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Effects of dietary blackberry syrup supplement on growth performance, antioxidant, and immunological responses, and resistance of Nile tilapia, *Oreochromis niloticus* to *Plesiomonas shigelloides*

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ABSTRACT

The present study investigated the effects of dietary blackberry syrup on 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 26.75 ± 2.67 g were used in the study; three of them were fed with blackberry syrup incorporated diets (7.5 g kg^{-1}- BBRY7.5, 15 g kg^{-1}- BBRY15, 30 g kg^{-1}- BBRY30), whereas an additive free basal diet served as the control. Additionally, the fifth group was an antibiotic medicated diet (0.02 g kg^{-1}- ABTC), prepared with the florfenicol. Dietary blackberry syrup especially at 15 g kg^{-1} significantly increased growth performance, respiratory burst activity, potential killing activity, phagocytic activity, phagocytic index, lysozyme activity, myeloperoxidase activity, total immunoglobulin levels, serum SOD activity and serum CAT activity ($p < 0.05$). Furthermore, dietary blackberry syrup increased the expression levels of immune [heat shock protein 70 (*HSP70*), interleukin 1, beta (*IL-1β*), tumor necrosis factor (*TNF-α*), interferon gamma (*IFN-γ*), immunoglobulin M (*IgM*)] and antioxidant [glutathione peroxidase (*GPx*)] related genes in the spleen of fish fed with especially 15 g kg^{-1} blackberry syrup ($p < 0.05$). At the end of the 20-day challenge period the survival rates were significantly higher in the BBRY15 and ABTC groups compared to all other treatment groups ($p < 0.05$). As a result, feeding Nile tilapia with a diet containing 15 g kg^{-1} blackberry syrup over a period of 90 days might be adequate to improve growth performance, fish immune parameters, antioxidant status, as well as survival rate against *P. shigelloides*, similar to antibiotic treatment. Hence, blackberry syrup can be used as an antibiotics replacer for controlling *P. shigelloides* in tilapia feed.

1. Introduction

Tilapia is an economically significant fish that can be cultivated almost anywhere in the world and whose global production reached 5.9 million tons [1]. Increased production in intensive culture conditions might result in a decrease in fish welfare because of the augmented stress environment, which in turn affects the fish health [2–4]. High mortality rates, and economic loss caused by infectious diseases often impede tilapia [5]. According to the past studies carried out on bacteria associated with tilapia farming (pond water, pond sediment, fish gill and intestine) practiced via culture-dependent methods, *Aeromonas hydrophila*, *Staphylococcus* spp. *Plesiomonas shigelloides*, *Pseudomonas fluorescens*, and *Bacillus* spp. were the prevalently found bacteria in the gills and intestine of tilapia [6]. These bacteria were also very commonly found in the pond sediment and rearing water. This demonstrates that resident bacteria in the pond water and sediment identically characterize the makeup of bacterial microbiota in the gills and intestine of tilapia which under stressful conditions may encourage the predominance of disease epizootics [6].

Recently identified as a potential human and animal pathogen belonging to the Enterobacteriaceae, *Plesiomonas shigelloides* is a motile, facultative anaerobic, gram-negative aquatic bacterium [7]. High mortality associated with *P. shigelloides* was reported in trout [8]. This pathogen was recognized as one of the major pathogens to cultured sturgeons in Beijing area [9]. During mass mortality of *Ctenopharyngodo nigellas* [10], *Orechromis niloticus* [11,12] and *Hypophthalmichthys molitrix* [7], *P. shigelloides* was also recently isolated from clinical cases of fish and was shown to be highly pathogenic for these cultured fishes.

Currently, it is possible to take bacterial diseases under control partially by administrating antibiotics [13]. Antibiotic treatment;

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however, is beyond being affordable for farmers in many undeveloped and developing countries [14]. It may also pose damage to the environment and human health. In the aquaculture sector, it is widely recognized that the use of antibiotics as feed additives could improve fish growth performance, however according to Ringo et al. [15] it's necessary to try new strategies in feeding and health management in fish aquaculture practice in order to improve the product quality. In this scope, a wide number of studies presented findings proving that alternative supplements could make alternative contributions in aquaculture, such as providing growth and resistance to diseases and fostering immune responses [16–20].

Blackberry is a fruit that creates interest with its rich content of anthocyanins and ellagitannins in addition to other phenolic compounds boosting its high antioxidant capacity [21,22]. These fruits are highly anti-proliferative and anti-inflammatory [23]. Today, blackberries are advertised as fruit rich in polyphenols, and as compounds of interest due to their antioxidant activity as radical scavengers, and their potential roles that can benefit human and animal health, such as diminishing the risk of cancer, cardiovascular disease and other pathologies [22,24–28].

Even though it is widely known that blackberries are beneficial and extraordinarily profitable, there are still questions to be answered regarding the antioxidant and immunostimulatory effects of blackberry in finfish diets.

Oxidative stress, immunological and haematological variables are important blood parameters useful for monitoring the conditions of fish breeding [29,30]. Further, some authors used these blood parameters for the evaluating the effects of feed additives on fish [31,32] [2]. Recently, immune-related gene expression changes have arisen interest as a research topic.

Additionally, the superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are significant antioxidant enzymes detoxifying oxygen free radicals and hydrogen peroxide, hampering oxidative damage [33,34]. The teleost spleen has an important duty in the catching and removal of foreign materials [35]. According to recent studies, immune-related genes are significantly differentially expressed in tilapia spleen after different immunostimulants are supplemented [36,37]. These observations demonstrate that the spleen is a significant organ to illuminate the effects of blackberry syrup in tilapia fish.

To best our knowledge, there was no any report on the effect of alternative additives instead of antibiotics on the disease resistance of tilapias exposed to *P. shigelloides* pathogen. Therefore, the aim of this study was to establish the growth stimulating, antioxidant and immune potentiating effects of blackberry syrup in tilapia (*Oreochromis niloticus*).

2. Materials and methods

2.1. Experimental diet

Blackberry syrup was obtained from farmers market in Kücükkuyu, Çanakkale, Turkey supplemented in the tilapia diets at rates of 7.5, 15 and 30 g kg^{-1}, and designated as BBRY7.5, BBRY15, and BBRY30, respectively. Dietary incorporation levels of blackberry syrup were determined according to the result obtained from the previous study conducted on rat [38]. In addition, an antibiotic diet (ABTC) was prepared with a 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 [39]. A control group (Contr) was prepared without any supplements. Experimental diets (see Table 1) were prepared and analyzed in our laboratory based on the procedure that was reported in our previous studies [40,41].

Table 1
Percentage and proximate composition of the experimental diets.

	Experimental Groups				
Ingredients (% dry matter)	Contr	BBRY7.5	BBRY15	BBRY30	ABTC
Fish meal (anchovy meal)[a]	310	310	310	310	310
Fish oil (anchovy oil) [a]	70	70	70	70	70
Soybean meal [a]	210	210	210	210	210
Wheat flour[a]	300	300	300	300	300
Wheat starch[a]	79.99	72.49	64.99	49.99	79.97
Blackberry syrup	0	7.5	15	30	0
Antibiotic	0	0	0	0	0.02
Vitamin mix[b]	10	10	10	10	10
Mineral mix[c]	20	20	20	20	20
BHT[a]	0.01	0.01	0.01	0.01	0.01
Total	1000	1000	1000	1000	1000
Chemical analyses (% DM)					
Protein	35.88	35.90	35.89	35.92	36.89
Fat	9.40	9.36	9.41	9.39	9.99
Ash	5.13	5.16	5.19	5.26	5.81
NFE[d]	37.01	36.99	36.97	39.98	37.10
Energy (kj/g)[e]	18.47	18.46	18.47	18.98	18.96

[a] Anchovy fish meal, Anchovy fish oil, Soybean meal, Wheat flour, Wheat starch, BHT. Sibal A.Ş. Sinop, Turkey.
[b] Vitamin Mix: Vitamin A. 18000 IU kg^{-1}feed; Vitamin D3. 2500 IU kg^{-1} feed; Vitamin E. 250 mg kg^{-1} feed Vitamin K3. 12 mg kg^{-1} feed; Vitamin B1. 25 mg kg^{-1} feed; Vitamin B2. 50 mg kg^{-1} feed; Vitamin B3. 270 mg kg^{-1} feed; Vitamin B6. 20 mg kg^{-1} feed; Vitamin B12.0.06 mg kg^{-1} feed; Vitamin C. 200 mg kg^{-1} feed; Folic acid. 10 mg kg^{-1} feed; Calcium D–pantothenate. 50 mg kg^{-1} feed; Biotin. 1 mg kg^{-1} feed; Inositol. 120 mg kg^{-1}feed; Choline chloride. 2000 mg kg^{-1}feed.
[c] Mineral Mix (mg kg^{-1}): Fe. 75.3 mg; Cu. 12.2 mg; Mn. 206 mg; Zn. 85 mg; I. 3 mg; Se. 0.350 mg; Co. 1 mg.
[d] Nitrogen-free extracts (NFE) = dry matter - (crude lipid + crude ash + crude protein).
[e] Energy was calculated according to 23.6 kJ/g protein, 39.5 kJ/g lipid, and 17.0 kJ/g NFE.

2.2. Fish and experimental design

Oreochromis niloticus were produced in the Faculty of Marine Sciences and Technology of Çanakkale Onsekiz Mart University. A visual inspection was performed on each fish externally as per the EPA guidelines to carry out a qualitative assessment on fish health [42]. The commercial diet *ad libitum* was applied to feed the fish ("see experimental diet" section) for ten days to ensure acclimation before the experiment started. The experiment was performed with 450 fish allocated into 140 L tank (30 fish/tank). Fish weighing 26.75 ± 2.67 g (mean ± S.D.) were randomly placed into 15 experimental tanks (triplicate design) in a recirculation water system. Water was changed daily at a rate of ~15% of the total volume. *Ad libitum* was provided to all fish twice daily (08.30 and 17.30 h) for 90 days. Photoperiod was adjusted to a 12L:12D light-dark cycle during the course of the study. Throughout the experiment, water parameters were measured as temperature 27.0 ± 0.5 °C, pH 7.32 ± 0.1, dissolved oxygen 7.5 ± 0.21 mg L^{-1}, conductivity 436 ± 3.1 µS, total ammonia 0.015 ± 0.0010 mg L^{-1}, nitrite 0.022 ± 0.001 mg L^{-1}, and nitrate 0.10 ± 0.1 mg L^{-1}.

2.3. Growth performance and biometric indices

Specific growth rate (SGR, % per day), feed conversion ratio (FCR) and relative growth rate (RGR, %) were calculated as follows [43]:

Relative growth rate (%) = 100 (final fish weight – initial fish weight) / initial fish weight, Specific growth rate (SGR, %/day) = 100 (ln final fish weight) – (ln initial fish weight) / experimental days,

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

2.4. Sampling

At the end of the 90-day feeding trial, blood samples were collected from the 9 fish (3 fish per tank). Fish were starved for 1 day prior to blood sampling. Blood sampling procedures were carry out in our laboratory accordance with the study by Yilmaz and Ergün [2]. After blood sampling, spleen tissues were collected and placed in RNAlater (Sigma-Aldrich, lot no. #R0901) solution at 4 °C overnight and then stored at −20 °C until the gene expression analysis [44].

2.5. Haematological parameters

Haematological parameters (RBC count, Hgb concentration and Hct ratio) were determined both manually and by using an automatic analyser (Mindray BC 3000 plus) as previously reported in similar experiments on fish [2,40,41].

2.6. Immune related parameters

Phagocytic activity, phagocytic index, potential killing activity, respiratory burst activity, myeloperoxidase activity, lysozyme activity and total immunoglobulin concentration were performed according to the methods as previously described in our laboratory [2]. Differently, *Plesiomonas shigelloides* (1.5×10^8 CFU mL^{-1} in PBS) was used instead of *Yersinia ruckeri* in the phagocytic activity, phagocytic index and potential killing activity tests.

2.7. Serum antioxidant enzymes

The serum SOD levels were determined using a SOD Assay Kit (Sigma, 19160) according to the manufacturer's instructions. Catalase (CAT) activity was determined spectrophotometrically by the method of Goth [45]. Briefly, 200 µL of serum was incubated with 1 mL of substrate (65 µmol per mL H_2O_2 in 60 mmoL/L sodium phosphate/potassium phosphate buffer, pH7.4), in 37 °C for 60 s. The enzymatic reaction was stopped with 1 mL of 32.4 mmoL/l ammonium molybdate and yellow complex of molybdate and hydrogen peroxide was read at 405 nm.

2.8. Bacteria and challenge experiment

The *Plesiomonas shigelloides* SY-PS16 used in this study was isolated from spleen of diseased *O. niloticus* before. *P. shigelloides* was produced overnight in BHB (Brain Heart Broth) at 37 °C, and then washed with PBS in order to set the density to 3×10^8 CFU mL^{-1}. The density of the pathogen was found in line with the LD50 value that was previously calculated for the *O. niloticus*.

When 90-day feeding trial came to an end, an insulin syringe was employed to inject a 100 µL bacterial suspension (3×10^8 CFU mL^{-1} in PBS) intraperitoneally into fish (75 fish/group). Dead *O. niloticus* were cleared from the tank on a daily basis and a daily mortality record was kept for 20 days. *P. shigelloides* was re-isolated with a view to ascertaining the mortality associated with the bacterial infection. 16S rDNA analysis was performed to identify isolates.

2.9. RT-qPCR analyses of gene expression

RT-qPCR analyses of gene expression was performed according to the method previously described in our laboratory [2]. Total RNA was extracted from the spleen using GeneMATRIX Kit (Cat. no. E3598, Poland) according to the manufacturer's instructions. The expression level of the genes *SOD*, *CAT*, *GPx*, *CC1*, *HSP70*, *IFN-γ*, *IL-8*, *IL-1β*, *TNF-α* and *IgM* (Table 2) was determined with an Applied Biosystems 7500 Sequence Detection system (USA). β-actin was used as the internal control. Gene expression levels were analyzed using $2^{-\Delta\Delta Ct}$, and β-actin was used as reference to normalize the RNA input [46].

2.10. Ethics statement

Fish experiments were performed in accordance with the guidelines for fish research from the animal ethics committee at Canakkale Onsekiz Mart University (Protocol Number: 2018/05–09).

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. [83]
	Reverse	GCTGTGGAGAAGAACCAAGC		
IL-8	Forward	GCACTGCCGCTGCATTAAG	85	Ming et al. [84]
	Reverse	GCAGTGGGAGTTGGGAAGAA		
TNF-α	Forward	GAGGTCGGCGTGCCAAGA	119	Chen et al. [85]
	Reverse	TGGTTTCCGTCCACAGCGT		
IgM-heavy chain	Forward	AGGAGACAGGACTGGAATGCACAA	171	Pang et al. [86]
	Reverse	GGAGGCAGTATAGGTATCATCCTC		
IFN-γ	Forward	TGACCACATCGTTCAGAGCA	128	Chen et al. [85]
	Reverse	GGCGACCTTTAGCCTTTGT		
HSP70	Forward	TGGAGTCCTACGCCTTCAACA	238	Chen et al. [85]
	Reverse	CAGGTAGCACCAGTGGGCAT		
CC-hemokine	Forward	ACAGAGCCGATCTTGGGTTACTTG		Abo-Al-Ela et al. [87]
	Reverse	TGAAGGAGAGGCGGTGGATGTTAT		
SOD	Forward	GACGTGACAACACAGGTTGC	20	Xie et al. [88]
	Reverse	TACAGCCACCGTAACAGCAG		
CAT	Forward	TCAGCACAGAAGACACAGACA	21	Xie et al. [88]
	Reverse	GACCATTCCTCCACTCCAGAT		
GPx	Forward	CCAAGAGAACTGCAAGAACGA	21	Xie et al. [88]
	Reverse	CAGGACACGTCATTCCTACAC		
β-Actin	Forward	CAGCAAGCAGGAGTACGATGAG	62	Pang et al. [86]
	Reverse	TGTGTGGTGTGTGGTTGTTTTG		

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

Table 3
Growth performance and survival rate of Nile tilapia fed diets supplemented with increasing levels (0, 7.5, 15 or 30 g kg^{-1} diet) of blackberry syrup (BBRY), and -antibiotic (ABTC) for a period of 90 days.

	Contr	BBRY7.5	BBRY15	BBRY30	ABTC
Mean initial fish weigh (g)	26.84 ± 0.76^a	27.28 ± 0.50^a	26.46 ± 0.28^a	26.41 ± 0.34^a	26.88 ± 0.72^a
Mean final fish weight (g)	133.83 ± 0.10^b	138.07 ± 0.04^b	145.14 ± 2.81^a	135.45 ± 0.92^b	137.69 ± 0.27^b
RGR (%)	399.50 ± 14.62^b	406.46 ± 9.51^b	448.53 ± 4.80^a	412.96 ± 3.07^b	413.03 ± 2.84^b
SGR (%/day)	1.78 ± 0.03^b	1.80 ± 0.02^b	1.89 ± 0.01^a	1.82 ± 0.01^b	1.82 ± 0.01^b
FCR	1.31 ± 0.02^a	1.26 ± 0.02^a	1.18 ± 0.01^b	1.28 ± 0.01^a	1.26 ± 0.01^a
Survival (%)	100	100	100	100	100

Values (mean ± SEM, n = 3) with same superscript letters in the same line are not significant different within groups ($p < 0.05$). RGR, relative growth rate; SGR,specific growth rate; FCR, feed conversion ratio.

2.11. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) and the obtained values were presented as Means ± Standard Error of Mean (SEM). In case of homogeneity of variances, Tukey's Multiple Comparison test was performed; otherwise, Tamhane post hoc test was run for serum SOD activities, total immunoglobulin levels and Hct ratios. Kruskal-Wallis test was followed for values of respiratory burst activity, potential killing activity, phagocytic activity and phagocytic index in case there was no assumption of normality variances. Student's t-test was run in order to determine the significance of differences between the manual and automatic haematological values. In each challenge treatment group, Kaplan-Meier analysis was used to estimate the survival of fish, whereas the log-rank (Mantel-Cox) test for pairwise comparisons was used to identify the differences amongst the groups. Value of significance level was accepted as 0.05, and SPSS 19.0 (SPSS Statistics) was used for the analysis.

3. Results

3.1. Growth performance

All experimental diets were well accepted by the treatment fish and no mortality or any signs of disease were observed in all treatment groups. Growth performance and survival rate of Nile tilapia fed different experimental diets are given in Table 3. The best final fish weight, relative growth rate (RGR), feed conversion ratio (FCR) and specific growth rate (SGR) were obtained with BBRY15 group compared to all other experimental treatment groups ($p < 0.05$).

3.2. Haematological variables

Haematological parameters evaluated with both manual and automatic methods did not present any statistical differences ($p > 0.05$; Table 4). The RBC count, Hgb concentration, and Hct ratio in the treatment groups did not vary significantly from the values observed for the Contr group ($p > 0.05$).

3.3. Immune related parameters

Results for the immunological parameters are given in Figs. 1–3. Significantly higher respiratory burst activity and potential killing activity were seen in the BBRY7.5 and BBRY15 groups compared to all other experimental treatment groups ($p < 0.05$). The phagocytic activity and phagocytic index increased significantly in the BBRY15 group compared to all other experimental treatment groups ($p < 0.05$). In addition, significantly higher phagocytic activity was found in the BBRY30 group over those in the Contr group ($p < 0.05$). The phagocytic index found for the BBRY30 group was significantly higher than the BBRY7.5, BBRY15 and ABTC groups ($p < 0.05$). Significantly higher lysozyme activity was noted in the BBRY7.5, BBRY15 and BBRY30 groups compared to the Contr and ABTC treatment groups ($p < 0.05$). In addition, significantly higher total immunoglobulin was found in the BBRY15 group over those in the Contr group ($p < 0.05$). The lysozyme activity and total immunoglobulin were significantly lower in the group ABTC than those in the remaining groups ($p < 0.05$). Significantly higher serum myeloperoxidase activity was seen in the BBRY7.5, BBRY15, BBRY30 and ABTC groups compared to Contr group ($p < 0.05$).

3.4. Serum antioxidant enzymes

The significantly higher serum superoxide dismutase activity and catalase activity were seen in the BBRY7.5, BBRY15, BBRY30 and ABTC groups compared to Contr group ($p < 0.05$; Fig. 4).

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

The expression profiles of the ten genes in spleen of *O. niloticus* after dietary blackberry syrup and antibiotic treatments are shown in Fig. 5A–C. At the end of the 60-day feeding period, the *IL-8*, *CC1*, *SOD* and *CAT* were found to be similar between the experimental groups ($p > 0.05$). The *IL-1β* and *TNF-α* genes expression levels (Fig. 3) were higher in the BBRY15 and ABTC groups than the Contr group

Table 4
Haematological parameters in Nile tilapia fed experimental diets supplemented with different concentrations (0, 7.5, 15 or 30 g kg^{-1} diet) of blackberry syrup (BBRY), and -antibiotic (ABTC) for a period of 90 days.

	Contr	BBRY7.5	BBRY15	BBRY30	ABTC
RBC (x10^6 mm^{-3})	A:3.68 ± 0.05	3.83 ± 0.05	3.81 ± 0.03	3.80 ± 0.04	3.88 ± 0.07
	M:3.63 ± 0.03	3.88 ± 0.06	3.78 ± 0.04	3.81 ± 0.02	3.85 ± 0.05
Hgb (g dL^{-1})	A:11.13 ± 0.32	12.04 ± 0.25	12.10 ± 0.26	12.04 ± 0.30	11.05 ± 0.50
	M:11.10 ± 0.10	12.20 ± 0.15	12.05 ± 0.11	12.10 ± 0.15	11.10 ± 0.22
Hct (%)	A:34.56 ± 0.95	35.71 ± 0.58	35.80 ± 0.48	36.03 ± 0.50	37.01 ± 0.60
	M:34.44 ± 0.58	35.55 ± 0.58	35.78 ± 0.48	36.11 ± 0.51	37.00 ± 0.58

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; A, automatic analysis results; M, manual analysis results.

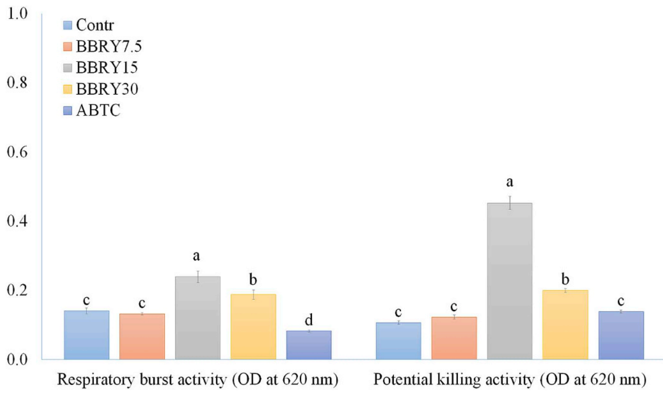

Fig. 1. Respiratory burst activity and potential killing activity of Nile tilapia, *Oreochromis niloticus* fed diets supplemented with different concentrations (0, 7.5, 15 or 30 g blackberry syrup kg^{-1}) of blackberry syrup, and antibiotic. Data are represented as mean ± SEM (n = 9 per group). Values with different superscript letters are significantly different ($p < 0.05$).

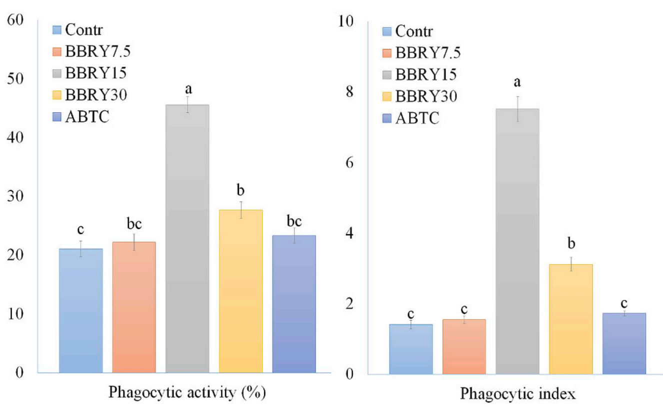

Fig. 2. Phagocytic activity and phagocytic index of Nile tilapia, *Oreochromis niloticus* fed diets supplemented with different concentrations (0, 7.5, 15 or 30 g blackberry syrup kg^{-1}) of blackberry syrup, and antibiotic. Data are represented as mean ± SEM (n = 9 per group). Values with different superscript letters are significantly different ($p < 0.05$).

($p < 0.05$), but no significant differences were found between the remaining groups ($p > 0.05$). The *HSP70* gene expression level (Fig. 4) was higher in the BBRY7.5 and BBRY15 groups than the other experimental groups ($p < 0.05$). Higher *IgM* and *IFN-γ* genes expression levels were found (Fig. 3) in the BBRY15 group compared to the other experimental groups ($p < 0.05$). The *GPx* gene expression level (Fig. 4) was higher in the BBRY15 group than the other experimental groups ($p < 0.05$).

3.6. Challenge test with *Plesiomonas shigelloides*

After the feeding trial for a period of 90 days, experimental fish were challenged with *Plesiomonas shigelloides* and cumulative survival was recorded for 20 days (Fig. 6). Clinically infected fish displayed darkened color, haemorrhage on skin, dark-red spleen, yellow liver and ascitic fluid in the abdomen. At the end of the 20 day challenge period, fish survival rates were significantly higher in the ABTC and BBRY15 groups compared to all other treatment groups ($p < 0.05$), but no significant differences were found between the remaining groups ($p > 0.05$).

4. Discussion

Findings in the present study demonstrated that dietary

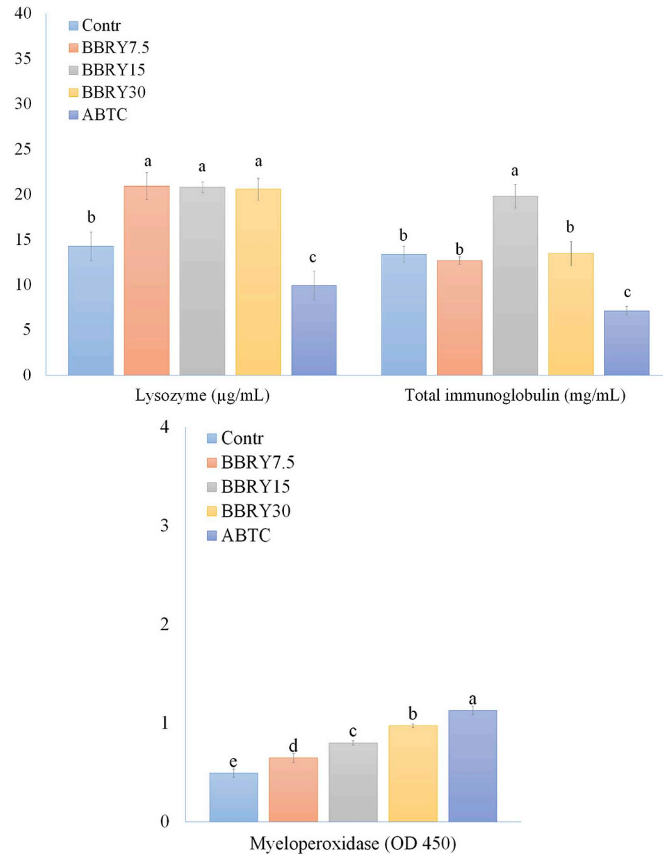

Fig. 3. Lysozyme activity, total immunoglobulin levels and myeloperoxidase activity of Nile tilapia, *Oreochromis niloticus* fed diets supplemented with different concentrations (0, 7.5, 15 or 30 g blackberry syrup kg^{-1}) of blackberry syrup, and antibiotic. Data are represented as mean ± SEM (n = 9 per group). Values with different superscript letters are significantly different ($p < 0.05$).

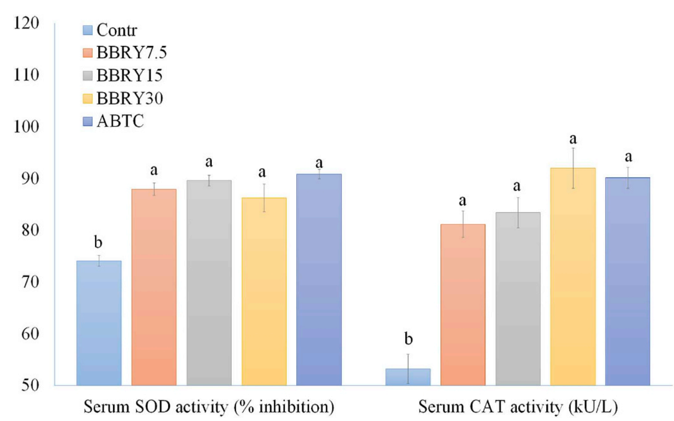

Fig. 4. Superoxide dismutase (SOD) and catalase (CAT) activities of Nile tilapia, *Oreochromis niloticus* fed diets supplemented with different concentrations (0, 7.5, 15 or 30 g blackberry syrup kg^{-1}) of blackberry syrup, and antibiotic. Data are represented as mean ± SEM (n = 9 per group). Values with different superscript letters are significantly different ($p < 0.05$).

incorporation of blackberry syrup significantly affected growth and feed efficiency in Nile tilapia (*Oreochromis niloticus*) through the improvement of nutrient digestibility -and utilization. Blackberry is reported to contain a variety of bioactive compounds such as anthocyanins, flavonols, or ellagitannins [47]. A stimulation of digestion and enhanced protein assimilation in the intestinal tract of fish via various active compounds of herbs has been reported by Chakraborty et al.

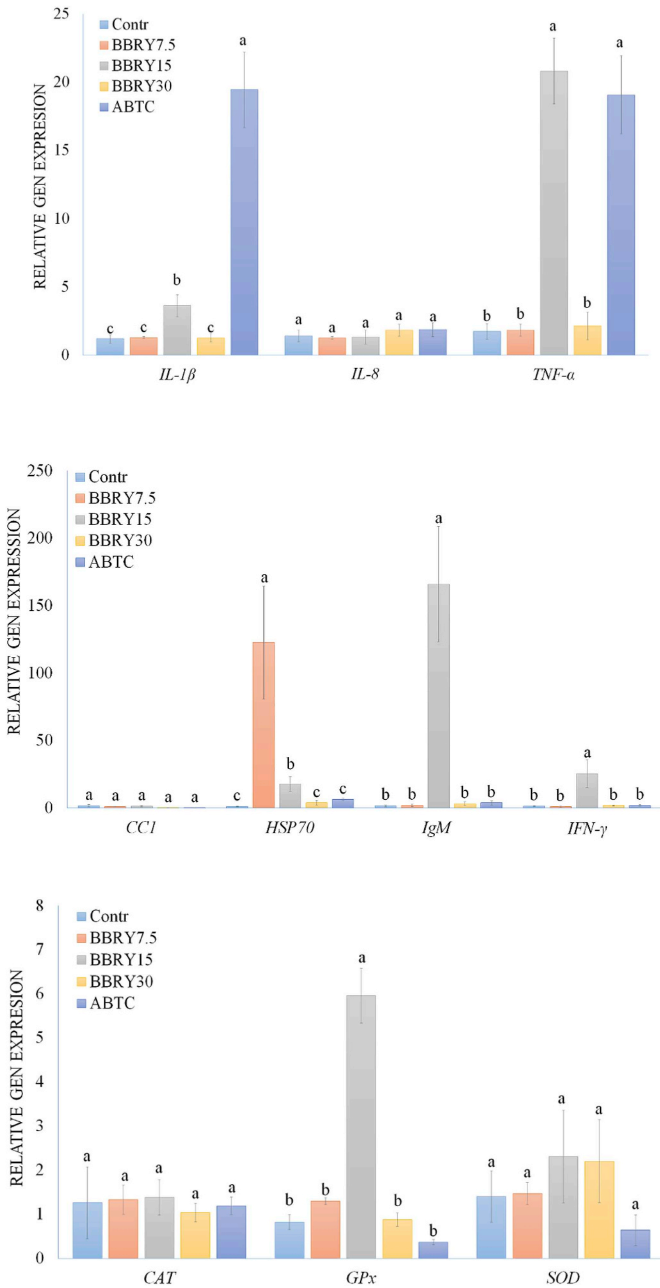

Fig. 5. Gene expression profiles in spleen of interleukin 8 (*IL-8*), interleukin 1, beta (*IL-1β*), interferon gamma (*IFN-γ*) and tumor necrosis factor (*TNF-α*), CC-chemokine (*CC1*), heat shock protein 70 (*HSP70*), immunoglobulin M (*IgM*), superoxide dismutase (*SOD*), catalase (*CAT*) and glutathione peroxidase (GPx) of Nile tilapia, *Oreochromis niloticus* fed diets supplemented with different concentrations (0, 7.5, 15 or 30 g blackberry syrup kg^{-1}) of blackberry syrup, and antibiotic. Data are represented as mean ± SEM (n = 9 per group). Values with different superscript letters are significantly different ($p < 0.05$).

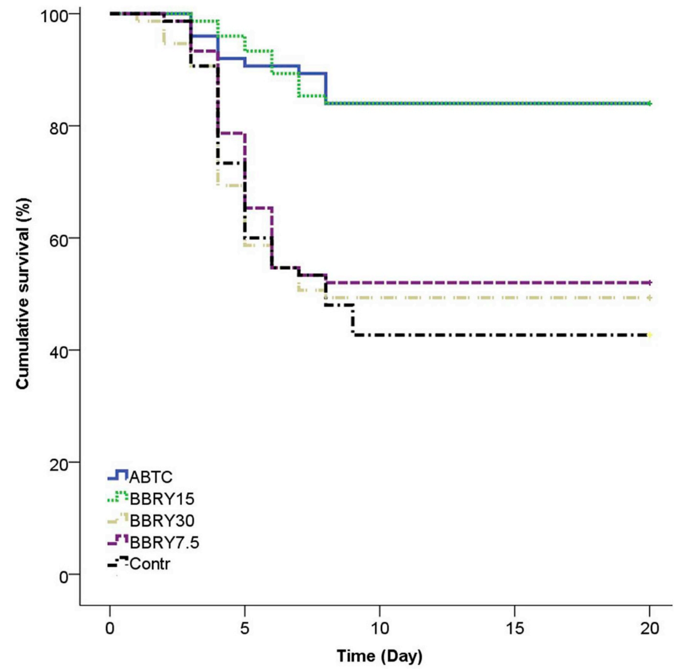

Fig. 6. Kaplan–Meier survivorship curves (cumulative survival [%] over time [Days 0, 5, 10, 15, 20]) for Nile tilapia after challenge with *P. shigelloides*; the fish were fed with blackberry syrup (0, 7.5, 15 or 30 g blackberry syrup kg^{-1} feed; Contr, BBRY7.5, BBRY15 and BBRY30, respectively) and antibiotic (20 mg kg^{-1} florfenicol; ABTC) supplemented diets prior to bacterial challenge.

[16]. Further, Yılmaz et al. [18] reported that herbs, such as thyme, rosemary and fenugreek can stimulate digestion in Sea Bass (*Dicentrarchus labrax*) through an increased bile production, or a possible stimulation of the pancreas and increased secretion of digestive enzymes as well. Similarly, some reports declared that the dietary phytochemicals rich plants enhanced the growth performance of fish [48–50]. In addition, some reports in hybrid tilapia (*Oreochromis niloticus* × *O. aureus*) showed that the dietary supplementation of florfenicol did not change growth performance in fish [39,51], which is in close agreement with our findings in the present study on Nile tilapia.

Haematological and serum biochemical parameters are used for monitoring the effects of feed supplements in fish studies [2,40,41]. In the present study, reference values of haematological parameters determined for Nile tilapia were similar to those reported in an earlier study [52]; RBC: 0.7–28 × $10^6 mm^{-3}$, Hgb: 6.58–15.98 g dL^{-1} and Hct: 15–45%. Moreover, haematological parameters did not changed in Nile tilapia fed with blackberry syrup supplemented test diets in this study. Not having a negative effect on the haematological parameters and physiological condition of fish is a vital factor to choose a healthy immunostimulant [18,53,54]. Similarly, *Ducrosia anethifolia* essential oil was added to rainbow trout feeds at a rate of 0.001, 0.01 and 0.1% and no change in RBC and Hct values were reported at the end of 30 days feeding trial [53].

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 [55]. 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 [56]. In this study, serum SOD and CAT increased with the addition of blackberry syrup and antibiotic.

Similarly, the dietary inclusion of *Hericium caput-medusae* polysaccharide [57], *Psidium guajava* leaf extract [58] and 1,8-cineole [59] in fish feed significantly increased serum SOD and/or CAT levels. However, blackberry syrup and florfenicol did not show an effect on *SOD* and *CAT* expression levels in spleen of Nile tilapia. This could be attributed to the different response of various tissues to different dietary blackberry syrup levels. Similarly, *SOD* and *CAT* expressions in head kidney were not influenced by dietary arginine levels [60]. Hoseinifar et al. [61] also reported that dietary *Gracilaria gracilis* powder supplementation showed differences in *SOD* and *CAT* expression in zebrafish whole body.

Unlike our study, Caipang et al. [62] reported that the expression of

CAT significantly decreased in the blood of the florfenicol-fed Atlantic cod at the 10th day of feeding with the antibiotic. Moreover, the expression of the *GSH-Px* in the blood of the florfenicol-fed Atlantic cod significantly increased until the 10th day post-withdrawal [62]. This can explain the impacts created by the florfenicol on gene expressions depending on time and dose.

Glutathione peroxidase (GPx) is a selenoenzyme that reduces H_2O_2 to $2H_2O$ gathering the needed reducing equivalents from glutathione (GSH) [63]. In our study, significantly higher *GPx* expressions in spleen were observed in fish fed with BBRY15 group, which showed optimal dietary blackberry syrup level ($15\,g\,kg^{-1}$) improved the ability of scavenging free radical in Nile tilapia. Similarly, optimal dietary *Mespilus germanica* leaf extract [64] and *Ferula assafoetida* powder [65] levels could improve *GPx* expression in liver of common carp.

In our study, increased respiratory burst activity, potential killing activity, phagocytic activity, phagocytic index, lysozyme activity, myeloperoxidase activity and total immunoglobulin levels particularly in the BBRY15 treatment group endorsed the immunomodulatory effects of blackberry syrup in fish. The spleen, head kidney, and kidney are major immune tissues in fish [66]. In this study, increases were recorded for the immune response gene (*IL-1β, TNF-α, HSP70, IgM and IFN-*γ) levels in the spleen with the addition of blackberry syrup to tilapia diets. Similar to our study, non-specific immune responses as well as variations in tissue gene expression were reported earlier in finfish fed diets containing different feed additives as organic acids [2,67], probiotics [68,69], herbs or phytogenic compounds [70,71].

In the present study, spleen immune-related gene expression of *IL-1β* and *TNF-α* levels increased in fish fed with $0.02\,g\,kg^{-1}$ 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 [62].

In recent years, different feed additives were provide resistance to pathogenic bacteria *Aeromonas hydrophila* [20,72], *Streptococcus agalactiae* [73,74] and *Streptococcus iniae* [17,19,75–78] in tilapias. However, to our knowledge so far, there was no any report on the effect of alternative additives instead of antibiotics on the disease resistance of tilapias exposed to *P. shigelloides* pathogen. In this study, the survival rate against *P. shigelloides* observed in the BBRY15 group was significantly higher than the other groups (except ABTC). In agreement with our study, fish fed different immune-stimulants were found to be similar in mortality rates with those of the control group, irrespective to the dose levels, i.e. above or below the optimum dose [2,17,19,75,78].

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 [79]. An earlier study revealed positive correlation between the antioxidant enzyme activity and innate immune response in finfish or shellfish fed diets supplemented by various feed additives.

For instance, *Bacillus subtilis* and Mannan oligosaccharide-supplemented diets given to *Cirrhinus mrigala* enhanced the antioxidant status and innate immune responses of fish [80]. *Megalobrama amblycephala* fed diets containing $15.8\,g\,kg^{-1}$ threonine significantly improved immune responses and regulated gene expressions of antioxidant-immune-cytokine-related signaling molecules [81]. In addition, feeding *Danio rerio* with $2.5\,g\,kg^{-1}$ yeast nucleotide resulted in increased serum killing percentage, lysozyme activity, SOD activity, GPx activity, and immune related gene (*TGF-β*, *IL-1β*, and *TNF-α*) expression responses [82]. As observed in the current study, when blackberry syrup 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 blackberry syrup 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 $15\,g\,kg^{-1}$ blackberry syrup over a period of 90 days might be adequate to improve fish growth, immune parameters, antioxidant status, as well as survival rate against *P. shigelloides*, similar to antibiotic treatment. Hence, blackberry syrup can be used as an antibiotics replacer for controlling *P. shigelloides* in tilapia feed. Nevertheless, the responses of fish are likely to change depending on different doses. Therefore further investigations are encouraged in order to evaluate and understand the effects of different levels of dietary blackberry syrup on various finfish species, as well as their reactions to different fish pathogens.

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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.2018.11.012.

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