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Effects of dietary caffeic acid supplement on antioxidant, immunological and liver gene expression responses, and resistance of Nile tilapia, *Oreochromis niloticus* to *Aeromonas veronii*

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ABSTRACT

The present study investigated the effects of dietary caffeic acid on haematological, serum biochemical, non-specific immune and liver gene expression responses of Nile tilapia, *Oreochromis niloticus*. Five experimental groups of fish with mean weights of 89.85 ± 2.5 g were used in the study; three of them were fed with caffeic acid incorporated diets (1 g kg^{-1}-Caf1, 5 g kg^{-1}-Caf5, 10 g kg^{-1}-Caf10), whereas an additive free basal diet served as the control. Additionally, the fifth group was an antibiotic medicated diet (0.02 g kg^{-1}-AMF), prepared with the florfenicol. Dietary caffeic acid especially at 5 g kg^{-1} significantly increased phagocytic index, potential killing activity, respiratory burst activity, serum myeloperoxidase activity and serum catalase activity. Furthermore, increased levels of immune expression [heat shock protein 70 (*HSP70*), interleukin 1, beta (*IL-1β*), tumor necrosis factor (*TNF-α*), CC-chemokine (*CC1*), interleukin 8 (*IL-8*), toll-like receptor 7 (*tlr-7*), interferon gamma (*IFN*-γ) and immunoglobulin M (*IgM*)] and antioxidant related genes [superoxide dismutase (*SOD*), catalase (*CAT*) and glutathione peroxidase (*GPx*)] in the liver of fish fed with 5 g kg^{-1} caffeic acid. At the end of the 20-day challenge period the survival rates were significantly higher in the Caf5 and AMF groups compared to all other treatment groups.

As a result, feeding Nile tilapia with a diet containing 5 g kg^{-1} caffeic acid over a period of 60 days might be adequate to improve fish immune parameters, antioxidant status, as well as survival rate against *A. veronii*, similar to antibiotic treatment. Thus caffeic acid can be suggested as a dietary substitute for antibiotic to prevent *A. veronii* in tilapia.

1. Introduction

The tilapia is an economically important fish species cultured mainly in China, Indonesia and Egypt, with a global production around 5.9 million tones [1]. As a result of the increased production in intensive culture conditions, fish welfare might be reduced due to increased stress environment, thus affecting fish health. Tilapia is often hampered by high mortality rates, and economic loss due to infectious diseases such as Edwardsiellosis, Flavobacteriosis, Motile Aeromonas Septicemia, Staphylococcosis and Streptococcosis [2]. In tilapia, influences of aeromonads (*Aeromonas hydrophila*, *Aeromonas sobria*, *Aeromonas dhakensis* [3], and *Aeromonas veronii* [4,5]) have been reported earlier. Through the use of antibiotics such as oxytetracycline, nifurpirinol and sulfadimethoxine/ormetoprim, it is partly possible to control aeromonasis [6]. Nevertheless, in undeveloped or developing countries, antibiotic treatment is a not an affordable way for many farmers, and it may harm both the environment and human health. Hence, it has been proved by many studies that organic acids can help the improvement in aquaculture facilities through the increase in fish growth and their resistance against illnesses and promoting immune responses [7].

Florfenicol has a broad antibacterial spectrum similar to chloramphenicol and is a widely used antibiotic in fish culture. It has currently been approved presently for use in Turkey, Europe, South Korea, Japan, South Korea, Norway, Canada, Chile, and the United Kingdom [8]. In a recent study carried out in our laboratory, Yılmaz and Ergün [9] reported that *in vitro* experiments showed that the florfenicol or chloramphenicol have antimicrobial effects against *Aeromonas veronii*. Hence, in the present study, florfenicol was used as a positive control group in order to clarify if caffeic acid could properly play an alternative role and replace the antibiotic.

Caffeic acid is produced from plants and considered as a pharmacologically safe organic compound with anti-carcinogenic, anti-microbial, anti-inflammatory, anti-oxidant and immunomodulatory effects

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[10]. Some earlier studies explained beneficial effects of caffeic on animals. For instance, Nardini et al. [11] reported that dietary supplementation of caffeic acid (0.2 and 0.8% w/w) in rats resulted in a statistically significant increase of α-tocopherol both in plasma and lipoprotein. In addition, caffeic acid significantly reduced lipid peroxidation and restored the levels of antioxidant defense in the nickel induced rat livers [12]. These results prove the physiological relevance of caffeic acid and its antioxidant action *in vivo,* through both a direct contribution to the antioxidant defense system and a sparing effect on α-tocopherol [11]. Moreover, some studies have declared caffeic acid to be an inducer of apoptosis in cancer cell lines and capable of tumor growth inhibition and regression in animals. Although there is abundant information that caffeic acid is beneficial and highly profitable, there are still many questions to be answered regarding their immunostimulary effects in finfish diets.

For the determination of dietary efficiencies of feed additives in fish, some of the significant indicators for the health status can be listed as haematological, serum biochemical and innate immune parameters [7]. In recent years, another significant research area is the alteration in immune-related gene expressions.

In this study, eleven genes related to immune and antioxidant systems were assigned to evaluate the effect of caffeic acid on tilapia fish. It is well known that the CC-chemokine (*CC1*), heat shock protein 70 (*HSP70*), interferon gamma (*IFN-γ*), interleukin 8 (*IL-8*), interleukin 1, beta (*IL-1β*), tumor necrosis factor (*TNF-α*), immunoglobulin M (*IgM*) and toll-like receptor 7 (*tlr-7*) are used for the assessment of innate, acquired and adaptive immune responses in teleost fish species [13–16]. In addition, the superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are significant antioxidant enzymes which detoxify oxygen free radicals and hydrogen peroxide, preventing oxidative damage [17,18].

The liver has highly significant roles for both the intermediate metabolism of an individual and the metabolism of xenobiotics. Therefore, it is considered as a good indicator of the health status in fish [19]. In recent studies, *IgM*, *IFN-γ*, *TNF-α*, *IL-1β* and *IL-8* genes were expressed in tilapia liver after the supplementation of resveratrol, and a continuous increase was revealed until the end-point of exposure [20,21]. These observations show the importance of liver as an organ in the investigation of the effects of caffeic acid in tilapia fish.

So far, there is only one study available reporting no adverse influences on growth or survival rate of tilapia exposed to a single dose caffeic acid treatment (5 g kg^{-1}) [22]. However, different than the present study no information was given on haematological, serum biochemical and immunological responses, and gen expressions at molecular basis in earlier reports. Therefore, the aim of this study was to establish the antioxidant and immune potentiating effects of caffeic acid in tilapia (*Oreochromis niloticus*).

2. Materials and methods

2.1. Experimental diet

Caffeic acid used in the present study was obtained from Carl Roth Gmbh & Co. KG in Germany, provided with ≥98% purity, and was supplemented in test diets at 1.0, 5.0 and 10.0 g kg^{-1}, which were then named as Caf1, Caf5, and Caf10 groups, respectively. Dietary incorporation levels of caffeic acid were determined according to the result obtained from the previous study conducted on *Oreochromis niloticus* [22]. In addition, an antibiotic medicated diet (AMF) was prepared with a commercial product of FLORMIS AQUA® (500 mg g^{-1} florfenicol; Mistav, Ankara, Turkey). In tilapia fish the recommended dose of florfenicol is 0.02 g kg^{-1} feed for 16 weeks [23]. Fish fed on a diet without supplementations of both caffeic acid and antibiotic, served as a control (Con) group. All ingredients (Table 1) were mixed using a laboratory blender and a pelleting machine (La Monferrina P3, Italy) with a 2-mm die to form the pellets, which were then dried in a drying cabinet at 40 °C until the moisture content of pellets declined to 10%. Thereafter, the pellets were stored in plastic bags and kept in a deep freezer at −20 °C until used.

Table 1
Percentage and proximate composition of the experimental diets.

Ingredients (% dry matter)	Con	Caf1	Caf5	Caf10	AMF
Fish meal (anchovy meal)	300	300	300	300	300
Fish oil (anchovy oil)	70	70	70	70	70
Soybean meal	240	240	240	240	240
Wheat flour	300	300	300	300	300
Wheat starch	49.99	48.99	44.99	39.99	49.79
Carboxymethyl cellulose	10	10	10	10	10
Caffeic acid	0	1	5	10	0
Antibiotic	0	0	0	0	0.02
Vitamin mix	10	10	10	10	10
Mineral mix	20	20	20	20	20
BHT	0.01	0.01	0.01	0.01	0.01
Total	1000	1000	1000	1000	1000
Chemical analyses (% DM)					
Protein	36.34	36.33	36.35	36.36	36.37
Fat	10.55	10.57	10.55	10.54	10.55
Ash	4.75	4.85	5.25	5.74	4.77
NFE[a]	37.26	37.17	36.83	37.00	37.02
Energy (kj/g)[b]	19.08	19.07	19.01	19.03	19.04

[a] Nitrogen-free extracts (NFE) = dry matter - (crude lipid + crude ash + crude protein).
[b] Energy was calculated according to 23.6 kJ/g protein, 39.5 kJ/g lipid, and 17.0 kJ/g NFE.

Proximate analyses of the diets and whole body fish samples (visceral organs and visceral fats excluded) have been conducted according to standard methods [24]. Methanol/chloroform extraction was performed for the crude fat analysis [25].

2.2. Fish and experimental design

Oreochromis niloticus were produced in the Faculty of Marine Sciences and Technology of Canakkale Onsekiz Mart University. Each fish was visually monitored externally in accordance with the EPA guidelines in order to qualitatively assess fish health [30]. Before initiating the experiment, fish were acclimatized to the experimental tank conditions for 15 days and fed until satiation with commercial feeds. A total of 450 fish with average weight of 89.85 ± 2.5 g (mean ± SD) were randomly allotted into 15 experimental tanks (30 fish/tank) for the five treatment groups in triplicate design. Feeding was performed two times a day until satiation at 08.00 and 17.00 h throughout the 60-days feeding trial conducted in a 12L:12D light-dark cycle photoperiod.

A heater/chiller was used to control the temperature in the recirculating system. Daily measurements of temperature (28.5 ± 0.3 °C), dissolved oxygenation (7.2 ± 0.18 mg L^{-1}), conductivity (441 ± 6.8 µS) and pH (7.18 ± 0.1) were performed while weekly measurements were conducted for total ammonia (0.017 ± 0.0013 mg L^{-1}), nitrite (0.029 ± 0.002 mg L^{-1}) and nitrate (0.9 ± 0.2 mg L^{-1}). Each of the experimental fiberglass tanks were filled with 140 L and supplied with 155 L h^{-1} aerated freshwater in a recirculating system.

2.3. Sampling

After termination of the 60-day feeding trial, blood samples were taken from 9 fishes (3 fish per tank). Before blood sampling, the fish were deprived from feed for one day. Fish were randomly selected and removed from the tanks as soon as possible with minimum disturbance and anesthetized in clove oil at 20 mg L^{-1}. Fish were cleaned using alcohol with special care around the anus in order to avoid any contamination of mucous membrane with the blood samples. Then blood was taken from the caudal vein via a 2.5 mL plastic syringe. For the analyses of some haematological and immune-related parameters, a proportion of the sampled blood was inserted into tubes with K_3EDTA. The other proportion of the blood was put into serum tubes and centrifuged at 5000 *g* for 10 min [7]. The acquired serum samples were kept at −80 °C for lysozyme, myeloperoxidase and serum biochemical analyses. After the blood sampling, overdose clove oil (200 mg L^{-1}) was used to euthanize the fish. Right after, liver tissues were collected and transferred into RNAlater solution (4 °C) and kept overnight at −20 °C until performing analyses of gene expression [7].

2.4. Haematological, biochemical and immune related parameters

Haematological parameters, serum biochemical parameters and immune related parameters were performed according to the methods previously described in our laboratory [7].

2.5. Serum antioxidant enzymes

The inhibition rate of percent-reaction of enzyme with water soluble tetrazolium dye substrate and xanthine oxidase using a SOD Assay Kit (Sigma, 19160) through tin was used to measure SOD activity in accordance with the producer's instructions. The method described by Goth [26] was used for the determination of catalase (CAT) activity in a spectrophotometer. As a short description, 200 μL serum was incubated with 1 mL of substrate (65 μmol/mL H_2O_2 in 60 mmol/L sodium phosphate/potassium phosphate buffer, pH7.4), at 37 °C for 60 s 1 ml of 32.4 mmol/l ammonium molybdate was used to stop the enzymatic reaction and readings for yellow complex of molybdate and hydrogen peroxide was done at 405 nm.

2.6. Bacteria and challenge experiment

Previously isolated *A. veronii* SY-AV10 (GenBank accession no. MG563680) from diseased *O. niloticus* was used in the present study. *A. veronii* was produced overnight in Brain Heart Broth at 37 °C, and then washed twice with PBS to adjust the density to 2×10^8 CFU mL^{-1}. The LD50 value was previously calculated according to Finney's probit analysis method [27].

After termination of the growth trial at day-60, fish (75 fish/group) were intraperitoneally injected with 100 μL bacterial suspension (2×10^8 CFU mL^{-1} in PBS) using an insulin syringe. Fish in tank was monitored daily and the morts were removed from the tank when seen, and the mortality rate was recorded over a 20-days period. In order to be sure that the mortalities were due to the bacterial infection, *A. veronii* was re-isolated. To identify isolates 16S rDNA analysis were performed.

2.7. RT-qPCR analyses of gene expression

Gene expression analyses of RT-qPCR was conducted according to the method previously described in our laboratory [7]. GeneMATRIX Kit (E3598, Poland) was used to extracted total RNA was from the liver according to the manufacturer's instructions. The expression levels of the genes (Table 2) were determined using an “Applied Biosystems 7500 Sequence Detection system (USA)”. β-actin was used as the internal control. For the normalization of RNA input, gene expression levels analyzed with $2^{-\Delta\Delta Ct}$, and β-actin was used as reference [28].

2.8. Ethics statement

All experiments were conducted according to the guidelines for fish studies provided by the Committee of Animal Ethics of Canakkale Onsekiz Mart University with the Protocol Number of 2018/01–04.

2.9. Statistical analysis

For the data analysis, one way analysis of variance (ANOVA) was used, and the acquired data was given as Means ± Standard Error of Mean (SEM). Tukey's multiple comparison test was selected when homogeneity of variances occurred; in other cases, a Tamhane post hoc test was used. After Dunn's post hoc test, Kruskal-Wallis test was applied where normality variances were not considered. In order to specify the importance of differences between the manual and automatic haematological values, student's t-test was preferred. In each challenge treatment group, Kaplan-Meier analysis was done according to the method of Yılmaz and Ergün [7] In order to make analysis, SPSS 19.0 (SPSS Statistics) was used with 0.05 value for the significance level.

3. Results

3.1. Haematological and serum biochemical variables

In the present study, both manual or automatically measured haematological parameters did not present any statistical differences ($p > 0.05$; data not shown). Haematological variables measured using automatic analyzer is given in Table 3. The RBC count, Hgb concentration, and Hct ratio in the treatment groups were statistically similar to the values recorded for the control group ($p > 0.05$). At the end of the feeding trial, no significant differences were found among all serum biochemical variables with the exception in serum triglyceride, glutamic oxaloacetic transaminase and alkaline phosphatase levels as shown in Table 3. Significantly lower serum triglyceride level was seen in the Caf10 group compared to Con group ($p < 0.05$). The glutamic oxaloacetic transaminase found for the Caf10 group however was significantly lower than the Con, Caf5 and AMF treatment groups ($p < 0.05$). Significantly higher serum alkaline phosphatase level was seen in the AMF group compared to all other experimental treatment groups ($p < 0.05$).

3.2. Immune related parameters

Findings for the immunological variables are given in Fig. 1. No significant differences were seen in terms of phagocytic activity and lysozyme activity among the experimental groups ($p > 0.05$). Significantly lower respiratory burst activity was seen in the Con group compared to all other experimental treatment groups ($p < 0.05$). The respiratory burst activity found for the Caf1 and Caf5 groups was significantly higher than the other experimental groups ($p < 0.05$). Significantly higher potential killing activity was noted in the Caf5 group compared to the Con and Caf10 treatment groups ($p < 0.05$), but the remaining groups did not present any significant difference ($p > 0.05$). However, significantly higher phagocytic index was recorded in the Caf5 group compared to Con and Caf10 treatment groups ($p < 0.05$). The myeloperoxidase activity increased significantly in the Caf5 group ($p < 0.05$) compared to all other experimental treatment groups except the Caf10 group. In addition, significantly higher myeloperoxidase activity was found in the Caf10 group over those in the Con and Caf1 groups ($p < 0.05$).

3.3. Serum antioxidant enzymes

Results for the antioxidant parameters are given in Fig. 2. The serum superoxide dismutase activity found for the Caf10 group was significantly lower than the other experimental groups ($p < 0.05$). Significantly higher serum catalase activity was seen in the Caf1, Caf5, Caf10 and AMF groups compared to Con group ($p < 0.05$).

3.4. Immune and antioxidant gene expression in the liver of Nile tilapia

The effects of dietary caffeic acid and antibiotic treatments on

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. [58]
	Reverse	GCTGTGGAGAAGAACCAAGC		
IL-8	Forward	GCACTGCCGCTGCATTAAG	85	Ming et al. [59]
	Reverse	GCAGTGGGAGTTGGGAAGAA		
TNF-α	Forward	GAGGTCGGCGTGCCAAGA	119	Chen et al. [60]
	Reverse	TGGTTTCCGTCCACAGCGT		
IgM-heavy chain	Forward	AGGAGACAGGACTGGAATGCACAA	171	Pang et al. [61]
	Reverse	GGAGGCAGTATAGGTATCATCCTC		
IFN-γ	Forward	TGACCACATCGTTCAGAGCA	128	Chen et al. [60]
	Reverse	GGCGACCTTTAGCCTTTGT		
HSP70	Forward	TGGAGTCCTACGCCTTCAACA	238	Chen et al. [60]
	Reverse	CAGGTAGCACCAGTGGGCAT		
CC-hemokine	Forward	ACAGAGCCGATCTTGGGTTACTTG		Abo-Al-Ela et al. [62]
	Reverse	TGAAGGAGAGGCGGTGGATGTTAT		
SOD	Forward	GACGTGACAACACAGGTTGC	20	Xie et al. [63]
	Reverse	TACAGCCACCGTAACAGCAG		
CAT	Forward	TCAGCACAGAAGACACAGACA	21	Xie et al. [63]
	Reverse	GACCATTCCTCCACTCCAGAT		
GPx	Forward	CCAAGAGAACTGCAAGAACGA	21	Xie et al. [63]
	Reverse	CAGGACACGTCATTCCTACAC		
tlr7	Forward	TCAGCAGGGTGAGAGCATAC	143	Salah et al. [64]
	Reverse	ACATATCCCAGCCGTAGAGG		
β-Actin	Forward	CAGCAAGCAGGAGTACGATGAG	62	Pang et al. [61]
	Reverse	TGTGTGGTGTGTGGTTGTTTTG		

profiles of the immune related genes expressions found in the liver of *O. niloticus* are given in Figs. 3 and 4. Significantly higher expression level of *IL-8* gene (Fig. 3) was found in the Caf1, Caf5, Caf10 and AMF groups compared to the control group ($p < 0.05$). The *IL-8* gene expression level was also higher for the fish in the AMF group than the control, Caf1 and Caf10 groups ($p < 0.05$). It was recorded that the *IL-1β* gene expression levels were significantly higher for all caffeic acid treatment groups and the antibiotic group when compared to the control group without caffeic acid treatments ($p < 0.05$) (Fig. 3). The expression levels of *IFN-γ* gene was significantly higher in the Caf5 group then the other treatment groups, with an exception for the AMF group ($p < 0.05$) (Fig. 3). Higher *IFN-γ* gene expression was also found in the AMF group over the control and the Caf1 groups ($p < 0.05$). In the Caf1, Caf5 and AMF groups, significantly higher *TNF-α* gene expressions were recorded when compared to the control group ($p < 0.05$) (Fig. 3). Further, the Caf5 group also presented higher levels of *TNF-α* gene expressions then the control, Caf1 and Caf10 groups ($p < 0.05$).

The Caf1, Caf5 and AMF groups demonstrated higher expression levels for the *CC1* gene compared to the control or the Caf10 groups ($p < 0.05$) (Fig. 4). Higher levels of the *HSP70* gene expression was found in the Caf5 and AMF groups over those of the other test groups ($p < 0.05$) (Fig. 4). Significantly higher levels of *tlr-7* gene expression was noted in the Caf1, Caf5 and AMF groups compared to the control group ($p < 0.05$) (Fig. 4). This was also higher for the AMF group than the control, Caf1 and Caf10 groups ($p < 0.05$). In the Caf5 group, the *IgM* gene expression level was higher than the other experimental groups ($p < 0.05$) (Fig. 4).

In the AMF group, the *SOD* gene expression level was significantly higher compared to the other experimental groups ($p < 0.05$) (Fig. 5). In all caffeic acid treatment groups and the antibiotic group the *CAT* gene expression levels were significantly higher than the control group ($p < 0.05$) (Fig. 5).

Higher expression levels for the *GPx* were found in the Caf5 and AMF groups compared to the other experimental groups ($p < 0.05$). It was also higher in the Caf5 compared to the other treatment groups ($p < 0.05$) (Fig. 4).

3.5. Challenge test with *A. veronii*

After the feeding trial for a period of 60 days, experimental fish were challenged with *A. veronii* and cumulative survival was recorded for 20 days (Fig. 6). Clinically infected fish displayed abnormal

Table 3
Effect of dietary caffeic acid and antibiotic on haematological and serum biochemical parameters in Nile tilapia fed different experimental diets for 60 days.

	Con	Caf1	Caf5	Caf10	AMF
RBC (x10^6 mm^{-3})	3.26 ± 0.10	3.29 ± 0.06	3.25 ± 0.07	3.28 ± 0.07	2.98 ± 0.05
Hgb (g dL^{-1})	10.39 ± 0.31	10.38 ± 0.22	10.31 ± 0.29	10.73 ± 0.26	9.69 ± 0.17
Hct (%)	36.19 ± 0.91	35.91 ± 0.58	35.28 ± 0.68	35.16 ± 0.56	34.56 ± 0.87
GLU (mg/dL)	65.34 ± 4.26	76.82 ± 3.72	71.71 ± 5.12	71.63 ± 3.62	79.33 ± 3.66
Tprot (g/dL)	7.92 ± 0.64	8.49 ± 0.70	7.69 ± 0.51	7.59 ± 0.49	7.84 ± 0.46
ALB (g/dL)	0.16 ± 0.02	0.10 ± 0.02	0.15 ± 0.02	0.14 ± 0.01	0.13 ± 0.02
GLO (g/dL)	7.76 ± 0.63	8.38 ± 0.70	7.54 ± 0.49	7.45 ± 0.48	7.71 ± 0.45
TRIG (mg/dL)	56.26 ± 1.67^{a}	53.85 ± 6.10^{ab}	45.40 ± 6.92^{ab}	33.90 ± 4.91^{b}	50.60 ± 4.66^{ab}
COL (mg/dL)	234.37 ± 20.79	249.13 ± 25.73	195.23 ± 12.63	197.33 ± 9.11	200.87 ± 13.88
GOT (U/L)	23.06 ± 1.73^{a}	22.72 ± 1.70^{ab}	23.40 ± 1.46^{a}	16.81 ± 1.26^{b}	24.37 ± 1.15^{a}
GPT (U/L)	7.41 ± 1.33	7.72 ± 1.16	8.00 ± 0.79	6.36 ± 1.15	8.12 ± 0.45
LDH (U/L)	439.77 ± 41.20	455.14 ± 29.35	363.32 ± 42.74	384.72 ± 31.63	433.57 ± 24.83
ALP (U/L)	15.89 ± 1.10^{b}	21.56 ± 2.05^{b}	18.74 ± 2.37^{b}	15.62 ± 1.07^{b}	35.50 ± 5.62^{a}

Values (mean ± SEM, n = 9) with different superscript letters in the same line are significantly different within groups ($p < 0.05$). RBC = red blood cell count, Hct = hematocrit, Hgb = hemoglobin, GLU = glucose, Tprot = total protein, ALB = albumin, GLO = globulin, TRIG = triglyceride, COL = cholesterol, GOT = glutamic oxaloacetic transaminase, GPT = glutamic pyruvic transaminase, LDH = lactate dehydrogenase and ALP = alkaline phosphatase.

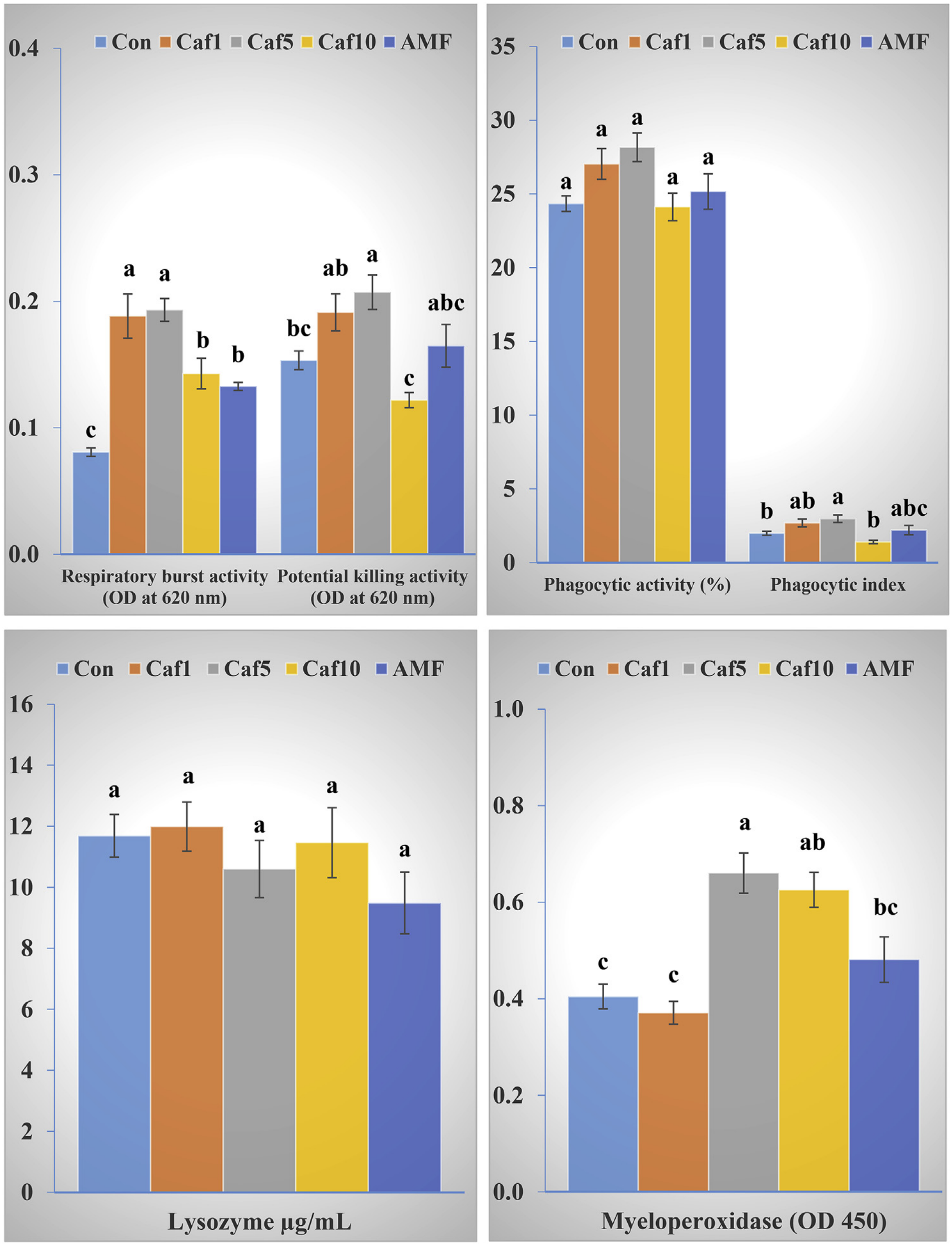

Fig. 1. Respiratory burst activity, potential killing activity, phagocytic activity, phagocytic index, lysozyme activity and myeloperoxidase activity of Nile tilapia, *Oreochromis niloticus* (mean ± SEM, n = 9) fed diets supplemented with different concentrations (0, 1, 5 or 10 g caffeic acid kg^{-1}) of caffeic acid, and antibiotic.

swimming, darkened color and loss of appetite. Internally, severe haemorrhage on the surfaces of the liver and inflamed intestine were noted. Fish were fed each experimental diet continuously from 4 days post-challenge. At the end of the 20 day challenge period, fish survival rates were significantly higher in the AMF and Caf5 groups compared to all other treatment groups ($p < 0.05$; Table 4), but no significant differences were found between the remaining groups ($p > 0.05$).

4. Discussion

Haematological and serum biochemical are considered as important parameters for assessing stress conditions, or health status of fish under culture conditions [29]. Our results in terms of reference values for haematological parameters found for Nile tilapia are in close agreement with previous report [30]; RBC: 0.7–28 × $10^6 mm^{-3}$, Hgb: 6.58–15.98 g dL^{-1} and Hct: 15–45%. Moreover, in the present study, haematological parameters were not influenced by dietary caffeic acid supplementation in Nile tilapia. Similarly, dietary inclusion of *trans*-cinnamic acid at levels (250, 500, 750 and 1500 mg kg^{-1}) were investigated in rainbow trout where no differences were reported for RBC, Hct and Hgb values in an earlier study [7].

Serum biochemical parameters are used as health indicators in fish studies [29,31]. In this study, no effects on serum glucose, cholesterol, triglyceride, GOT, GPT, ALP and LDH levels were recorded in fish fed caffeic acid-incorporated diets, especially in the 1 and 5 g kg^{-1} supplemented diet groups, which is in agreement with an earlier study on *O. mykiss* fingerlings fed with BioAcid Ultra® [32]. In the present study, serum triglyceride and GOT levels decreased at high-dose dietary caffeic acid (10 g kg^{-1}) levels. It is well known that, organic acid type and dose may show different effects on serum biochemistry of fish [7]. For instance, hybrid *Oreochromis* sp. fed with different types of sodium salt of organic acids (acetate, butyrate, formate, and propionate) at a rate of 2%, demonstrated increased levels of serum glucose and GPT with dietary inclusion of sodium formate, while the incorporation of other

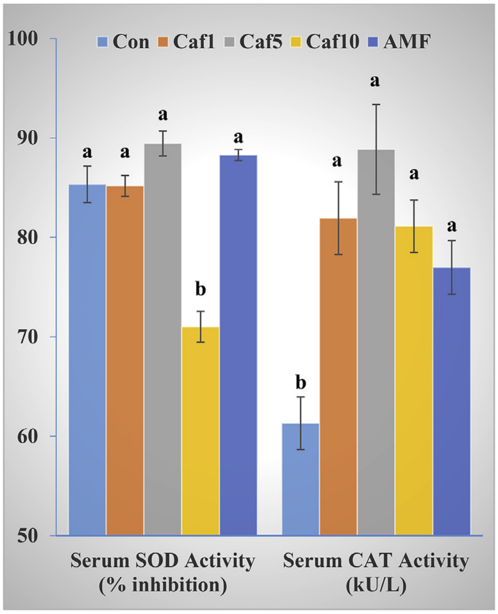

Fig. 2. Serum superoxide dismutase (SOD) and catalase (CAT) activities of Nile tilapia, *Oreochromis niloticus* (mean ± SEM, n = 9) fed diets supplemented with different concentrations (0, 1, 5 or 10 g caffeic acid kg^{-1}) of caffeic acid, and antibiotic.

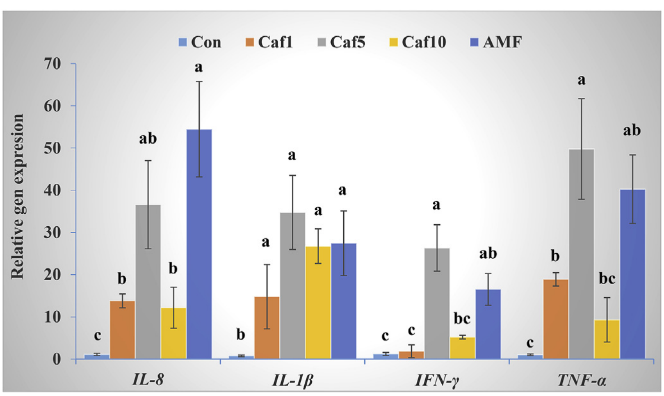

Fig. 3. Gene expression profiles in liver of interleukin 8 (*IL-8*), interleukin 1, beta (*IL-1β*), interferon gamma (*IFN-γ*) and tumor necrosis factor (*TNF-α*) of Nile tilapia, *Oreochromis niloticus* (mean ± SEM, n = 9) fed diets supplemented with different concentrations (0, 1, 5 or 10 g caffeic acid kg^{-1}) of caffeic acid, and antibiotic. Values with different superscript letters are significantly different ($P < 0.05$).

organic acid additives did not show any influence on serum glucose or GPT levels [33]. In addition, our current data showed that serum ALP levels increased significantly in fish fed florfenicol supplemented diet. This is probably related to the damage on the biliary tract [34]. Similar findings were reported in Atlantic cod [35] and rainbow trout [36].

In recent years, there is a growing interest for the prophylactic features of organic acids on bacterial challenge in fish [7,37,38]. In our study, increased respiratory burst activity, potential killing activity, phagocytic index, myeloperoxidase activity, immune response gene levels in the liver and survival rate against *A. veronii* particularly in the Caf5 treatment group endorsed the immunomodulatory effects of caffeic acid in fish. Parallel with our study, Cao et al. [39] reported that a significant increase of survival rate was recorded for snakehead fish, *Ophiocephalus argus* fed with probiotic (*Bacteriovorax* sp), when fish were exposed to *A. veronii*. In this study, the survival rate against *A. veronii* observed in the Caf5 group was significantly higher over the control and the other caffeic acid-treated groups. However, similar

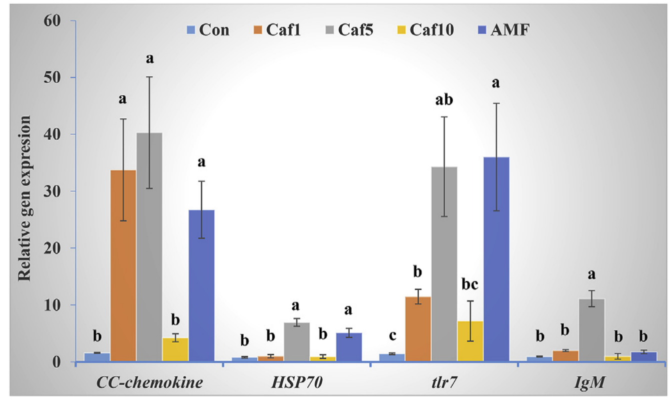

Fig. 4. Gene expression profiles in liver of CC-chemokine (*CC1*), heat shock protein 70 (*HSP70*), toll-like receptor 7 (*tlr-7*) and immunoglobulin M (*IgM*) of Nile tilapia, *Oreochromis niloticus* (mean ± SEM, n = 9) fed diets supplemented with different concentrations (0, 1, 5 or 10 g caffeic acid kg^{-1}) of caffeic acid, and antibiotic. Values with different superscript letters are significantly different ($P < 0.05$).

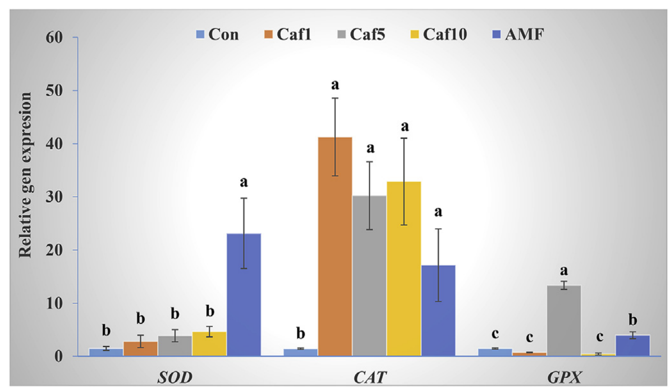

Fig. 5. Gene expression profiles in liver of superoxide dismutase (*SOD*), catalase (*CAT*) and glutathione peroxidase (*GPx*) of Nile tilapia, *Oreochromis niloticus* (mean ± SEM, n = 9) fed diets supplemented with different concentrations (0, 1, 5 or 10 g caffeic acid kg^{-1}) of caffeic acid, and antibiotic. Values with different superscript letters are significantly different ($P < 0.05$).

results for survival rates were recorded in the other treatment groups with increasing or decreasing doses when compared to the control. In agreement to our results, the *Oreochromis* sp. fed diets with 0.5% incorporation of organic acid mixture augmented the survival rate against *Streptococcus agalactiae*, whereas survival was not affected when dietary incorporation of the mixture of organic acid increased [40]. In a different study, non-specific immune responses as well as variations in tissue gene expression and resistance were reported earlier in finfish fed diets containing organic acid, namely, diet containing 250 or 500 mg kg^{-1} *trans*-cinnamic acid were tested in *O. mykiss* and increased non-specific immune response, expression levels of the immune related genes and survival against *Yersinia ruckeri* were recorded [7]. Feeding *Epinephelus fuscoguttatus* with sodium salt of alginic acid at 1.0 or 2.0 g kg^{-1} levels resulted in increased non-specific immune response, enhanced by respiratory burst, phagocytic activity, ACH50, and resistance against *Streptococcus* sp [41]. The addition of 1.0 and/or 2.0 g kg^{-1} formic and propionic acid/salt mixture to *O. niloticus* feeds remarkably regulated the liver *IL-1β* and *TNF-α* genes and survival rate against *Aeromonas sobria* after 60 days of a feeding experiment [38]. However, unlike our study, *IL-1β*, *TNF-α* and *IL-8* gene expressions levels for the liver and intestines in *D. labrax* fed with feed containing 0.2% sodium butyrate for 8 weeks remained unchanged [42].

In the present study, liver immune-related gene expression levels and respiratory burst activity increased in fish fed with 0.02 g kg^{-1}

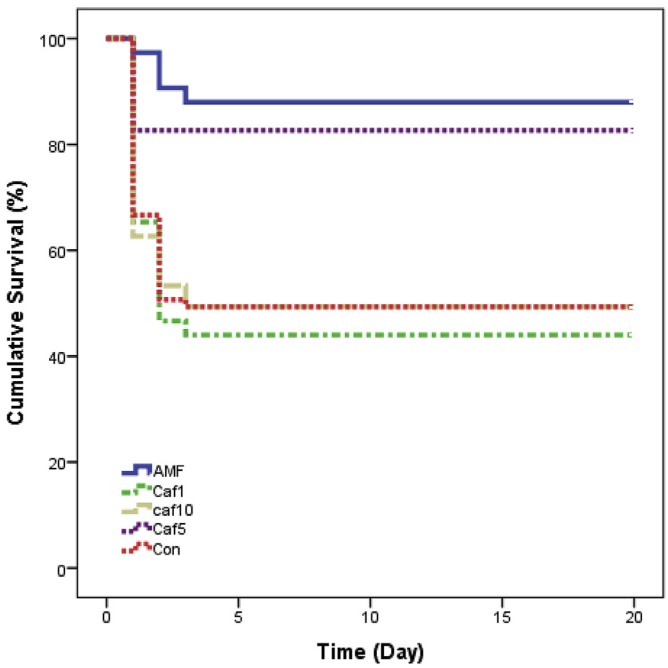

Fig. 6. Kaplan–Meier survivorship curves (cumulative survival [%] over time [Days 0, 5, 10, 15, 20]) for Nile tilapia after challenge with *Aeromonas veronii*; the fish were fed with caffeic acid (0, 1, 5 or 10 g caffeic acid kg^{-1} feed; Con, Caf1, Caf5 and Caf10, respectively) and antibiotic (20 mg kg^{-1} florfenicol; AMF) supplemented diets prior to bacterial challenge.

Table 4
Mortality rate, survival and relative percentage survival (RPS) of infected Nile tilapia fed with caffeic acid and antibiotic at different ratios.

	No. of challenged fish	Mortality Rate (%)[a]	Survival Rate (%)[a]	RPS
Con	75	50.67[a]	49.33[b]	
Caf1	75	56.00[a]	44.00[b]	−10.53
Caf5	75	21.33[b]	82.67[a]	57.89
Caf10	75	50.67[a]	49.33[b]	0.00
AMF	75	12.00[b]	88.00[a]	76.32

[a] Values with different superscript letters in the same column are significantly different within groups ($p < 0.05$). RPS = (1-(% experimental mortality/% control mortality)) x 100.

florfenicol addition. Similarly, the expressions of *IL-1β* and *IL-8* genes in the florfenicol-fed Atlantic cod significantly increased at the 3rd day until the 10th day post-withdrawal of the antibiotic [35]. In our study, florfenicol did not significantly influence the potential killing activity, phagocytic activity, phagocytic index, lysozyme activity and myeloperoxidase activity of tilapia at 0.02 g kg^{-1}. Similarly, hybrid tilapia fed on florfenicol supplemented diet at 0.02 g kg^{-1} diet for 16 weeks were unaffected by serum lysozyme activity, head kidney macrophage phagocytic index [23]. Moreover, in *O. niloticus*, lysozyme activity increased when fish were fed a diet with 5 mg kg^{-1} florfenicol inclusion for 15 days [43]. However, some previous reports showed that the negative effects of antibiotics on the immune system of fish [44,45]. The immunosuppressive effects of florfenicol have been reported earlier for *Cyprinus carpio* [46] and rainbow trout [44]. This might explain the time and dose-effective influences caused by florfenicol on gene expressions or immune responses.

Superoxide dismutase (SOD) and catalase (CAT) are known as main enzymes which may detoxify reactive oxygen species (ROS). The dismutation of highly reactive O_2^- into less reactive H_2O_2 are catalyzed by SOD and is among the main antioxidant defense mechanism against oxidative stress [47]. Catalase is a kind of ROS scavenger enzyme, which is capable to decompose H_2O_2 into O_2 and H_2O by removing H_2O_2 from the body. Therefore, CAT is considered as essential enzymes for the biological defense system of living organisms [48]. In this study, caffeic acid did not show an effect on serum SOD levels in fish when fed diets incorporated with caffeic acid up to 5 g kg^{-1}. Similarly, SOD activities in liver were not dramatically influenced by dietary arachidonic acid levels [49]. However, serum SOD decreased with the addition of high-dose caffeic acid. This could be attributed to the different response of various tissues to different dietary caffeic acid levels. For instance, in *Lateolabrax japonicus* fed with increasing levels (0.08%, 0.22%, 0.36%, 0.56%, 1.33% and 2.12%) of arachidonic acid for 12 weeks, enhanced serum SOD levels were observed particularly in the 0.36% and 0.56% treatment groups, whereas no significant difference were seen in the increasing or decreasing doses [50]. In the present study, CAT activity was significantly increased in the serum of tilapia fed antibiotic and caffeic acid incorporated diets, and this correlated well with the significantly higher *CAT* expression levels in the liver. Caffeic acid has also been reported to up-regulate zinc-responsive antioxidant genes (*MTA*, *MTB*, *GST* and *G6PD*) in *O. mykiss* gill cells *in vitro* [51]. Unlike our study, Caipang et al. [35] reported significant decline of postprandial *CAT* expression in the blood of the florfenicol-fed Atlantic cod at day 10 after feeding with antibiotic. On the other hand, similar to our finding, the expression of the *GSH-Px* in the blood of the florfenicol-fed Atlantic cod significantly increased until the 10th day post-withdrawal [35].

The balance of oxidant and antioxidant is fundamental for immune cell function since it preserves cell membrane integrity and functionality, cellular proteins, and nucleic acids [52]. Chung et al. [51] reported that caffeic acid may inhibit the DNA fragmentation and caspase-3 activity when exposed to ROS and pre-incubation with 50 μM caffeic acid protect against sodium nitroprusside (SNP)-induced toxicity in *O. mykiss* gill tissues. Therefore, it might be suggested that SNP induced cell death was caused by caspase-3 related apoptosis and caffeic acid protect cells from apoptosis. Hoseinifar et al. [53] reported that the supplementation of organic acids or their salts in the diets of fish resulted in the modulation of the immune responses of the host. The fish responded favourably to these feed additives resulting in the up-regulation of the beneficial immune components. An earlier study also revealed positive correlation between the antioxidant enzyme activity and innate immune response in finfish or shellfish fed diets supplemented by various organic acids [17,53–55]. For instance, sodium propionate-supplemented diets (10 or 20 g kg^{-1}) given to common carp enhanced the expression of immune and antioxidant related genes and improved innate immune responses of fish [54]. *Epinephelus fuscoguttatus* fed diets containing 1.0 or 2.0 g kg^{-1} sodium alginate increased respiratory burst activity, phagocytic, and SOD activities [41]. Moreover, feeding *O. niloticus* with 3.0 g kg^{-1} potassium diformate resulted in increased phagocytic activity, phagocytic index, nitroblue tetrazolium reduction test and serum/gut mucous lysozyme activity [56] and survival rate against *Aeromonas hydrophila* [57]. As observed in the current study, when caffeic acid was applied, fish could generate antioxidant defense, as well as produce more innate components as a consequence. Thus, the better anti-oxidative status following supplementation with caffeic acid observed in the present study may indicate higher capacity of diseases prevention in fish.

5. Conclusion

In conclusion, findings of the present study indicate that feeding Nile tilapia with a diet containing 5 g kg^{-1} caffeic acid over a period of 60 days might be adequate to improve fish immune parameters, antioxidant status, as well as survival rate against *A. veronii*, similar to antibiotic treatment. Hence, caffeic acid can be suggested as a dietary substitute for antibiotic to prevent *A. veronii* in tilapia.

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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.2018.11.068.

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