups fed with anthocyanin compared with the control group of Nile tilapia. These results indicate that the addition of anthocyanin to feed of fish positively affects fish immunity. Similarly, in white shrimp (*Litopenaeus vannamei*) fed with a water extract of *Gynura bicolor* feed (containing 47.6 mg g^{-1} anthocyanin), the respiratory burst activity was increased significantly compared with the shrimp fed with the control feed [49]. In another study, respiratory burst activity, potential killing activity, phagocytic activity, phagocytic index, lysozyme activity, myeloperoxidase activity, and total immunoglobulin levels increased in tilapia fed with blackberry syrup with a high anthocyanin content [46]. In the present study, the high mortality rate observed in the Antho160 group may be related to the decrease in total immunoglobulin level.

Like other vertebrates, fish have an antioxidant defence system to reduce the negative effects of reactive oxygen species (ROS). SOD, CAT, and GPx enzymes represent a cellular defence mechanism against the toxicity of ROS [50]. SOD converts superoxide radicals into hydrogen peroxide, thus providing protection against damage caused by free radicals [51]. CAT and GSH-Px catalyse H_2O_2 to create water and O_2 and control free radicals [52]. The activities of antioxidant enzymes and the levels of antioxidant substances have been found to be associated with various physiological and pathological conditions, including oxidative stress [53]. In our study, respiratory burst activity was increased along with the increase in *T. SOD* and *CAT* enzymes. This indicates that the immune response was stimulated by the addition of anthocyanin rather than by oxidative stress. Other studies have found that increased *T. SOD* and *CAT* enzyme activities were an indication of increased immune response in fish [54,55].

TNF-α is a member of the TNF/TNFR cytokine superfamily and is used as a marker for the detection of innate immune system function and tissue damage [56–58]. IL-1β and IL-8 are other important cytokines involved in the fish immune system that mediate the response to infectious diseases [59]. The HSP70 group of HSPs are found in many organisms, from microbes to mammals, and its expression is notably induced in opposition to ambient stresses including UV, heat shock, γ-irradiation, and chemical exposure [60]. Interferons (IFNs) regulate the innate and adaptive immune reaction through multifactorial transcriptional regulation. IFN-γ is in the type II IFN family, which is generated by T lymphocytes and natural killer cells in opposition to bacterial, viral, and parasite antigens [61]. In our study, expression levels of *IL-1β, IL-8, TNF-α, HSP70*, and *IFN*-γ genes were found to be significantly higher in the groups fed with anthocyanin compared with the control group. A similar increase in the expression levels of the *IL-1β, IL-8, TNF-α, HSP70*, and IFN-γ genes in the spleen was obtained by Yilmaz [46], in a study which added blackberry syrup with high contents of anthocyanin to tilapia feed. In aquatic species, an increase in the amount of ammonia in the environment causes oxidative stress and excessive free radical formation, especially of endogenous ROS [62,63]. These ROS can be deactivated by the antioxidant enzymes SOD and CAT, known as the scavengers of ROS. In addition, the molecular stress protein HSP70 can directly increase endogenous peroxidase activity to catalyse the conversion of ROS [64,65]. We found that the gene expression levels of *SOD* and *CAT* increased in tilapia fed with diets containing anthocyanin. Furthermore, when fish exposed to high ammonia stress were fed with diets containing anthocyanin at 20 and 40 mg kg^{-1}, the survival rates increased. Similarly, Jin et al. [66] observed an increase in survival rates in white shrimp fed with 1% yeast hydrolysate and 1% brewer's yeast, then exposed to ammonia stress. In another study, *HSP70* and *SOD* gene expression levels were increased in succinic acid-fed shrimp (*Litopenaeus vannamei*) and the survival rate also increased after ammonia stress for 48 h [67].

5. Conclusions

Our results show that inclusion of dietary anthocyanin at 20 mg kg^{-1} can increase antioxidant capacity, non-specific immune responses, and expression of immune-related genes and thereby improve the survival rate of Nile tilapia against high ammonia stress. Therefore, the addition of anthocyanin to fish feed appears to be beneficial. However, this study is a single experimental model and further research is necessary using feed supplemented with various concentrations of anthocyanin in different fish species. Changes in the response to anthocyanin-supplemented feed in relation to different doses and further blood parameters should be investigated.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.08.033.

Values (mean ± SEM, n = 3) with the same superscript letters within the same line indicate no significant difference between the groups ($p < 0.05$). RGR: Relative growth rate, SGR: specific growth rate, FCR: feed conversion ratio.

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