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The effects of berberine powder supplementation on growth performance, skin mucus immune response, serum immunity, and disease resistance of Nile tilapia (*Oreochromis niloticus*) fingerlings

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ABSTRACT

Nile tilapia (*Oreochromis niloticus*) cultured commercially often suffer from several stressors that increases its susceptibility to infectious diseases. In the present study, five grades of berberine were added to the basal dietary; 0, 1, 3, 6 and 9 g kg^{-1}, of Nile tilapia (11.61 ± 0.09 g $fish^{-1}$) fingerlings in triplication. At weeks 4 and week 8, the fish were collected to evaluate the effects of berberine on growth parameters, skin mucosal, and serum immunity. Challenge test utilizing *Streptococcus agalactiae* infection was implemented at the close of the feeding trial. Fish fed berberine displayed statistically significant enhanced skin mucus lysozyme and peroxidase activities; as well as improved serum lysozyme, peroxidase, alternative complement (ACH50), phagocytosis, and respiratory burst activities compared with the control ($P < .05$). The challenge test revealed that the survival rates of the berberine-fed fish were significantly higher ($P < .05$) by 80% (Diet 2), 63.33% (Diet 3), 56.68% (Diet 4), and 53.33% (Diet 5). Fish that did not survive demonstrated symptoms of abnormal swimming, decreased appetite, darkness, exophthalmia, pair-fins basal haemorrhage, and pale liver. The relative percent survival (RSP) was 73.91%, 52.17%, 43.48, and 39.13% within the 1, 3, 6, and 9 g kg^{-1} berberine inclusions, respectively. The addition of berberine significantly ($P < .05$) increased final body weight, weight gain, and specific growth rate; while a reduction in the feed conversion ratio was recorded at 1 g kg^{-1} berberine after four and eight weeks. In conclusion, diets supplemented with berberine 1 g kg^{-1} boosted mucosal and serum parameters, growth rates, and disease resistance of Nile tilapia.

1. Introduction

World aquaculture has played a significant role in the globalised economy, and the demand for seafood is increasing worldwide (Ahmed and Thompson, 2019; Liu et al., 2018a). Today, aquaculture contributes > 50% of global seafood production and has been responsible for remarkable development of protein sources for human consumption (FAO, 2018). Tilapia, one the most widely cultivated fish worldwide (Delphino et al., 2019; Machimbirike et al., 2019), is farmed in > 100 countries; owing to its rapid growth, adaptiveness, and high market value (Prabu et al., 2019). However, the intensification and expansion of tilapia farming has caused extreme pressures upon cultured water quality, and raised the prevalence of infective diseases, particularly bacterial related diseases (Assefa and Abunna, 2018; Chen et al., 2019); leading to the high death rate of farmed fish and serious financial losses (Chen et al., 2019). Among pathogenic bacteria, *Aeromonas* spp. and *Streptococcus* spp. are the most common groups that cause substantial economic loss in tilapia farming (AlYahya et al., 2018; Fawzy et al., 2014; Neamat-Allah et al., 2019; Zahran et al., 2019). Antimicrobial substances and chemotherapeutics have been intensively applied worldwide to prevent and handle these diseases (Okocha et al., 2018; Santos and Ramos, 2018; Watts et al., 2017). However, the excessive use of these substances has generated the development of antimicrobial-resistant bacterial, antimicrobial residue in fish products, creating environmental threats, and changing the microflora structure and diversity of aquacultural environments (Done et al., 2015; Hong

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et al., 2018). Hence, an environmentally friendly approach to disease control in aquaculture is necessary.

Several methods have been established to deal with infectious diseases and improve the fish growth rate in aquaculture (Assefa and Abunna, 2018; Gratacap et al., 2019; Lieke et al., 2019). Natural products have gained considerable attention in aquacultural practice to boost fish immunity and consequently enhance their resistance against diseases, as well as stimulate growth rates (Awad and Awaad, 2017; Wang et al., 2017). Medicinal plants have been proven to possess beneficial effects toward feed palatability, growth enhancement, and immune system trigger (Awad and Awaad, 2017; Pu et al., 2017; Wang et al., 2017). Likewise, medicinal herbs have gained considerable attention as potential treatments for the stimulation of immune responses and prevention of fish disease (Chakrabarti et al., 2014). A wide range of medicinal plants has been adopted in aquaculture as efficacious antimicrobial, enhanced nourishment compounds, and growth promoters (Gabriel, 2019; Van Hai, 2015), including berberine. Berberine is a type of essential alkaloid, known as a traditional herb, that has been used in China since ancient time (Chen et al., 2016). Berberine contains multiple pharmacological properties; such as anti-inflammatory, antihypertensive, and anti-proliferative (Feng et al., 2019; Ji et al., 2012), which are capable of counteracting pathogens and have been applied as a clinical treatment as nonprescription medicines (Chen et al., 2016). Kim et al. (2009) also demonstrated its role in preventing heart failure, lowering cholesterol, enhancing insulin resistance, and improving anti-inflammatory properties. Hydrochloride, a natural substance found in berberine, has also been shown to control fat (Chang et al., 2010), and has been applied to treat hyperlipidemia and hepatopancreas dysfunction (Huang et al., 2006). Natural antimicrobial property of berberine has been considered as a powerful weapon to deal with *Streptococcus agalactiae* and *Staphylococcus aureus* infections (Chu et al., 2016; Peng et al., 2015). In aquaculture, berberine is regarded as a significant antibiotic for the control of enteritis and other diseases via the co-supplementation with other clinical medicines (Wang et al., 2017). It was also tested for the prohibition and handling of fish diseases caused by several types of pathogenic bacteria (Ji et al., 2012; Zhang et al., 2010). The present study addresses the possible effects of berberine on growth performance, skin mucus immune response, serum immunity, and disease resistance against *S. agalactiae* in Nile tilapia, *O. niloticus* fingerlings.

2. Materials and methods

2.1. Experimental diets

The modification of the basal diet followed the specifications of an earlier publication of Van Doan et al. (2019b); which was designed for tilapia. The experimental diets were produced via a pellet extruder machine and kept in plastic bags at 4 °C. Proximately analysis of each diet followed the AOAC (1995) protocol; and consisted of crude protein, crude lipid, crude ash, and crude fibre (Table 1).

2.2. Fish and experimental design

Male tilapia fingerlings were purchased from the Chiang Mai Pathana Farm Co., Ltd. After arrival, the fish were released into a cage and fed commercial pellets (CP, 9950) for two months, followed by the supplemented basal diets for a period of two weeks. At the start of the experiment, twenty fish were randomly selected for health inspection via the examination of their external appearance, gills, and internal organs. Three hundred with an average weight of 11.61 ± 0.09 g fish^{-1}, were then distributed into 15 glass tanks (150 l of water per tank^{-1}), resulting in 20 fish tank^{-1}. Berberine (Berberine Extract Powder – Giftlover Natural Herbal) was incorporated into the basal diet at 0, 1, 3, 6, and 9 g kg^{-1} feed (Chen et al., 2016). The experiment was laid out in a completely randomised design, in which the five dietary treatments were randomly assigned to the tanks in triplicate for a period of eight weeks. The growth and immune parameters were computed every four weeks, and ten fish from each tank were randomly challenged with the bacterium *S. agalactiae*. The fish in each replication were given experimental diets ad libitum, twice a day, at 8:30 a.m. and 5:30 p.m. Water temperature and pH were maintained at 27.5 ± 0.8 °C and 7.79 ± 0.15, respectively, and dissolved oxygen in each tank was maintained at a minimum of 5 mg litre^{-1}.

Table 1
The experiment's formulation and proximate compositions (g kg^{-1}).

Ingredients	Diets (g kg^{-1})				
Fish meal	150	150	150	150	150
Corn meal	200	200	200	200	200
Soybean meal	390	390	390	390	390
Wheat flour	70	70	70	70	70
Rice bran	150	150	150	150	150
Berberine	0	1	3	6	9
Cellulose	20	19	17	14	11
Soybean oil	5	5	5	5	5
Premix[a]	10	10	10	10	10
Vitamin C[b]	5	5	5	5	5
Proximate composition of the experimental diets (g kg^{-1} dry matter basis)					
Crude protein	324.06	324.33	324.76	324.88	324.99
Crude lipid	69.75	69.88	69.95	70.09	70.22
Fibre	49.77	50.48	50.88	51.01	51.11
Ash	100.55	100.79	100.99	101.08	101.22
Dry matter	916.15	916.49	916.79	917.03	917.22
GE (Cal/g)[c]	4105	4095	4081	4066	4055

[a] Vitamin and trace mineral mix supplemented as follows (IU kg^{-1} or g kg^{-1} diet): retinyl acetate 1,085,000 IU; cholecalciferol 217,000 IU; D, L-a-tocopherol acetate 0.5 g; thiamin nitrate 0.5 g; pyridoxine hydrochloride 0.5 g; niacin 3 g; folic 0.05 g; cyanocobalamin 10 g; Ca pantothenate 1 g kg^{-1}; inositol 0.5 g; zinc 1 g; copper 0.25 g; manganese 1.32 g; iodine 0.05 g; sodium 7.85 g.
[b] Vitamin C 98% 8 g;
[c] GE = Gross energy.

2.3. Immunological assays

2.3.1. Skin mucus collection

Three fish from each replication were randomly retrieved from each tank for mucus collection. The anesthetized fish with clove oil (5 mL litre^{-1} of water) was put in a polyethene bag containing 10 mL of 50 mM NaCl. Fish were gently rubbed inside the bag for two minutes. Afterward, the solution was immediately released into a 15 mL sterile tube and centrifuged at 1500 *g* at 4 °C for ten minutes (5810R Eppendorf, Engelsdorf, Germany). Then, 500 μL of supernatant were gathered and kept at −80 °C for further analysis.

2.3.2. Collection of serum

Serum was separated and gathered from the collected blood of three fish per tank, as mentioned in Van Doan et al. (2019a). Briefly, 1 mL of blood was taken from the caudal vein of each fish via a 1 mL syringe. The blood was then immediately moved to a 1.5 mL Eppendorf tube (without anticoagulant) and incubated at room temperature for one hour, and 4 °C for 4 h. It was then centrifuged at 10,000 RPM for five minutes at 4 °C. The anticipated serum was gathered and preserved at −80 °C for further assays.

2.3.3. Leukocytes isolation

Leukocytes were separated from the peripheral blood with minor modifications, as outlined in Chung and Secombes (1988). Briefly, 1 mL of blood obtained from each fish (three fish tank^{-1}) was combined with 2 mL RPMI 1640 (Gibthai) in a 15 mL tube. The mixture was then

cautiously loaded into a 15 mL tube containing 3 mL of *Histopaque* (Sigma, St. Louis, MO, USA). The tube was centrifuged at 400 *g* for 30 min at 25 °C. Subsequently, a buffy coat of leucocytes was collected and transferred to a sterile 15 mL tube. A phosphate buffer solution (PBS: Sigma-Aldrich, USA) was then applied to each tube (3 mL per tube) and gently aspirated. The tube was then centrifuged twice at 250 *g* for ten minutes (25 °C) to remove any *Histopaque* residual. The cells obtained were re-suspended in the PBS and adjusted to the desired cell number for the measurements of the phagocytic and respiratory burst activities.

2.3.4. Serum and skin mucus lysozyme assays

Lysozyme activity of serum and skin mucus was calculated following the protocol of Parry et al. (1965) with slight modifications in detail, previous specified in Van Doan et al. (2019a). Briefly, 25 μL of serum and 100 μL of skin mucus from each fish were loaded into 96 well-plates, in triplication. *Micrococcus lysodeikticus* (175 μL, 0.3 mg mL^{-1} in 0.1 M citrate phosphate buffer, pH 5.8; Sigma-Aldrich, USA) solution was loaded into each well and gently mixed. The changes in turbidity were recorded every 30 s for 10 min at 540 nm, 25 °C using a microplate reader. The sample's equivalent unit of activity was determined and compared with the standard, and expressed as μg mL^{-1} serum.

2.3.5. Serum and skin mucus peroxidase activities

Measurement of the peroxidase activity was followed the procedure of Quade and Roth (1997); and Cordero et al. (2016) with slight variation as specified in the publication Van Doan et al. (2019a). In short, 5 μL of serum or skin mucus from each fish was loaded into 96 flat-bottomed well-plates in triplicate. Then, 45 μl of *Hank's Balanced Salt Solution* (without Ca^{+2} or Mg^{+2}) and 100 μL of solution (contains 40 mL of distilled water +10 μL of H_2O_2, 30%; Sigma Aldrich + one pill of 3,3′,5,5′-tetramethylbenzidine, TMB; Sigma Aldrich) were added into each well. Once the reaction color turned blue (30–60 s), 50 μl of 2 M H_2SO_4 was added to each well. The optical density was read at 450 nm by a microplate reader (Synergy H1, BioTek, USA). Samples not containing serum or skin mucus were considered to be blanks. A single unit was defined as the amount which produces an absorbance change, expressed as units (U) mL^{-1} of serum or mucus.

2.3.6. Alternative complement pathway activity (ACH50)

ACH50 was measured following the procedure of Yano (1992) with details, as mentioned in our previous study Van Doan et al. (2019a). Briefly, rabbit red blood cells (R-RBC) were washed with PBS through centrifugation at 3000 rpm, in 0.01 M ethylene glycol tetra-acetic acid-magnesium-gelatin veronal buffer (0.01 M – EGTA-Mg-GVB). After this, the R-RBC concentration was adjusted to 2×10^8 cells mL^{-1} in the 0.01 M – EGTA-Mg-GVB buffer. The 100 μL of the R-RBC suspension was then lysed with 3.4 mL of distilled water. Hemolysate absorbance was measured at 414 nm vs. distilled water as a blank and was adjusted to reach 0.740.

For the ACH50 test, serial two-fold dilution was conducted with 100 μL of serum diluted with 400 μL of 0.01 M-EGTA-Mg-GVB. The tubes were maintained on ice to retard the complement reaction throughout the preparation. The R-RBC suspension (100 μL) was loaded into each tube and incubated at 20 °C for 1.5 h with occasional shaking. Post incubation, 3.15 mL of cold saline solution (0.85% NaCl) was placed into each tube to stop the reaction, and then centrifuged at 1600 *g* for 5 min. After centrifugation, 100 μL of supernatant in each dilution was loaded into 96-well plates and read at 414 nm. The degree of hemolysis was calculated by dividing the corrected absorbance 414 value by the corrected absorbance 414 of the 100% hemolysis control. The degree of hemolysis and the serum volume were plotted on a log-log paper. The volume of serum that presented 50% hemolysis was used for calculating the ACH50, through the following formula: ACH50 (units/mL) = 1/K × r × ½. Where K is the amount of serum giving 50% hemolysis, r is the reciprocal of the serum dilution, and ½ is the correction factor. The assay was performed on a ½ scale of the original method.

2.3.7. Phagocytic activity

Phagocytic activity was determined via the approach of Yoshida and Kitao (1991) with minor changes, as delineated in the previous study of Van Doan et al. (2019a). In brief, 200 μL of leucocyte cells (2×10^6 cells mL^{-1}) were placed on a coverslip in duplicate and incubated at 25 °C for two hours. Afterward, the coverslips were washed with 3 mL of RPMI-1640 to remove any non-adherent cells. Then, a solution of 200 μL of fluorescence latex beads (2×10^7 of beads mL^{-1}) (Sigma-Aldrich, USA) was loaded into each coverslip and re-incubated at 25 °C for 1.5 h. After incubation, the coverslips were then rewashed with 3 mL of RPMI- 1640 and then fixed with methanol, followed by staining with Diff-Quik (Sigma-Aldrich, USA) for ten seconds per solution. After staining, the coverslips were cleaned via PBS (pH 7.4) and allowed to dry at 25 °C, and then attached to the slides with Permount (Merck, Germany). The number of phagocyted cells was later counted microscopically (300 cells per coverslip). The phagocytic index (PI) was created through the following equation: PI = average number of beads per cell divided by the number of phagocytizing cells.

2.3.8. Respiratory burst

The procedure following Secombes (1990) was used to determine respiratory burst activity, with slight modifications as detailed in the study Van Doan et al. (2019a). Briefly, 175 μL PBS cell suspension at a concentration of 6×10^6 cells mL^{-1} was loaded into the 96 well plates in triplication. We then added 25 μL of nitro blue tetrazolium (NBT) at a concentration of 1 mg mL^{-1} to each well and incubated the solution for two hours at room temperature. We later discarded the supernatant in each well, and then added 125 μL of 100% methanol into each well for five minutes, in order to fix the cells. After that, 125 μL of 70% methanol $well^{-1}$ were twice added into each well for clean-up. The plates were then dried for thirty minutes at room temperature. The second solution of 125 μL of 2 N KOH and 150 μL of DMSO was then added to each well. Afterward, the plates were measured at 655 nm via microplate-reader, according to the following: Spontaneous O2– production = (absorbance NBT reduction of the sample) – (absorbance of blank).

2.4. Challenge test

Isolation and preparation of *Streptococcus agalactiae* were achieved via the specifications outlined in Van Doan et al. (2019a). Ten fish from each replication were randomly caught for the challenge test against *Streptococcus agalactiae*. The fish were intraperitoneally injected with 0.1 mL of 0.85% saline solution containing 10^7 CFU mL^{-1} of *S. agalactiae* (Wang et al., 2016). The fish were inspected for disease syndromes, and dead fish were eliminated daily. The tilapia's fatality rates (%) were estimated for each tank, 15 days post-feeding. The relative percentage of survival (RPS) was also determined per the equation of Amend (1981): RPS = (1- % mortality in vaccinated/ % mortality in control) × 100.

2.5. Measurement of growth parameters

Growth parameters and survival rate were computed after four and eight weeks of feeding, as outlined in Van Doan et al. (2019a).

2.6. Statistical analysis

Data normality was checked via the Kolmogorov- Smirnov test, and significant differences among treatments were computed via the use of one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test) through SAS Computer Program (SAS, 2003). Significant different

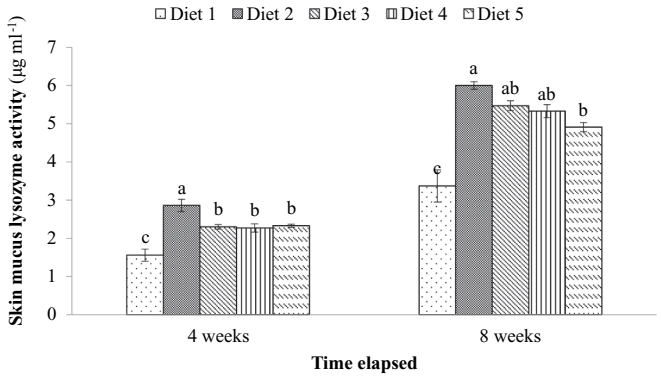

Fig. 1. Skin mucus lysozyme activity of *O. niloticus* after 8 weeks feeding trial fed different concentrations of dietary berberine (mean ± S.E., $n = 3$): Diet 1 (0 - control), Diet 2 (1 g kg^{-1} berberine), Diet 3 (3 g kg^{-1} berberine), Diet 4 (6 g kg^{-1} berberine), and Diet 5 (9 g kg^{-1} berberine). Columns sharing the same superscript letter are not significantly different ($P < .05$) (by Duncan's Multiple Range Test).

mean values ($P < .05$) and other data are displayed as means ± standard deviation.

3. Results

3.1. Skin mucosal parameters

3.1.1. Skin mucus lysozyme

Skin mucus lysozyme activity (SMLA) was significantly higher in fish fed berberine powder compared to that of the control after 4- and 8-weeks post feeding ($P < .05$; Fig. 1). The highest value was recorded in fish fed Diet 2; following by Diet 3, Diet 4, Diet 5, and Diet 1. However, no significantly differences in SMLA were found in fish fed Diets 2,3, 4 ($P > .05$; Fig. 1).

3.1.2. Skin mucus peroxidase

Skin mucus peroxidase activity (SMPA) was significantly greater in fish fed berberine in comparison to the control after 4- and 8-weeks post feeding ($P < .05$; Fig. 2). The peak value was noted in fish fed Diet 2, followed by Diet 3, Diet 4, Diet 5, and Diet 1 ($P > .05$; Fig. 2).

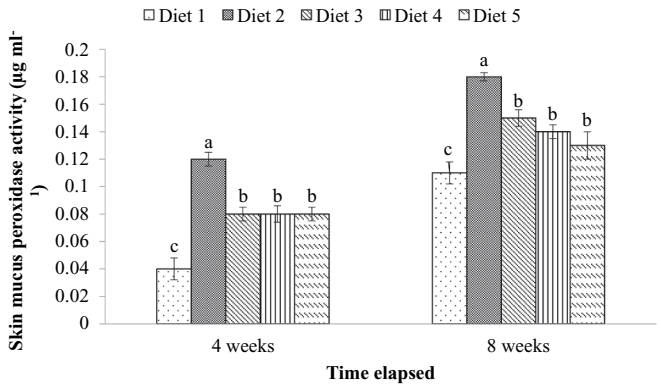

Fig. 2. Skin mucus peroxidase activity of *O. niloticus* after 8 weeks feeding trial fed different concentrations of dietary berberine (mean ± S.E., n = 3): Diet 1 (0 - control), Diet 2 (1 g kg^{-1} berberine), Diet 3 (3 g kg^{-1} berberine), Diet 4 (6 g kg^{-1} berberine), and Diet 5 (9 g kg^{-1} berberine). Columns sharing the same superscript letter are not significantly different ($P < .05$) (by Duncan's Multiple Range Test).

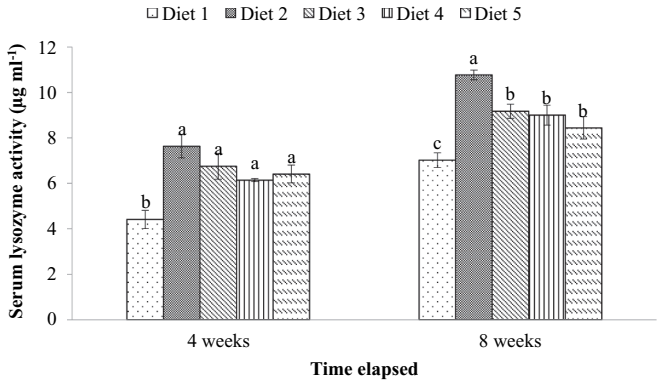

Fig. 3. Serum lysozyme activity of *O. niloticus* after 8 weeks feeding trial fed different concentrations of dietary berberine (mean ± S.E., n = 3): Diet 1 (0 - control), Diet 2 (1 g kg^{-1} berberine), Diet 3 (3 g kg^{-1} berberine), Diet 4 (6 g kg^{-1} berberine), and Diet 5 (9 g kg^{-1} berberine). Columns sharing the same superscript letter are not significantly different ($P < .05$) (by Duncan's Multiple Range Test).

3.2. Serum immune responses

3.2.1. Serum lysozyme activity (SL)

Fluctuations in SL were observed in both the berberine fed fish and the control diet (Fig. 3). Those fish fed the berberine diets produced significantly enhance SL in comparison to the control fed fish ($P < .05$; Fig. 3). The maximum level was found in the fish fed Diet 2 (1 g kg^{-1}); however, no significant gap was recorded among supplemented diets after 4 weeks post-feeding ($P > .05$; Fig. 3).

3.2.2. Serum peroxidase (SP)

Significant enhanced SP was detected in fish fed berberine after eight weeks of feeding ($P < .05$; Fig. 4). The highest value was observed in the fish fed Diet 2 (1 g kg^{-1}), followed by Diet 3,4, and 5; in which no significant discrepancies were displayed in fish fed Diet 3,4, and 5 ($P > .05$; Fig. 4).

3.2.3. Alternative complement activity (ACH50)

Dietary incorporation of berberine generated a significant boost in ACH50 when compared with the control treatment ($P < .05$; Fig. 5). The optimal level of berberine was noticed, again, in the fish fed Diet 2 (1 g kg^{-1}). No significant discrepancies were witnessed in fish fed Diet 2 and Diet 3 after four weeks; and among Diet 3, 4, and 5 after eight

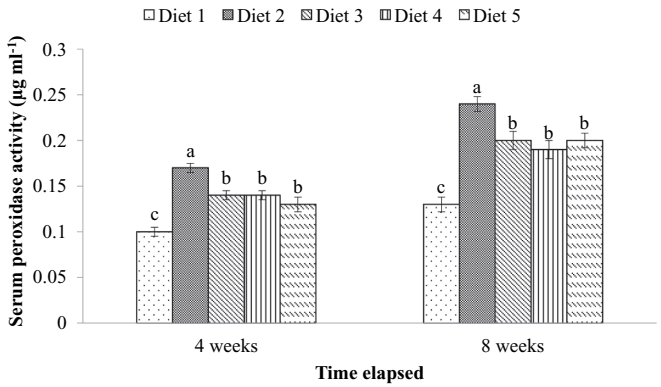

Fig. 4. Serum peroxidase activity of *O. niloticus* after 8 weeks feeding trial fed different concentrations of dietary berberine (mean ± S.E., n = 3): Diet 1 (0 - control), Diet 2 (1 g kg^{-1} berberine), Diet 3 (3 g kg^{-1} berberine), Diet 4 (6 g kg^{-1} berberine), and Diet 5 (9 g kg^{-1} berberine). Columns sharing the same superscript letter are not significantly different ($P < .05$) (by Duncan's Multiple Range Test).

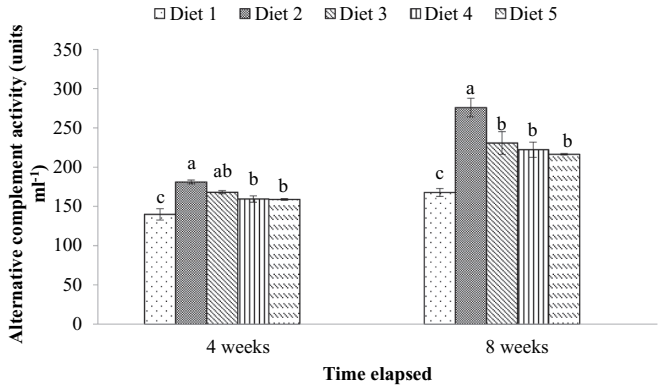

Fig. 5. Alternative complement activity (ACH50) of *O. niloticus* after 8 weeks feeding trial fed different concentrations of dietary berberine (mean ± S.E., n = 3): Diet 1 (0 - control), Diet 2 (1 g kg^{-1} berberine), Diet 3 (3 g kg^{-1} berberine), Diet 4 (6 g kg^{-1} berberine), and Diet 5 (9 g kg^{-1} berberine). Columns sharing the same superscript letter are not significantly different ($P < .05$) (by Duncan's Multiple Range Test).

weeks post-feeding ($P > .05$; Fig. 5).

3.2.4. Phagocytic activity (PI)

Berberine administrated diets boosted PI in comparison to the control group ($P < .05$; Fig. 6). Dietary incorporation of 1 g kg^{-1} berberine (Diet 2) illustrated the highest values compared to the control and other supplemented diets. No significant discrepancies in PI were evidenced in fish fed Diet 3,4, and 5 ($P > .05$; Fig. 4).

3.2.5. Respiratory burst activity (RB)

The incorporation of berberine brought about a significant increase in RB ($P < .05$; Fig. 7), in which the optimal dose of berberine proved to be Diet 2 (1 g kg^{-1}). Again, there were no significant differences in RB among the other administrated groups ($P > .05$; Fig. 7).

3.3. Challenge test

At the conclusion of the experiment, ten fish from each tank were injected with *S. agalactiae* for a period of 15 days. Fish fed berberine diets led to a significantly greater survival rate ($P < .05$; Fig. 8), in which the highest survival rate of 80% was achieved by Diet 2, followed by Diet 3 (63.33%), Diet 4 (56.67%), and Diet 5 (53.33%). The typical syndromes of death/dying fish included abnormal swimming, appetite

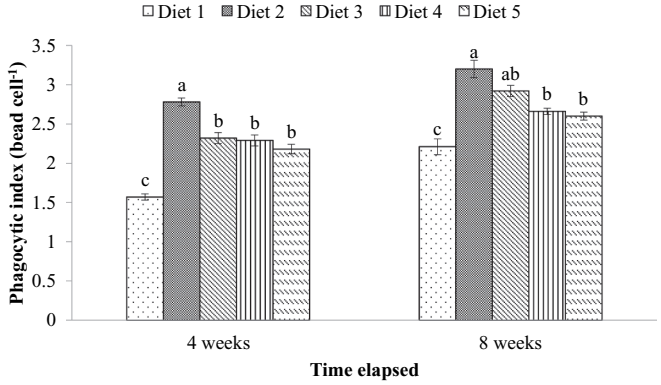

Fig. 6. Phagocytic activity of *O. niloticus* after 8 weeks feeding trial fed different concentrations of dietary berberine (mean ± S.E., n = 3): Diet 1 (0 - control), Diet 2 (1 g kg^{-1} berberine), Diet 3 (3 g kg^{-1} berberine), Diet 4 (6 g kg^{-1} berberine), and Diet 5 (9 g kg^{-1} berberine). Columns sharing the same superscript letter are not significantly different ($P < .05$) (by Duncan's Multiple Range Test).

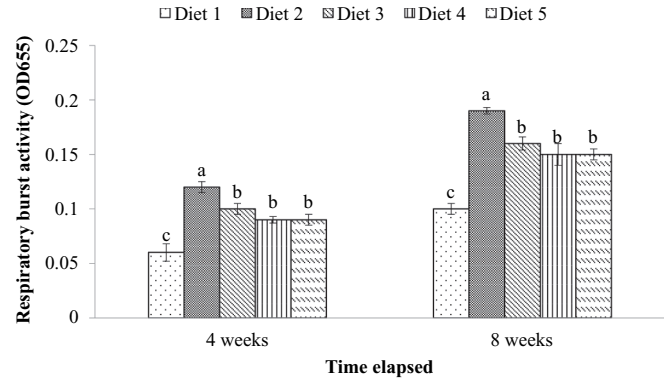

Fig. 7. Respiratory burst activity of *O. niloticus* after 8 weeks feeding trial fed different concentrations of dietary berberine (mean ± S.E., n = 3): Diet 1 (0 - control), Diet 2 (1 g kg^{-1} berberine), Diet 3 (3 g kg^{-1} berberine), Diet 4 (6 g kg^{-1} berberine), and Diet 5 (9 g kg^{-1} berberine). Columns sharing the same superscript letter are not significantly different ($P < .05$) (by Duncan's Multiple Range Test).

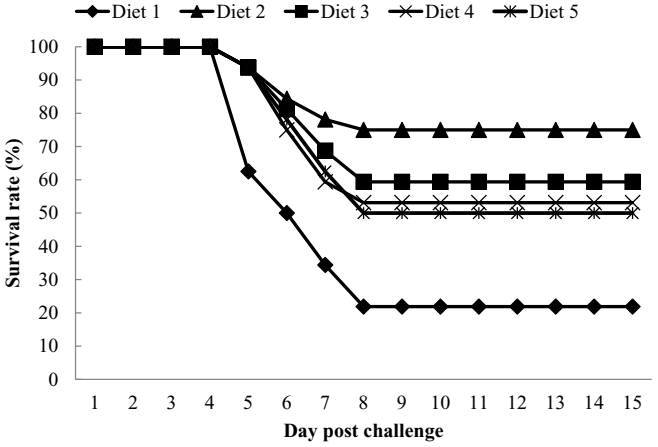

Fig. 8. Survival rate of *O. niloticus* after consumption of the experimental diets: Diet 1 (0 - control), Diet 2 (1 g kg^{-1} berberine), Diet 3 (3 g kg^{-1} berberine), Diet 4 (6 g kg^{-1} berberine), and Diet 5 (9 g kg^{-1} berberine).

suppression, darkness, exophthalmia, pair-fins basal haemorrhage, and a pale liver. The relative percent survival (RPS) was 73.91% (Diet 2), 52.17% (Diet 3), 43.48 (Diet 4), and 39.13% (Diet 5) in 1,3,6, and 9 g^{-1} kg berberine inclusion, respectively (Fig. 8). The highest RPS was demonstrated in the fish fed 1 g kg^{-1} (Diet 2) (Fig. 8).

3.4. Growth parameters

There was a significant increase in specific growth rate (SGR), weight gain (WG), and final weight (FW) in fish fed berberine diets after eight weeks post-feeding ($P < .05$; Table 2). The maximum SGR and WG levels were found in Diet 2 (1 g kg^{-1}) (Table 2). No significant discrepancies in SGR and WG were found within the 3, 6, and 9 g kg^{-1} berberine supplements ($P > .05$; Table 2). The feed conversion ratio (FCR) was lowest in fish fed 1 g kg^{-1} berberine when compared to other groups, except for those fish fed 3 g kg^{-1} berberine ($P < .05$); while fish fed the control diet gave the highest FCR value. Additionally, there were no significant discrepancies in survival rate between fish fed the control and berberine inclusion diets (Table 2).

4. Discussion

Overuse of antibiotics in an attempt to treat diseases increases chemical concentrations in culture systems; weakened the natural

Table 2
Growth performances and feed utilization (mean ± SE) of *O. niloticus* after 4 and 8 weeks feeding with experimental diets: Diet 1 (0 - control), Diet 2 (1 g kg^{-1} berberine), Diet 3 (3 g kg^{-1} berberine), Diet 4 (6 g kg^{-1} berberine), and Diet 5 (9 g kg^{-1} berberine). Different letter in a row denote significant difference ($P < .05$).

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
IW (g)	11.62 ± 0.04	11.63 ± 0.06	11.57 ± 0.04	11.62 ± 0.05	11.62 ± 0.05
FW (g)					
4 weeks	33.36 ± 0.40^{c}	39.60 ± 0.45^{a}	38.84 ± 0.16^{a}	36.78 ± 0.45^{b}	36.42 ± 0.24^{b}
8 weeks	75.07 ± 5.25^{c}	91.33 ± 1.52^{a}	88.24 ± 1.85^{ab}	83.65 ± 0.43^{b}	84.31 ± 0.48^{b}
WG (g)					
4 weeks	21.75 ± 0.36^{c}	27.97 ± 0.40^{a}	27.27 ± 0.15^{a}	25.16 ± 0.40^{b}	24.80 ± 0.29^{b}
8 weeks	63.45 ± 5.29^{c}	79.69 ± 1.51^{a}	76.67 ± 1.85^{ab}	72.03 ± 0.49^{b}	72.69 ± 0.47^{b}
SGR (%)					
4 weeks	3.52 ± 0.03^{c}	4.08 ± 0.03^{a}	4.04 ± 0.01^{a}	3.84 ± 0.03^{b}	3.80 ± 0.04^{b}
8 weeks	3.11 ± 0.05^{c}	3.43 ± 0.03^{a}	3.38 ± 0.03^{ab}	3.29 ± 0.02^{b}	3.30 ± 0.01^{b}
FCR					
4 weeks	1.52 ± 0.007^{a}	1.47 ± 0.005^{c}	1.48 ± 0.006^{bc}	1.49 ± 0.005^{bc}	1.50 ± 0.002^{b}
8 weeks	1.56 ± 0.01^{a}	1.51 ± 0.005^{c}	1.51 ± 0.01^{c}	1.54 ± 0.001^{b}	1.54 ± 0.001^{b}
SR (%)	97 ± 0.22	99 ± 0.57	99 ± 0.36	98 ± 0.12	98 ± 0.45
Price index	1.54	1.57	1.73	2.01	2.25

IW = Initial weight (g); FW = Final weight (g); WG = Weight gain (g); SGR = Specific growth rate (%); FCR = Feed conversion ratio; SR = Survival rate (%); Price index (USD) = cost of feed (per kg) x FCR (with the control feed costs 0.99 USD).

immunity of aquatic organisms subsequently increasing host susceptibility, creates an emergence of antibiotic resistant bacteria, and resists the transfer to other bacteria. Such adverse impacts of antimicrobial substances to both the environment and human well-being by residual antimicrobials call for environmentally friendly, health, safe, and reliable protection; such as medicinal herbs (Dawood et al., 2018; Singer et al., 2016). Traditional herbs are known to possess beneficial properties that impact growth parameters, immunity, and diseases resistance of fish and shellfish (Awad and Awaad, 2017; Dawood et al., 2018; Gabriel, 2019; Hai, 2015). Scientific experts, for this purpose, have sought appropriate feed supplements capable of boosting the fish immune systems and general health. We have, therefore, chosen to focus on the effects of berberine powder on the Nile tilapia (*O. niloticus*) health and well-being.

Skin mucus is secreted from goblet cells within a fish's skin (Sanahuja et al., 2019). It play a crucial role as physical -, biological -, and immunological-barrier against extraneous particles, external stressors, and naturally occurring microorganisms (Cordero et al., 2017; Esteban, 2012; Shephard, 1994). As a result, skin mucus has undergone intensive investigation as a reflection of a fish's immune status (Brinchmann, 2016; Carda-Diéguez et al., 2017; Cordero et al., 2017). A wide range of non-specific immunity parameters has been found in skin mucus; including proteases, antiproteases, peroxidases, esterases, alkaline phosphatase, lysozyme, and immunoglobulins (Cordero et al., 2016; Cordero et al., 2017; Guardiola et al., 2014; Neil et al., 2000). The results of our experiment indicate that the berberine incorporated diets significantly enhanced Nile tilapia's skin mucus lysozyme and peroxidase. Studies of improved mucosal immunity have been conducted in numerous fish species; such as common carp, *Cyprinus carpio* (Giri et al., 2019; Hoseinifar et al., 2019); rainbow trout, *Oncorhynchus mykiss* (Shakoori et al., 2019); Nile tilapia, *O. niloticus* (Doan et al., 2019; Vazirzadeh et al., 2019), and striped catfish (*Pangasianodon hypophthalmus*) (Nhu et al., 2019). The significant rise of skin mucus lysozyme and peroxidase activities recorded in the present study is due to the immunostimulant property of berberine. It is well-established that herbal plants are able to exhibit beneficial impacts on the fish's mucosal immune system of fish via gut-, skin-, and gill-associated lymphoid tissues (Caipang, 2015; Martin and Król, 2017; Vallejos-Vidal et al., 2016). Nevertheless, the exact mechanism to which berberine motivates a fish's mucosal immune immunity requires further investigation.

Serum immunity parameters were significantly enhanced in Nile tilapia fed berberine diets. As far as we know, no information about berberine effects on tilapia non-specific immune response is available. Nonetheless, in compliance with our results, significantly strengthened serum immunity was demonstrated in blunt snout bream, *Megalobrama amblycephala* (Chen et al., 2016; Xu et al., 2017). Although dietary berberine stimulates Nile tilapia's immunity, the mechanism of action has not obviously been proven. In recent study, Li et al., 2019 determined that berberine exhibited direct defensive effects upon the monoculture of enteric glial cells (EGCs), bone marrow-derived dendritic cells, T cells, and intestinal epithelial cells (IECs) in the stimulated inflammatory conditions. In addition, berberine can modulate gut EGCs-IECs-immune cell interactions within co-culture system.

S. agalactiae seriously influences aquacultural practice and causes enormous financial damages worldwide (Li et al., 2015). Thus, successfully protecting fish from *Streptococcus* infection is one of the principal purposes in aquaculture practice. Significant improvements through medicinal herbs diets have been proved in numerous fish species; such as snakehead, *Channa argus* (Li et al., 2019b); striped catfish, Pangasianodon hypophthalmus) (Nhu et al., 2019); gilthead sea bream, *Sparus aurata* and European sea bass, *Dicentrarchus labrax* (Bilen et al., 2019), and Nile tilapia, *O. niloticus*; (Doan et al., 2019; Van Doan et al., 2019a; Van Doan et al., 2019b). In aquaculture, immunostimulatory substances have been found to increase a fishes' natural resistance (Raa, 1996; Sakai, 1999; Wang et al., 2017); and, in the case of bacteria restriction, are generally connected to the enhancement of host's immunity (Kheti et al., 2017). In accordance with our results, the dietary inclusion of berberine stimulated the survival rate of grass carp, *Ctenopharyngodon idella* (Ji et al., 2012) and blunt snout bream, *M. amblycephala* (Xu et al., 2017). These findings imply that berberine can regulate humoral and cellular factors of the host during pathogen invasion. It is well-established that berberine inhibits a wide range of fish pathogens, namely *Edwardsiella ictaluri, Escherichia coli*, and *Streptococcus agalactiae* (Zhang et al., 2010). Recently, Liu et al. (2018b) reported that berberine prevented LPS-induced TLR4/TNF-α activation, and enhanced insulin receptor and insulin receptor substrate-1 expression in the liver; partially by improving the profusion of *Lactobacillus* and *Bifidobacterium*. In contrast, it reduced the abundance of *Escherichia coli* and *Enterococcus* spp.

The primary purpose of aquaculture practice is to archive the most rapid growth and lowest feed conversion ratio (FCR); both of which improved in Nile tilapia fed a berberine diet. Likewise, significantly improved growth performance and FCR were detected in blunt snout bream, *M. amblycephala* (Chen et al., 2016; Xu et al., 2017) fed diets supplemented with berberine. Interestingly, Nile tilapia fed berberine supplements of 3, 6, and 9 g kg^{-1} led to lower growth and

immunological parameters compared to the fish fed 1 g kg^{-1} berberine. This may be owing to the high levels of berberine in the diets, which have been known to create GI upset and ulceration, immunotoxicity, phototoxicity, neurotoxicity, cardiotoxicity, and jaundice in a dose dependent manner (Rad et al., 2017). Although the mode of action in which berberine enhanced growth performance in fish is not clear, it may be due to the beneficial microflora promotion effects of berberine. Zhang et al. (2012) reported that putative short-chain fatty acid (SCFA)-producing bacteria, including *Blautia* and *Allobaculum*, were selectively enriched, along with elevations of fecal SCFA concentrations following administration of berberine. Wang et al. (2018) determined that berberine was capable of prohibiting the proliferation of *Verrucomicrobia*. At the genus level, berberine may inhibit *Akkermansia* and upgrade several SCFA-producing bacteria. According to the more recent study of Tian et al. (2019); short-term berberine exposure varied gut microbiota through decreased *Clostridium* cluster XIVa and IV and bile salt hydrolase (BSH) activity, which led to the collection of taurocholic acid (TCA). The collection of TCA was linked to the trigger of gut FXR, which arbitrates bile acid, lipid, and glucose metabolism. In addition, berberine has been shown to play an essential role in modulating the structure and diversity of grass carp gut microbiota (Pan et al., 2019). Owing to the antibacterial activity of berberine, numerous plentiful bacteria, such as Firmicutes and Fusobacteria, were suppressed, excreting little food and niche resources. At the same time, other intestinal microorganisms; namely Bacteroidetes, Proteobacteria, and Actinobacteria may have consumed these resources, increasing the diversity of intestinal microflora (Pan et al., 2019).

In summary, berberine inclusion may stimulate Nile tilapia's mucosal and serum immunities, produce higher disease tolerance to *S. agalactiae*, as well as enhance growth rate and feed utilization. However, more mechanistic studies must be investigated prior to its application in aquaculture practice.

Ethical approval

The study was performed in accordance with the guidelines on use of animals for scientific purposes (Chiang Mai University).

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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