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Feeding-regimen of β-glucan to enhance innate immunity and disease resistance of Nile tilapia, *Oreochromis niloticus* Linn., against *Aeromonas hydrophila* and *Flavobacterium columnare*

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ABSTRACT

β-glucan is one of the most potent immunostimulants enhancing innate immune activity, disease resistance and growth performance of many aquatic organisms. Nevertheless, there are few studies on feeding regimens of β-glucan that correlate to immune response and disease resistance and are important considerations for practical β-glucan utilization. Thus, the effect of β-glucan and feeding duration on innate immunity and disease resistance was investigated to establish an optimal feeding regimen of β-glucan for Nile tilapia (*Oreochromis niloticus* Linn.). A variety of β-glucan feeding regimens were evaluated, including: i) feeding for 2 weeks, ii) feeding for 4 weeks, and iii) feeding every-other-week, with the objective of establishing the optimal feeding regimen that enhanced innate immunity and disease resistance. Innate immunity parameters were determined every week for eight weeks. Alternative complement activity of all β-glucan groups was significantly ($P < 0.05$) increased at the end of the first week, and then fluctuated but was not significantly ($P > 0.05$) different to the control until the end of the trial. Increased lysozyme activity was only detected at the end of the second week in all β-glucan-treated groups, and then decreased to the control level during most of the sampling periods. Phagocytosis percentage was increased and prolonged by β-glucan feeding, while the phagocytic index was not. Apart from innate immunity, β-glucan-fed fish demonstrated enhanced disease resistance against *Aeromonas hydrophila* and *Flavobacterium columnare* challenge at only the end of the fourth week of the trial. The growth performance of β-glucan-fed fish was not significantly ($P > 0.05$) different among the experimental groups and control. Taken together, the result indicated that all β-glucan-feeding regimens resulted in quite similar outcomes with respect to innate immunity stimulation, disease resistance and growth performance. This novel result suggests that an every-other-week regimen is the optimal choice for Nile tilapia cultivation as an economic cost saving benefit. This is the first study to determine the optimal feeding-regimen of β-glucan to enhance innate immunity and increase resistance to infection by pathogenic bacteria in Nile tilapia.

1. Introduction

Nile tilapia, *Oreochromis niloticus* (Linn.), is a commercial freshwater fish that has been farmed worldwide. Farmed tilapias are mostly cultured in earthen ponds and cages with intensive system to increase the production. However, the system was influenced by environmental factors such as temperature, pH, light, salinity, and disease that caused problem and damage to the culture [1,2]. Bacteria were major group of pathogens in aquaculture system, they cause disease and high mortality to cultivated aquatic animals that consequently lead to production losses. Although disease control carried out by antibiotic application may effectively eliminate infectious bacteria, the residuals of those antibiotics are a cause for concern in fisheries products and also for consumer health. Moreover, utilization of antibiotics may generate considerable problems such as antimicrobial resistance among bacteria [3] and dissemination of drug-resistance genes by horizontal gene

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transfer to naïve bacteria [4]. Therefore, an immunostimulant-supplemented diet is one of the alternative and promising methods in disease prevention and control [5]. It is proposed that this immunostimulant-supplemented diet will stimulate non-specific immunity, which could protect fish and shrimp from various diseases. The aim is to apply this diet in a large-scale cultivation system, which can stimulate immunity and limit stress to fish.

β-glucan is a polysaccharide immunostimulant constituting in cell wall of many organisms including bacteria, yeast, mycelial fungi (such as mushrooms), as well as plants; however, it is absent among vertebrates [6–8]. In aquaculture, β-glucan has been identified as an immunostimulant capable of enhancing the innate immunity of many aquatic species such as Pacific white shrimp (*Litopenaeus vannamei*) [9,10], black tiger shrimp (*Penaeus monodon*) [11], snapper (*Pagrus auratus*) [12] and common carp (*Cyprinus carpio*) [13]. However, the mechanism of immunity that was stimulated or enhanced by β-glucan in such cases remains unclear. Disease resistance enhanced by β-glucan administration has been demonstrated in a number of studies, including resistance to *Edwardsiella tarda* in common carp [14], *Aeromonas hydrophila* in walking catfish (*Clarias batrachus*) [15] and *Flavobacterium columnare* in rainbow trout (*Oncorhynchus mykiss*) [16]. Furthermore, application of β-glucan had a positive effect on growth performance in roho labeo (*Labeo rohita*) [17] and large yellow croaker (*Pseudosciaena crocea*) [18], but it had no effect on sea bass (*Dicentrarchus labrax*) [19].

Taken together, these results suggest that a β-glucan supplemented diet possibly could be useful as an immunostimulant for disease prevention and control in tilapia cultivation that was facing problem caused by bacterial disease. Nile tilapia industry have to contend with at least three pathogenic bacteria, including *Streptococcus agalactiae*, *A. hydrophila* and *F. columnare* [20–22]. These pathogens usually infect tilapia at the first month after stocking in natural ponds or cage culture. Based on this observation, the present study was designed to investigate the effect of dietary β-glucan on innate immunity and disease resistance of Nile tilapia when administered according to different feeding regimens. β-glucan feeding regimens included: i) feeding for 2 weeks, ii) feeding for 4 weeks, and iii) every-other-week, to establish the feeding regimen that enhanced innate immunity and disease resistance against those pathogenic bacteria. The objective was to identify an appropriate regimen for β-glucan feed-supplementation for the purpose of disease control in Nile tilapia aquaculture.

2. Materials and methods

2.1. Experimental fish

Total of 1560 apparently healthy Nile tilapia (*Oreochromis niloticus* Linn.) with average weight 30 g, obtained from commercial GAP (Good Aquaculture Practice) farm, Phetchaburi province, Thailand were used in this trial. The fish were randomly divided to one-hundred and thirty fish and put in twelve 1.5 cu m concrete tanks holding water of about 1000 L and acclimated for 14 days. During acclimatization, they were fed 6% body weight twice per day with basal diet. Water in experimental tanks were operated with re-circulating system and maintained temperature at 27–29 °C. Ammonia, dissolved oxygen, and pH were monitored at least three times a week. Water was changed twice a week or more if water qualities were poor.

2.2. β-glucan supplement diet preparation

The concentration of β-glucan used in this trial was 0.1% (1 g of β-glucan per 1 kg basal diet) as recommended by the manufacturer and previous reports [13,15,18,19,23]. β-glucan-supplemented diet at 0.1% was prepared by suspending β-glucan (DSM Nutritional Products [Thailand] Limited) with phosphate-buffer saline (PBS, pH 7.4) and spraying it on basal diet. Type of basal diet was commercial floating fish pellet feed with protein content of 32%. Subsequently, the diet was air dried to reduce moisture about 45 min at room temperature and stored in refrigerator at 4 °C until use. The supplemented diet was prepared daily for avoiding β-glucan degradation.

2.3. Bacterial culture and preparation

A. hydrophila and *F. columnare* isolated from diseased Nile tilapia were obtained from Aquatic Animal Health Management Laboratory, Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand. Bacterial identification was conducted by 16srRNA analysis [24] before use in the experiment.

A. hydrophila was cultured in Tryptone Soya broth at 31 °C for 24 h. Then, it was precipitated by centrifugation (2000 × *g*, 5 min, 25 °C) and washed twice with PBS (pH 7.4). The bacterium was resuspended into PBS (pH 7.4) and adjusted by optical density (OD_{600}) to 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9. Each OD was diluted by ten-fold dilution and spread on Tryptone Soya agar to estimate colony forming unit (CFU). CFU and OD values were plotted to create relative formula used to predict expected concentration of *A. hydrophila* for the challenge test. Differently, *F. columnare* was not suspended into PBS (pH 7.4), because it would be clumped during precipitation via centrifugation. Thus, after cultivation of *F. columnare* in Shieh broth at 31 °C for 24 h, the bacterial suspension was immediately adjusted by OD_{595} to 0.3 by aseptic Shieh broth. Adjusted suspension was diluted by ten-fold dilution and spread on Shieh agar to estimate CFU. The CFU was diluted to obtain lower concentration for *F. columnare* challenge.

For challenge, both bacteria were cultured and prepared as previously mentioned. *A. hydrophila* was adjusted to 3×10^8 CFU mL^{-1} predicted by relative formula and subsequently diluted to LD50 concentration of 3×10^7 CFU mL^{-1} (OD_{600} = 0.7) for peritoneal injection. On the other hand, *F. columnare* suspension was aliquoted to 3×10^6 CFU mL^{-1} (OD_{595} = 0.3) with aseptic Shieh broth and then added into tank with 1:100 ratio of water to obtain the LD50 concentration of 3×10^4 CFU mL^{-1} for immersion.

2.4. Experimental design

Feeding-regimen trial was conducted for eight weeks in which fish were fed with β-glucan supplemented diet for the first four weeks and following fed with basal diet for another four weeks. After acclimatization, fish were randomly separated into four groups with three replicates. One was control fed with basal diet. Another group was treatment fed with 0.1% β-glucan-supplemented diet following three different feeding regimens including feeding for 2 weeks, 4 weeks and every-other-week (Fig. 1). The fish were investigated for innate immune activity, disease resistance and growth performance.

Innate immune activity was determined from blood and head kidney of nine fish in each experimental group every week.

For disease resistance study, experimental fish were challenged by *A. hydrophila* and *F. columnare* at fourth and eight weeks of the trial. Forty fish from each tank were sampled and equally separated to two subgroups. One subgroup was injected with *A. hydrophila* (3×10^7 CFU mL^{-1}) and another was immersed with *F. columnare* (3×10^4 CFU mL^{-1}).

Growth performance was assessed from specific growth rate (SGR), average daily gain (ADG) and feed conversion ratio (FCR). These parameters were estimated from fish weight at initial and fourth week of trial. Survival rate was also recorded in the present study.

2.5. Sample collection

Blood was withdrawn from caudal vein and left to clot for 1 h at room temperature. Then, the clotted blood was transferred into 1.5 mL of microtube and centrifuged (2500 × *g*, 15 min, 25 °C) to obtain serum. The serum was immediately used to examine alternative complement and lysozyme activities. For phagocytic activity, phagocytes were

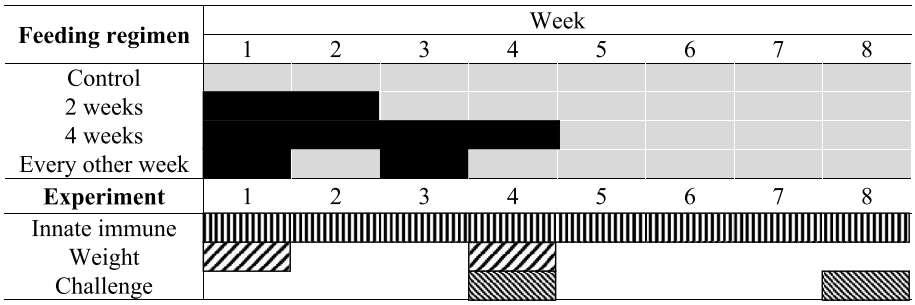

Fig. 1. Feeding regimen and experimental design during eight weeks of trial. Feeding regimen: ☐ fed basal diet and ■ fed β-glucan. β-glucan feeding was conducted only in first four weeks of trial. In experiment: ▥ investigated innate immune activity, ▨ measured fish weight and ▧ challenged fish with *Aeromonas hydrophila* and *Flavobacterium columnare*.

isolated from head kidney. After excision, head kidney was placed and minced on sterile petri dish containing RPMI-1640 medium (Hyclone, USA) for suspending head-kidney cells. Head-kidney debris was removed from cell-suspending RPMI by filtration through a fine cloth with mesh size of 0.12 mm. Subsequently, the RPMI was loaded on Lymphoprep (Fresenius Kabi, USA) and centrifuged (800 × *g*, 20 min, 25 °C) to separate phagocytes from other cells.

2.6. Alternative complement activity

Alternative complement activity (ACH) was determined according to the method modified from Sunyer and Tort [25]. Briefly, 100 μL of serum was added into second and third column of 96-well round-bottom microplate. Then, serum was diluted by ten-fold dilution from the third column till eleventh column with PBS (pH 7.4). First and last column of microplate represented as positive and negative control of the method, they were added 100 μL of PBS (pH 7.4) and distilled water, respectively. After that, 100 μL of 2×10^7 cells mL^{-1} of sheep red blood cell was filled into all well of microplate and incubated without shaking for 1 h. Microplate was centrifuged (1500 × *g*, 5 min, 25 °C) and supernatant was pipetted into 96-well flat-bottom microplate for OD_{415} measurement. OD_{415} value was used to estimate hemolysis percentage by the formula below. Hemolysis percentage and dilution were plotted to calculate ACH50 (Unit mL^{-1}).

$$\text{Hemolysis percentage} = \frac{\text{Absorbance (sample)} - \text{Absorbance (negative control)}}{\text{Absorbance (positive control)} - \text{Absorbance (negative control)}} \times 100$$

2.7. Lysozyme activity assay

Serum lysozyme activity was modified by turbidity method as described by Shugar [26]. 250 μL of *Micrococcus lysodeikticuc* suspension (0.2 mg mL^{-1} of the bacterium in 50 mM potassium phosphate buffer, pH 6.2) was added to 10 μL of fish serum in 96-well flat-bottom microplates. Control was set by adding bacterial suspension into 10 μL of potassium phosphate buffer. Turbidity reduction was measured after adding bacteria suspension at 1 and 4 min by OD_{540} measurement.

$$\text{Lysozyme activity (Unit/ml)} = \frac{\Delta\text{Absorbance(sample)} - \Delta\text{Absorbance(control)}}{0.001 \times 0.01}$$

2.8. Phagocytosis assay

Phagocytic activity was measured through method modified from Park and Jeong [27]. Phagocytes from head kidney were allowed to adhere on glass coverslips for 2 h. Then, coverslips were washed with PBS (pH 7.4), loaded with latex beads (L4530, Sigma, USA) that were adjusted to 1×10^7 beads mL^{-1} in PBS (pH 7.4), and incubated for 1 h. After incubation, excess beads were washed out. Existing phagocytes on coverslips were stained with Diff-Quik (MERCK, Thailand). 300 and 100 phagocytes were counted for calculate phagocytosis percentage and phagocytic index, respectively.

$$\text{Phagocytosis percentage} = \frac{\text{Number of engulfing phagocytes}}{\text{Number of phagocytes}} \times 100$$

$$\text{Phagocytic index} = \frac{\text{Number of engulfed beads}}{\text{Number of engulfing phagocytes}}$$

2.9. Disease resistance

Forty fish from each tank were divided into twenty fish of two subgroups. One subgroup was challenged with *A. hydrophila* by intraperitoneal injection with 0.1 mL of *A. hydrophila* suspension (3×10^7 CFU mL^{-1}). Another subgroup was challenged with *F. columnare* by immersion in tank containing 20 L of *F. columnare* suspension (3×10^4 CFU mL^{-1}) for 1 h with continuous aeration. Challenge tank was used only one batch of fish. After immersion, fish were allocated to 250 L tank filled with 200 L of water. Challenged fish was daily observed for 14 days or until mortality stopped. The cause of death was confirmed by bacterial isolation.

2.10. Growth performances

Growth parameters, including SGR, ADG and FCR, were estimated from fish weight measured at initial and fourth week of trial. For measurement, thirty percent of total fish in each tank (40 fish) was weighed individually.

(1) Specific growth rate (SGR) (%) = [(ln Weight (final) – ln Weight (initial))/Time] × 100
(2) Average daily growth (ADG) (g day^{-1}) = Weight gain/Time
(3) Feed conversion ratio (FCR) = Total feed given/Weight gain
(4) Survival rate (SR) (%) = (Number of fish (final)/Number of fish (initial)) × 100

2.11. Statistical analysis

Data were tested for normality by Kolmogorov-Smirnov test prior to determine statistical difference with one-way analysis of variance (ANOVA) and Duncan's new multiple range test at 5% ($P \leq 0.05$) level. Innate immune activity was presented as mean ± SE (standard error of mean), whereas disease resistance and growth parameters were displayed as mean ± SD (standard deviation).

3. Results

3.1. Alternative complement activity (ACH_{50})

ACH50 of all β-glucan-fed groups were significantly ($P < 0.05$) increased in the first week (247.01 ± 16.97 unit mL^{-1} for the 2-week feeding, 230.19 ± 15.75 unit mL^{-1} for the 4-week feeding and

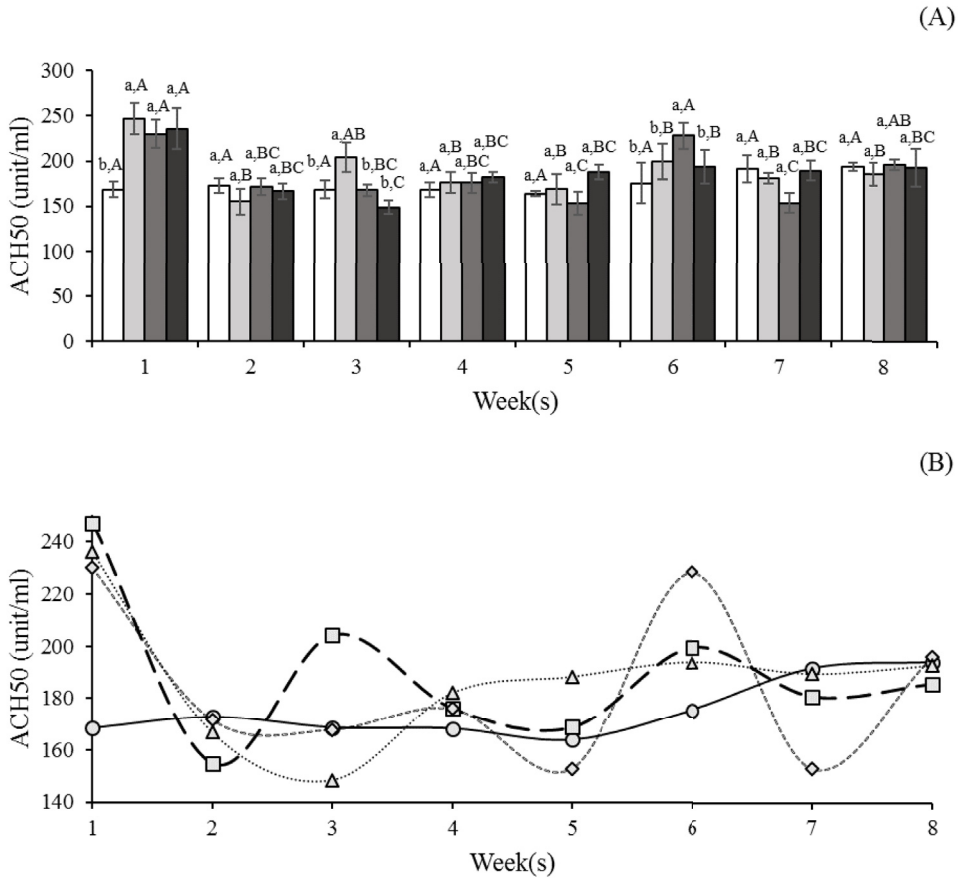

Fig. 2. Alternative complement activity (ACH50) of Nile tilapia fed with basal or β-glucan supplement diets (2 weeks, 4 weeks and every-other-week feeding). (A) Bar graph showed statistical comparison of ACH50 between groups within the same week (small superscripts) and within group at different weeks (capital superscripts) analyzed by one-way analysis of variance (ANOVA) and Duncan's new multiple range test at $p = 0.05$, $n = 9$. Different superscripts indicated significant differences ($P < 0.05$). Data were presented as mean ± SE (standard error of mean). A: □ control fed basal diet, ▨ fed β-glucan for 2 weeks, ▩ fed β-glucan for 4 weeks and ■ fed β-glucan for every other week. (B) Line graph displayed pattern of ACH50 in eight weeks of trial. B: –○–control fed basal diet, -□- fed β-glucan for 2 weeks, ··◇·· fed β-glucan for 4 weeks and ···△··· fed β-glucan for every other week.

235.96 ± 22.20 unit mL^{-1} for the every-other-week feeding), and then decreased in the second week (Fig. 2A). By the third week, the 2-week feeding regimen, which β-glucan was stopped, had significantly higher ACH_{50} (204.26 ± 16.04 unit mL^{-1}) than control and other treated groups ($P < 0.05$). The circumstance was similar to 4-week feeding, after the end of β-glucan feeding in the sixth week of the trial, ACH_{50} of the 4-week feeding were significantly increased and higher than control and other treatments ($P < 0.05$).

From line graph (Fig. 2B), ACH_{50} of control showed a steady level (average of 175.3 unit mL^{-1}) for all weeks of the trial. Differently, ACH_{50} of treatments was fluctuated with different patterns among them. ACH_{50} pattern of the 2-week feeding fluctuated considerably for the entire duration of the trial. Whereas, ACH_{50} of 4-week feeding varied during the fifth to seventh week. ACH_{50} pattern of the every-other-week feeding was quite different from other treatments, as after decreasing in the second and third week, it then gradually increased, though not significantly when compared to the control ($P > 0.05$).

3.2. Lysozyme activity

Lysozyme activity of fish fed β-glucan was significantly ($P < 0.05$) lower compared to the control at first week (Fig. 3A). However, the activity increased at the second week, but only the 2- and 4-week feeding (907.78 ± 43.39 and 902.78 ± 16.90 unit/ml, respectively) were significantly different when compared to the control ($P < 0.05$). After that, lysozyme activities of all β-glucan-feeding regimens decreased slightly and remained so until the end of the trial (Fig. 3B). Lysozyme activity of the control group showed a steady response with the value of 772.64 ± 42.03 unit mL^{-1} observed from the first week until the end of the trial.

3.3. Phagocytic activity

Phagocytic activity was comprised of phagocytosis percentage and phagocytic index. Phagocytosis percentage of control showed stable activity with an approximate average of 19.20% from the first week until the end of the trial (Fig. 4). Phagocytosis percentage of fish fed β-glucan were significantly ($P < 0.05$) higher compared to control, especially during the fifth to seventh week (Fig. 4A). During the fifth to seventh weeks, percentage of every-other-week feeding was highest ($P < 0.05$) compared to other treatment groups. Maximum percentages for each feeding regimen were recorded as 20.57% of control, 28.30% of 2-week feeding, 26.67% of 4-week feeding and 40.89% of every-other-week feeding.

Fig. 4B showed slightly higher phagocytosis percentages in all treatment groups in the first three weeks of trial. After that, 4-week feeding was a first treatment in which phagocytosis percentage was significantly ($P < 0.05$) higher than other treatments and control. The 2-week and every-other-week feeding then started to increase at the fifth week.

Phagocytic index of all groups was not significantly ($P > 0.05$) different for all weeks of trial (Fig. 5A). All experimental groups and control displayed a similar pattern of response during and after β-glucan feeding (Fig. 5B). The phagocytic index of all groups were close in range: 1.42–1.83 for control, 1.44–1.87 for 2-week feeding, 1.52–1.85 for 4-week feeding and 1.5–1.83 for every-other-week feeding.

3.4. Disease resistance

Fish fed β-glucan could significantly ($P \leq 0.05$) reduce percent mortality challenged by *A. hydrophila* and *F. columnare* compared to fish

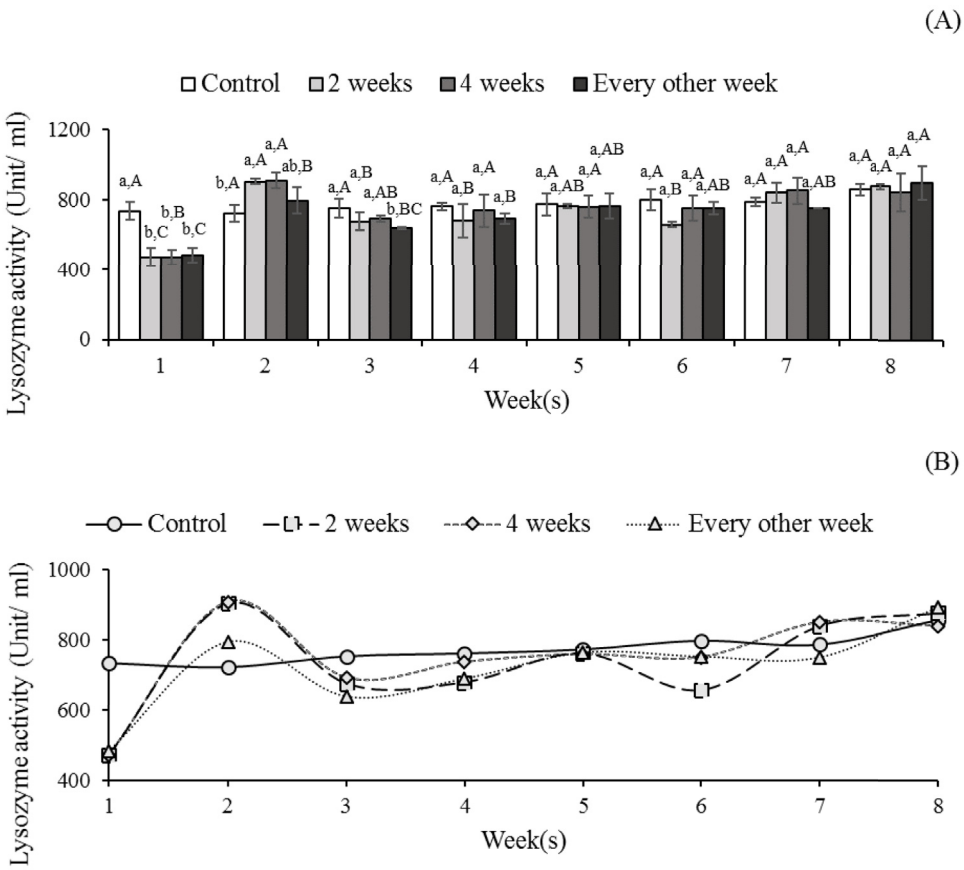

Fig. 3. Lysozyme activity of Nile tilapia fed with basal or β-glucan supplement diets (2 weeks, 4 weeks and every-other-week feeding). (A) Bar graph showed statistical comparison of lysozyme activity between groups within the same week (small superscripts) and within group at different weeks (capital superscripts) analyzed by one-way analysis of variance (ANOVA) and Duncan's new multiple range test at p = 0.05, n = 9. Different superscripts indicated significant differences ($P < 0.05$). Data were presented as mean ± SE (standard error of mean). A: □ control fed basal diet, ▨ fed β-glucan for 2 weeks, ▩ fed β-glucan for 4 weeks and ■ fed β-glucan for every other week. (B) Line graph displayed pattern of lysozyme activity in eight weeks of trial. B: –○–control fed basal diet, -□- fed β-glucan for 2 weeks, --◇-- fed β-glucan for 4 weeks and ···△··· fed β-glucan for every other week.

fed only basal diet (control) at first time of challenges (at fourth week), but not at second time (at eighth week) (Figs. 6A and 7A). However, the result did not show significant ($P \leq 0.05$) difference among percent mortalities of three β-glucan feeding regimens in both bacterial challenges. Accumulation mortality revealed high mortality of Nile tilapia infected with *A. hydrophila* and *F. columnare* highly increased since first two days of challenge and the dead fish in this time did not show clinical signs from infection (Figs. 6B and 7B). Clinical sign gradually appeared at fourth day of challenge. Fish infected by *A. hydrophila* showed abnormal swimming, opaque eyes, redness skin, haemorrhage and gallbladder and liver enlargement. On the other hand, fish challenged by *F. columnare* exhibited scale loss, fin rot, gill necrosis and lesion on skin.

3.5. Growth performances and survival

SGR, FCR, ADG and survival rate of all experimental groups and control were not significantly different at the end of the four weeks of β-glucan feeding ($P > 0.05$) (Table 1). However, the 4-week and every-other-week feeding groups showed a slight decrease in SGR and ADG compared to the control, while 2-week feeding showed a slight increase for all tested parameters.

4. Discussion

Nile tilapia is an important commercial freshwater fish for aquaculture and economy in many countries around the world. The fish plays an important role as an inexpensive and high-quality source of protein for human consumption. Furthermore, the culture period of tilapia is relatively short, which is beneficial for the farmer as many problems can therefore be avoided during the culture process. Nevertheless, many farmers are facing disease problems caused by various bacterial pathogens [20–22]. Such problems can cause critical damage to the fish culture and may lead to production loss. Vaccination is the best way to solve the disease problem; however, its protection may be limited due to pathogenic variation and diversity. Innate immunity is a primordial and primary protection mechanism of organisms against a broad spectrum of pathogens. Therefore, increasing innate immunity is an interesting possibility for the prevention of bacterial pathogens living in the culturing environment. Thus, we decided to use β-glucan, a polysaccharide immunostimulant, to stimulate innate immunity of Nile tilapia against two major bacterial pathogens of Nile tilapia, *A. hydrophila* and *F. columnare*.

In the present study, we focused on a feeding regimen for β-glucan application that can be implemented at farm scale in consideration of such factors as β-glucan usage, amount of β-glucan and cost of culture. The control group in the present study was fed with a normal basal diet, while treatment groups were fed with a β-glucan supplemented diet with three different feeding regimens; including feeding for 2 weeks, 4 weeks and every-other-week within a one-month trial. The effect of β-glucan and the feeding regimen was assessed from the perspectives of innate immune activity, disease resistance and growth performance.

Innate immunity was considered in terms of alternative complement, lysozyme and phagocytic activities. The results showed alternative complement activity in fish fed β-glucan significantly increased in the first week ($P < 0.05$) and some later weeks, including the third week of the 2-week feeding regimen and the sixth week of the 4-week feeding regimen (Fig. 2A). The result was different from other studies that reported the effect of β-glucan feeding on the alternative complement activity of fish. Some studies showed the complement activity was activated during the period of β-glucan feeding [17,28] while others reported β-glucan feeding had no effect on alternative complement activity [12,15,18]. These different results were very interesting, as it may indicate that there were other factors, such as species, length of

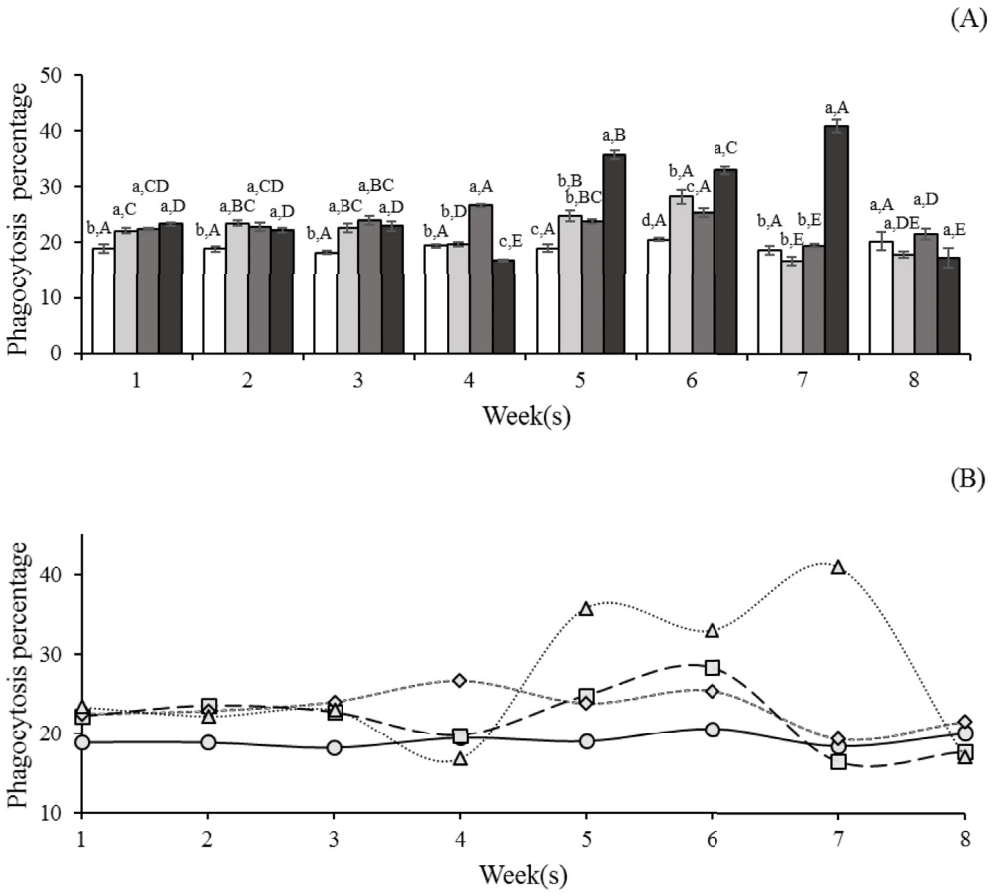

Fig. 4. Phagocytosis percentage of Nile tilapia fed with basal or β-glucan supplement diets (2 weeks, 4 weeks and every-other-week feeding). (A) Bar graph showed statistical comparison of phagocytosis percentage between groups within the same week (small superscripts) and within group at different weeks (capital superscripts) analyzed by one-way analysis of variance (ANOVA) and Duncan's new multiple range test at $p = 0.05$, $n = 9$. Different superscripts indicated significant differences ($P < 0.05$). Data were presented as mean ± SE (standard error of mean). A: □ control fed basal diet, ▨ fed β-glucan for 2 weeks, ▩ fed β-glucan for 4 weeks and ■ fed β-glucan for every other week. (B) Line graph displayed pattern of phagocytosis percentage in eight weeks of trial. B: –○–control fed basal diet, -□- fed β-glucan for 2 weeks, --◇-- fed β-glucan for 4 weeks and ···△··· fed β-glucan for every other week.

application and sampling time that can influence the activation of alternative complement activity. Complement system is generally understood as involving humoral immunity which immediately eliminates antigens by cytolysis and phagocytosis induction (opsonization). It also plays a central role in the inflammatory process and other processes including silent apoptosis and homeostasis [29,30]. Thus, the complement activity is tightly regulated by many inhibitors and regulators such as such as CD46, CD35, Factor H and Factor I [30]. The fact suggested that the complement activity was rapidly activated and suppressed, so value and pattern of the activity were inconclusive in long-term investigation (Fig. 2B).

Lysozyme activity of fish fed β-glucan was significantly decreased ($P < 0.05$) when compared with the control only at the first week of trial but then increased in the second week. After that, lysozyme activity of treatment and control were quite stable and not significantly different till the end of the eight week trial. In other studies on β-glucan and lysozyme activity of Nile tilapia, El-Boshy et al. [31] reported that lysozyme activity of the fish significantly increased after feeding β-glucan for three weeks, while Whittington, Lim and Klesius [32], found that the lysozyme activity of Nile tilapia fed with β-glucan was not affected. This different finding was similar to alternative complement. This suggested that humoral immunity may be tightly controlled or activated for a short time by an immunostimulant that was just like an antigen presentation but not a real infection. A study by Whittington, Lim and Klesius [32] also reported that the lysozyme activity of Nile tilapia in both control and treatment groups was increased after booster immunization or challenge compared to the normal state. This indicated that lysozyme activity was less activated in normal state even being fed with immunostimulant. On the contrary, the activity was highly increased when faced with critical state such as booster immunization or infection (challenge). Our findings present interesting possibilities for future study to explore the capacity of humoral immunity during infection and non-infection in fish fed with immunostimulant. Such investigations may provide more information about the activation and function of humoral immunity in cultured fish species.

An increasing of phagocytosis percentage in fish fed β-glucan was observed in the present and other studies [17,18]. It confirmed that β-glucan feeding has an activation effect on phagocytosis percentage of Nile tilapia and other fish species. Engstad [33] revealed that phagocytes of Atlantic salmon contained β-glucan-specific-receptor on cell membrane that indicated β-glucan was recognized by fish phagocytes and increasing of phagocytosis percentage was possibly influenced by β-glucan. Moreover, the present study showed phagocytosis percentage of β-glucan feeding groups slightly increased in the first three weeks and highly increased at the fifth and sixth week. The circumstance was similar to result of Misra et al. [17] who reported phagocytosis percentage of *Labeo rohita* was continuously elevated by β-glucan feeding. LeibundGut-Landmann et al. [34] reported that β-glucan was capable of increasing the number of mature dendritic cells in mice. Additionally, Cain et al. [35] found that monocytes percentage in the blood of Nile tilapia fed β-glucan was higher, especially at the sixth week of feeding. These experimental results indicated that β-glucan had ability to continuously increase phagocytosis percentage in fish and possibly in other animals. Comparison among feeding regimens of β-glucan showed "every-other-week" could increase phagocytosis percentage of Nile tilapia greater than the control and other regimens.

Phagocytic index was in contrast to phagocytosis percentage, it showed non-significant difference among experimental groups and control ($P < 0.05$). Likewise, phagocytic index of all groups increased and decreased in similar pattern at range of 1.42–1.87. Interestingly, the phagocytosis index values from our study were similar to the

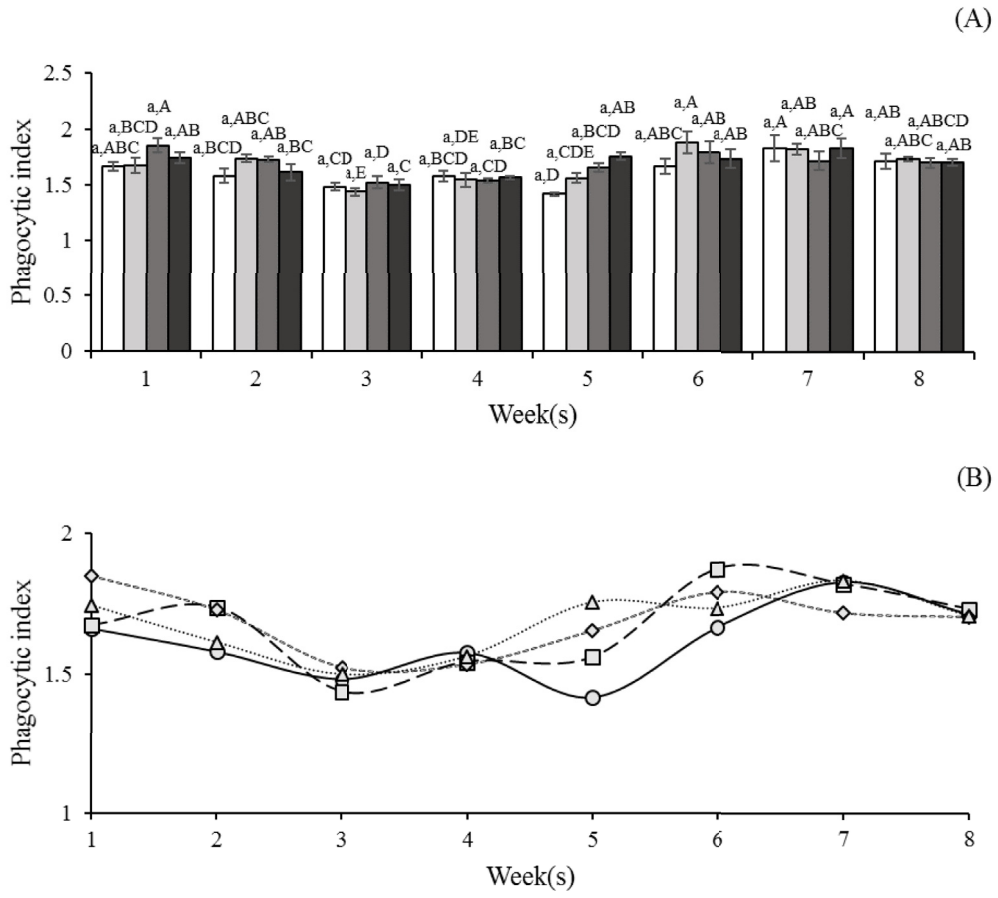

Fig. 5. Phagocytic index of Nile tilapia fed with basal or β-glucan supplement diets (2 weeks, 4 weeks and every-other-week feeding). (A) Bar graph showed statistical comparison of phagocytic index between groups within the same week (small superscripts) and within group at different weeks (capital superscripts) analyzed by one-way analysis of variance (ANOVA) and Duncan's new multiple range test at $p = 0.05$, $n = 9$. Different superscripts indicated significant differences ($P < 0.05$). Data were presented as mean ± SE (standard error of mean). A: □ control fed basal diet, ▒ fed β-glucan for 2 weeks, ▓ fed β-glucan for 4 weeks and ■ fed β-glucan for every other week. (B) Line graph displayed pattern of phagocytic index in eight weeks of trial. B: –○–control fed basal diet, -□- fed β-glucan for 2 weeks, --◇-- fed β-glucan for 4 weeks and ···△··· fed β-glucan for every other week.

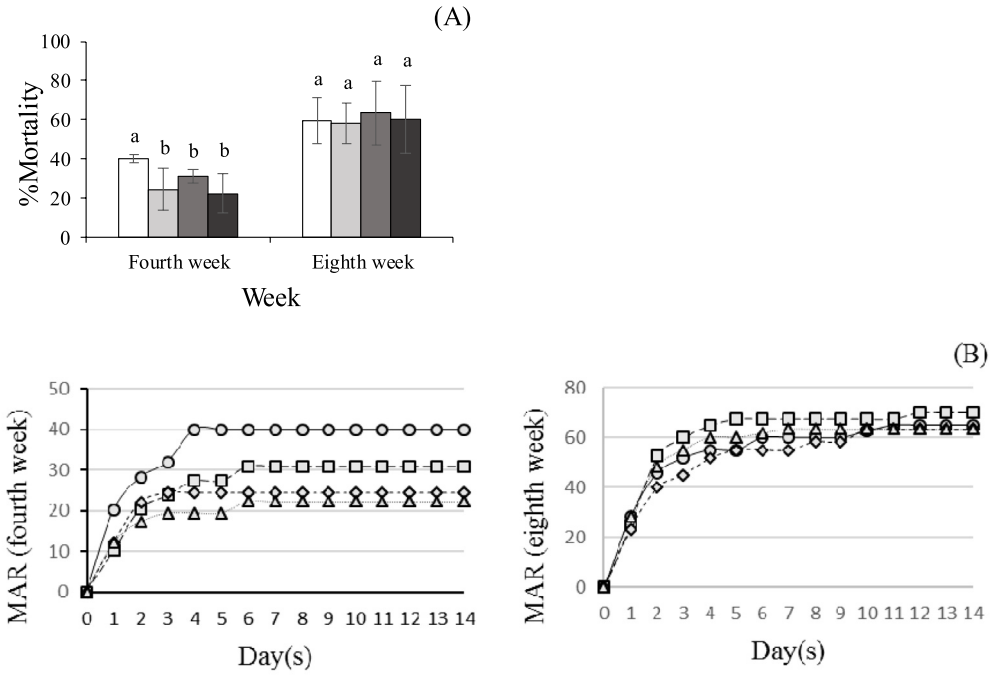

Fig. 6. Mortality of Nile tilapia challenged with *Aeromonas hydrophila* at the fourth and eighth week of trial. (A) Final percentage mortality was presented on bar graph as mean ± SE (standard deviation). A: □ control fed basal diet, ▒ fed β-glucan for 2 weeks, ▓ fed β-glucan for 4 weeks and ■ fed β-glucan for every other week. Statistical comparison was analyzed by one-way analysis of variance (ANOVA) and Duncan's new multiple range test at $p = 0.05$, $n = 20$. Different superscripts on the bar within the same challenge week indicated significant differences ($P < 0.05$). (B)Mortality accumulation rates (MAR) in the fourth and eighth week. B: –○–control fed basal diet, -□- fed β-glucan for 2 weeks, --◇-- fed β-glucan for 4 weeks and ···△··· fed β-glucan for every other week. Accumulation rates were recorded for 14 days after challenge.

reports of other studies, such as Park and Jeong [27] reported their index as 1.42 ± 0.56, Tellez-Bañuelos et al. [36] as 0.894 ± 0.059 and El-Boshy et al. [31] as 1.82 ± 1.1. Thus, we would like to propose the normal value of phagocytic index of Nile tilapia to be in range of 0.894–1.87. However, type of antigen used for investigation should be considered [37].

Besides phagocytosis percentage and phagocytic index, there were reports studying other activities relating to phagocytosis of fish fed with

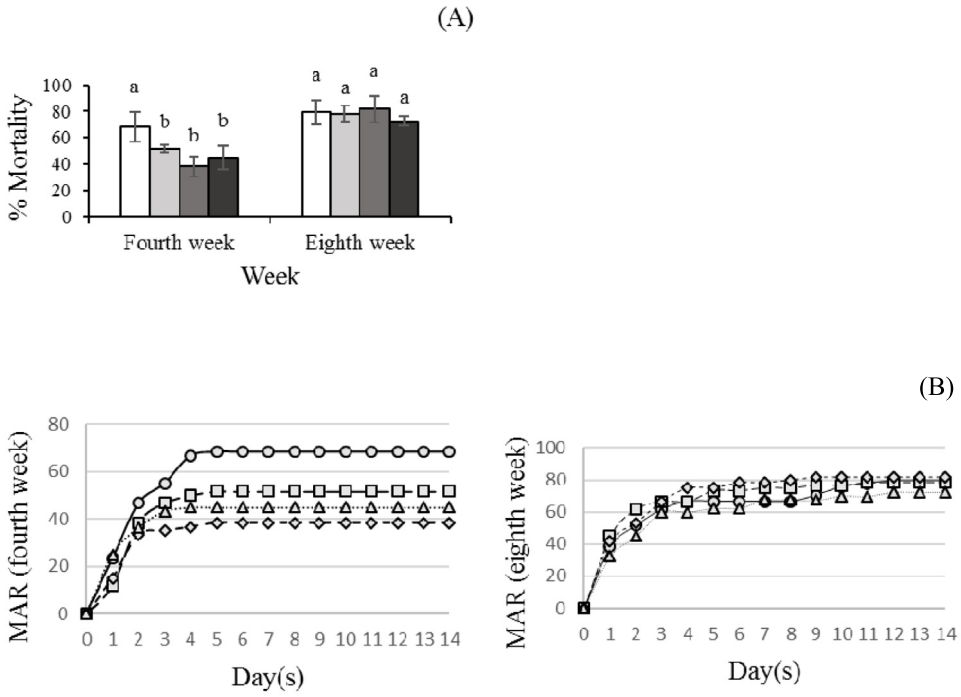

Fig. 7. Mortality of Nile tilapia challenged with *Flavobacterium columnare* at the fourth and eighth week of trial. (A) Final percentage mortality was presented on bar graph as mean ± SE (standard error of mean). A: □ control fed basal diet, ▨ fed β-glucan for 2 weeks, ▩ fed β-glucan for 4 weeks and ■ fed β-glucan for every other week. Statistical comparison was analyzed by one-way analysis of variance (ANOVA) and Duncan's new multiple range test at $p = 0.05$, $n = 20$. Different superscripts on the bar within the same challenge week indicated significant differences ($P \leq 0.05$). (B) Mortality accumulation rates (MAR) in the fourth and eighth week. B: -○-control fed basal diet, -□- fed β-glucan for 2 weeks, --◇-- fed β-glucan for 4 weeks and -△- fed β-glucan for every other week. Accumulation rates were recorded for 14 days after challenge.

β-glucan such as superoxide production [12,17], peroxidase [15] and respiratory burst [16,18,38,39], which were found to be increased by β-glucan.

In the remaining part of this study, a disease challenge was conducted twice at the fourth and eighth week of trial by two pathogenic bacteria, *A. hydrophila* and *F. columnare*. The result clearly indicated that fish fed β-glucan during first four-week period showed significantly better resistance to both bacteria when compared to the control ($P < 0.05$). This result indicated that β-glucan could enhance the disease resistance of Nile tilapia against both septicemia (*A. hydrophila*) and external infection (*F. columnare*). Activating innate immunity might be one of mode of actions of β-glucan to enhance disease resistance. Many and our studies reported innate immune activity of fish were improved by β-glucan and the fish had higher disease resistance compared to normal fish on challenge test [12–16,37,39]. Besides activating innate immune activity, β-glucan probably had other capacities to enhance disease resistance. Rørstad, Aasjord and Robertsen [40] and Selvaraj, Sampath and Sekar [41] reported β-glucan could increase antibody production against *A. salmonicida* in *Salmo salar* and *A. hydrophila* in *Cyprinuscarpio*, respectively. Moreover, β-glucan was also reported to improve antioxidant capacity against *A. hydrophila* infection [42]. These data suggested that β-glucan could enhance disease resistance in fish and the enhancement was possibly associated with many functions activated by β-glucan.

Protection duration of Nile tilapia fed β-glucan was also investigated in the present study. A comparison of β-glucan-fed groups showed that their mortality percentages after bacterial challenges were not significantly different in both first and second challenge ($P > 0.05$). Thus, we assumed all feeding-regimens similarly induced disease protection of Nile tilapia and the mortality result could be used to predict protection duration activated by β-glucan feeding. The 2-week feeding regimen was conducted at the first two weeks of the trial; however, it still provided protection by reducing mortality percentage at first challenge (fourth week) in which the percentage was not significantly different compared to other feeding groups ($P > 0.05$). This meant that the protection provided by β-glucan remained effective even feeding was ended for two weeks. Additionally, on the second challenge, mortality result showed non-significance between the control and all feeding regimens. This suggested that even with the 4-week feeding regimen, the protective effect of β-glucan did not last for four weeks after stopping. From this finding, we propose β-glucan could prolong protection approximately two weeks and not exceeded four weeks after feeding.

Growth performance of Nile tilapia was not affected by β-glucan feeding in the present study. This result was similar to other studies on *O. niloticus* [32] and *Dicentrarchus labrax* [19]. However, many studies have reported that β-glucan feeding significantly ($P < 0.05$) increased growth in fish [10,17,18,43]. Although conclusion about effect of β-glucan on growth performance was unclear, the present study revealed β-glucan feeding did not cause negative effect on growth performance.

In summary, the present study indicated the three feeding-regimens of β-glucan could enhance innate immune activity and disease resistance in similar way without the negative effect on growth performance. This novel finding suggested that "every-other-week" feeding of

Table 1
Growth performances and survival of Nile tilapia with different β-glucan feeding regimens.

	Control	2 weeks	4 weeks	Every-other-week
Survival rate (%)	98.46 ± 0.77	98.72 ± 0.44	97.95 ± 0.89	97.69 ± 1.33
Specific growth rate (%)	4.07 ± 0.08	4.10 ± 0.07	3.93 ± 0.14	3.98 ± 0.06
Food conversion ratio	1.33 ± 0.03	1.34 ± 0.03	1.35 ± 0.05	1.37 ± 0.09
Average daily growth (g day^{-1})	5.19 ± 0.13	5.15 ± 0.10	5.11 ± 0.21	4.84 ± 0.01

Data were presented as mean ± SD (standard deviation). Statistical analysis was conducted by one-way analysis of variance (ANOVA) and Duncan's new multiple range test at $p = 0.05$. Different superscripts indicated significant differences ($P < 0.05$).

β-glucan was the appropriate feeding-regimen for Nile tilapia culture.

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