

Effects of Dietary Supplementation with Broccoli Sprouts (*Brassica oleracea*) on the Hematology and Immunological Responses of Nile Tilapia (*Oreochromis niloticus*)

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ABSTRACT

We analyzed the effects of dietary administration of broccoli sprouts (*Brassica oleracea*) at varying concentrations (0, 10, 15, 20 and 25 g·kg⁻¹ diet, for one and two weeks) on the hematology and select immunological parameters of Nile tilapia (*Oreochromis niloticus*). Subsequent results showed that the hematocrit level and red blood cell count remained unchanged following ingestion of broccoli sprouts for one and two weeks. However, the fish fed the supplemented diets of 20 and 25 g·kg⁻¹ for two weeks displayed significant increase in white blood cell counts. Further analyses revealed that Nile tilapia fed with 10, 15, 20 and 25 g broccoli sprouts·kg⁻¹ for two weeks displayed significant ($p < 0.05$) improvement in phagocytic activity compared to that of the control group. In addition, serum bactericidal activity against *Aeromonas hydrophila* and *Streptococcus agalactiae* was enhanced in broccoli sprout-fed groups as early as one week after feeding. These results suggest that broccoli sprouts can enhance immune response in Nile tilapia by increasing total white blood cells and stimulating both cellular and humoral immunity.

Keywords: Broccoli sprouts, Hematological, Nile tilapia, Phagocytic activity

INTRODUCTION

Recently, antibiotic residues in food have received much consideration, following rising food safety and public health concerns. Of particular interest in aquaculture is the application of functional dietary supplements, such as prebiotics, probiotics, immunostimulants, and natural phytochemical compounds (Ringo *et al.*, 2010; Van Hai and Fotedar, 2010; Barman *et al.*, 2013), and specifically, the utilization of plant extracts as immunomodulators for cultured fish. Plants with identified immunomodulatory effects in fish include prickly chaff flower (*Achyranthes aspera*) (Rao *et al.*, 2004; Rao and Chakrabarti,

2005), Chinese herbs (*Astragalus radix* and *Scutellaria radix*) (Yin *et al.*, 2006), nimtree (*Azadirachta indica*) and turmeric (*Curcuma longa*) (Harikrishnan *et al.*, 2009), Chinese herbs (*Radix astragalin seu Hedysari* and *Radix angelicae sinensis*) (Jian and Wu, 2003; 2004), basil (*Ocimum sanctum*) (Logambal *et al.*, 2000), nettle (*Urtica dioica*) and ginger (*Zingiber officinale*) (Dugenci *et al.*, 2003).

Broccoli (*Brassica oleracea*) is rich in sulforaphane (Cheung and Kong, 2010; Li *et al.*, 2014), and a significant quantity of this compound was found specifically in broccoli sprouts (Bessler and Djaldetti, 2018). Sulforaphane, a natural

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phytochemical compound, has been confirmed to display various biological effects, such as neuroprotection (Tarozzi *et al.*, 2013), reduction of anti-oxidative stress (Danilov *et al.*, 2009), antimetastatic activity (Thejass and Kuttan, 2006), suppression of inflammation (Lin *et al.*, 2008), immunomodulatory activity (Thejass and Kuttan, 2007) and also regulation of phagocytosis, which is a key mechanism in cellular defense (Saganuma *et al.*, 2011). These biological effects of sulforaphane, however, were mostly observed in humans or mice. There is very little information on the effects of sulforaphane on the immune systems of fish.

Nile tilapia (*Oreochromis niloticus*) is one of the most popular cultured freshwater fish worldwide due to its hardiness and rapid growth. According to the Food and Agriculture Organization of the United Nations (FAO), the global aquaculture production of Nile tilapia was estimated at 3.2 million tonne in 2017 (FAO, 2017). However, a problem that occurs frequently in most production systems of farmed tilapia is disease. Therefore, strengthening the immune system is one approach used to address disease problems. For the reasons mentioned above, the present study was therefore undertaken to examine the effects of dietary supplementation of different doses of broccoli sprouts in Nile tilapia on selected hematological parameters, phagocytic activity and serum bactericidal activity against bacterial pathogens. In addition, we wanted to determine the optimum duration of feeding with the broccoli sprout supplement.

MATERIALS AND METHODS

Preparation of broccoli sprouts

Broccoli seeds were first soaked in water overnight. The seeds were then rinsed with water and transferred to glass bottles for germination, and kept at room temperature. The seeds were rinsed with water twice daily for seven days. Seven-day-old broccoli sprouts were collected and chopped into small pieces, dried at 60 °C for 24 h, and then ground.

Feed preparation

The commercial fish feed powder used in this study was purchased from Grobest, Thailand. Ground broccoli sprouts were mixed thoroughly with the feed powder at 10, 15, 20 and 25 g·kg⁻¹ of the commercial feed powder. The mixture was pelleted through a mincer to obtain pellets of 2.5 mm diameter and 5.0-8.0 mm length, and dried at 60 °C for 24 h. The supplemented feed was then kept in sealed plastic containers at 4 °C until use.

Bacterial strain

The *Aeromonas hydrophila* and *Streptococcus agalactiae* strains were kindly provided by the Coastal Aquatic Animal Health Research Center, Department of Fisheries, Songkhla Province, Thailand. These bacteria were isolated from diseased fish.

Experimental animals and feeding

Fingerlings of Nile tilapia were purchased from the Aquaculture Genetics Research and Development Center, Department of Fisheries, Chumphon Province, Thailand. These fish were cultured in the pond of King Mongkut's Institute of Technology Ladkrabang, Prince of Chumphon Campus, Chumphon Province, for three months. The fish were placed in 500-L plastic tanks and acclimatized for two weeks before the trial period. Only those with a mean body weight of 114.57±4.42 g were used in the experiment. The fish were then assigned randomly to five treatments comprised of control (fed only with commercial diet) and diets with broccoli sprouts at four concentrations as previously indicated. Each treatment was replicated using three tanks, each containing six fish. The fish were fed twice daily, 07.00 AM and 05.00 PM, at 3 % of their body weight. Cleaning of the tanks, including water change, was done daily.

Blood sampling

Blood samples were collected from two fish randomly selected from each tank at one and two weeks after feeding with trial diets. Blood (1.5 mL) was drawn from each fish using 3-mL

syringes and 27-gauge needles pre-rinsed with 0.5 M EDTA as an anticoagulant (Ajax-Finechem (Univar), Australia). The total red blood cell count, total white blood cell count, hematocrit, and phagocytic activity were measured. Blood samples (1.0 mL) were also collected without anticoagulant and allowed to clot at room temperature. Blood serum was collected by centrifugation at $3,000\times g$ for 15 min (Hettich Rotina 380 R, America) and kept at -20°C until measurement of serum bactericidal activity, as described by Aly *et al.* (2008).

Hematocrit values

The blood was drawn into hematocrit capillary tubes about two-thirds of their length and centrifuged at 12,000 rpm for 5 min using a hematocrit centrifuge (Gemmy KHT-430b, Taiwan). The hematocrit (as percentage) was determined by a hematocrit reader (Panase *et al.*, 2018).

Red blood cell and white blood cell counts

To count the total red blood cells, blood samples were initially diluted at 1:250, using Dacie's fluid as diluent. For total white blood cells, blood was diluted at 1:100 with Turk's fluid as diluent. After dilution, the cells were counted immediately in a Neubauer chamber (Fazio *et al.*, 2013).

Phagocytic activity

Leukocytes for phagocytosis assay were obtained through density gradient separation. First, 3 mL of the lymphoprep (density 1.077) was dispensed into Corning centrifuge tubes. One milliliter of blood containing 2 mL of RPMI medium was carefully layered on the top. The prepared samples were then centrifuged at 2,200 rpm for 30 min at 25°C . Next, leukocytes were carefully taken out and transferred into new Corning tubes containing 1 mL RPMI medium. Leukocytes were washed twice in RPMI medium and diluted to a concentration of 2×10^6 cells $\cdot\text{mL}^{-1}$. To measure phagocytic activity, 200 μL of the leukocyte suspension was dispensed onto a slide and left at room temperature for 1 h. Afterwards, the slides were washed gently three times with RPMI medium, then mixed with 200 μL of latex beads

(2×10^7 cells $\cdot\text{mL}^{-1}$). The slides with latex beads were incubated again at room temperature for 1 h, then were gently washed again with RPMI medium and fixed with methanol 96 % (v/v). The slides were left to dry at room temperature for 5 min. The dried slides were stained using Dip Quick staining. The number of ingested latex beads and the number of phagocytizing cells were counted from 200 leukocytes at random on each slide under a microscope. The leukocyte phagocytic activity (PA), phagocytic index (PI) and average number of latex beads ingested/cell (ABPC) were calculated according to the following formulas (Itami *et al.*, 1994; Rengpipat *et al.*, 2000):

$$\text{PA} = \left(\frac{\text{number of cells that phagocytosed latex beads}}{\text{number of cells observed}} \right) \times 100$$

$$\text{PI} = \left(\frac{\text{number of cells that phagocytosed latex beads}}{\text{number of cells observed}} \right) \times \left(\frac{\text{number of latex beads ingested}}{\text{number of cells observed}} \right) \times 100$$

$$\text{ABPC} = \frac{\text{number of latex beads ingested}}{\text{number of cells that phagocytosed latex beads}}$$

Serum bactericidal activity

One loop of both *A. hydrophila* and *S. agalactiae* were cultured in 100 mL of tryptic soy broth (Difco™, France) at 37°C for 24 h. The bacterial cultures were centrifuged at 5,000 rpm for 10 min at 25°C (Hettich Rotina 380 R, America) and then the supernatant was discarded. The pellets were washed once and adjusted to the optical density of 0.5 at 546 nm (Thermo Scientific-Evolution 201, America) using sterile phosphate-buffered saline (PBS, pH 7.4). The bacterial suspensions were then serially diluted 10-fold, five times with PBS. The serum bactericidal activity measurement was conducted by incubating 100 μL of the diluted bacterial suspensions with 100 μL of the serum in a 96-well plate for 1 h at 37°C . Then, a 50- μL aliquot was removed from each well and spread on tryptic soy agar (Difco™, France) plates and kept at 37°C for 24 h. After culturing, the number of viable bacteria was observed by counting the

colonies (modified from Vasudeva Rao *et al.*, 2006). PBS replaced the serum in the bacterial control group. Percentage inhibition was calculated using the following equation (Adams, 1991):

$$100 - \left(\frac{\text{mean of colonies sample}}{\text{mean of colonies control}} \right) \times 100$$

Statistical analysis

Statistical differences among treatments were evaluated by analysis of variance followed by Duncan's Multiple Range Test, with p-values less than 0.05 deemed significant. Standard errors of the means were estimated. The statistical analyses were performed using the Statistical Analysis Systems (SAS) program.

RESULTS

Hematological parameters

Hematocrit values and total red blood cell counts in the group fed with broccoli sprout-supplemented diets for one and two weeks showed no significant difference ($p > 0.05$) from the control (Figure 1). However, white blood cell counts in the groups fed for two weeks with broccoli sprout-supplemented diets at 20 and 25 g·kg⁻¹ concentrations were significantly ($p < 0.05$) higher than that of the control; total white blood cells in fish fed the supplement at 10 and 15 g·kg⁻¹ for two weeks were also slightly higher than the control, but the result was not statistically ($p > 0.05$) different (Figure 2).

Phagocytic activity

Induced leukocytes taken from whole blood of fish showed the ability to phagocytose latex beads (Figure 3). Fish fed with broccoli sprout-supplemented diets for one week displayed leukocyte phagocytic ability (Figure 4a) and phagocytic index (Figure 4b) that were not different ($p > 0.05$) from those of the control fish. However, for fish fed with broccoli sprouts for two weeks, phagocytic ability and phagocytic index were significantly ($p < 0.05$) higher compared to the control. No significant difference ($p > 0.05$) was found among treatments in the average number of latex beads ingested/cell at one or two weeks of feeding (Figure 4c).

Serum bactericidal activity

Serum samples collected from fish fed for one week with broccoli sprouts and the control group displayed bactericidal activity against both *A. hydrophila* and *S. agalactiae*. The serum of all the broccoli sprout-fed fish showed higher percentage inhibition of both bacterial species compared with the control. Further, the percentage inhibition increased with the increase in broccoli sprout concentration in the diet. Serum bactericidal activity of fish fed broccoli sprouts for two weeks showed results similar to those fed for one week. Statistical analysis showed that serum of fish fed broccoli sprout-supplemented diets with concentrations of at least 15 g·kg⁻¹ for one week showed significantly ($p < 0.05$) higher percentage inhibition of both bacteria than the control (Table 1).

Table 1. Serum bactericidal activity (percent inhibition) against pathogenic bacteria of Nile tilapia fed with diets supplemented with broccoli sprouts for one and two weeks. Means±SD with same letter/s within the same column are not significantly different ($p > 0.05$).

Treatment (g·kg ⁻¹ diet)	One week		Two weeks	
	<i>A. hydrophila</i>	<i>S. agalactiae</i>	<i>A. hydrophila</i>	<i>S. agalactiae</i>
0	22.67±11.24 ^c	33.33±13.02 ^d	21.60±17.04 ^c	47.43±3.63 ^c
10	28.00±9.43 ^c	50.00±10.00 ^c	54.29±5.82 ^b	53.47±1.05 ^c
15	52.00±8.91 ^b	58.33±3.33 ^{bc}	63.99±8.07 ^{ab}	56.49±9.97 ^{bc}
20	65.33±13.63 ^{ab}	66.67±3.33 ^b	63.43±10.80 ^{ab}	66.16±9.08 ^b
25	84.00±8.43 ^a	100.00±0.00 ^a	76.73±2.88 ^a	83.38±4.56 ^a

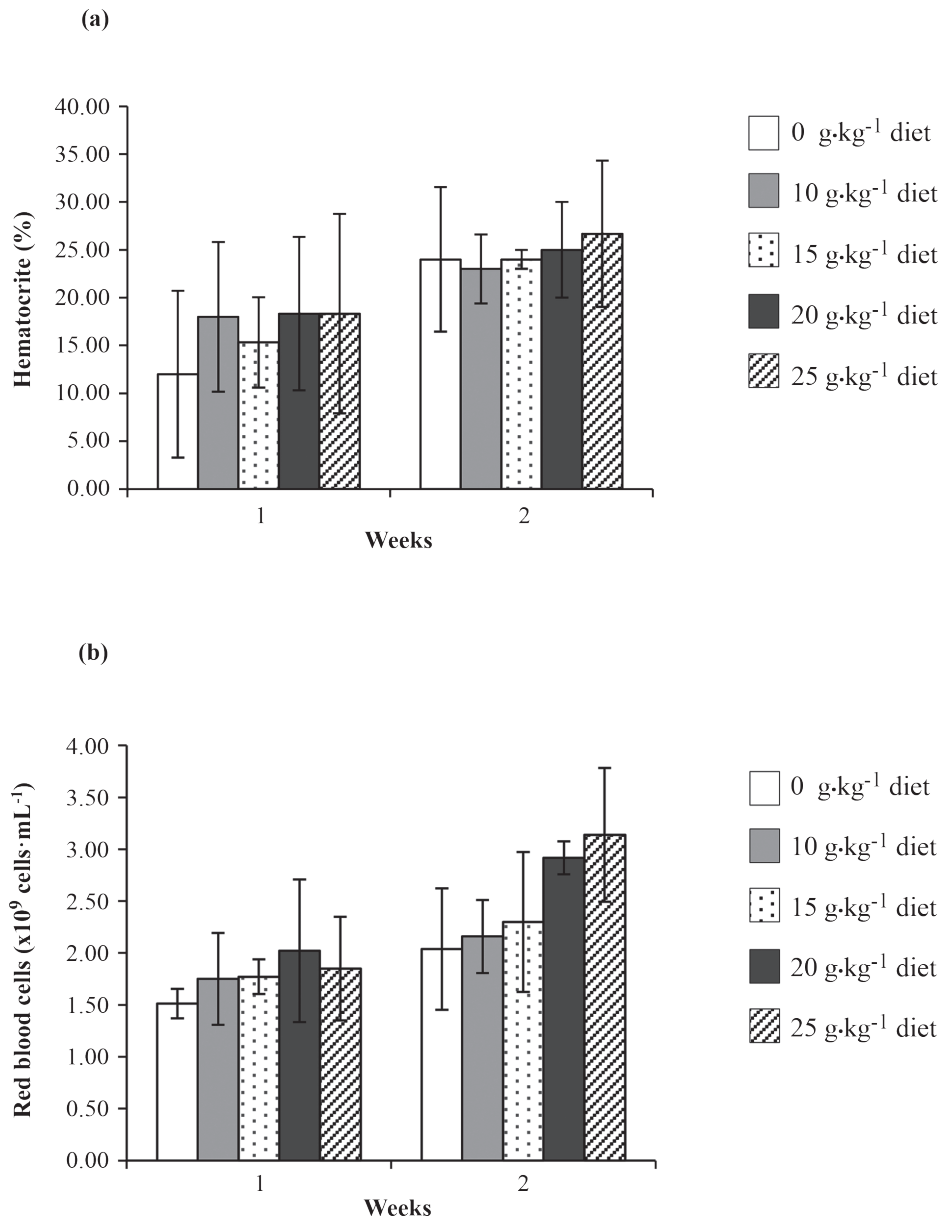


Figure 1. Hematocrit values (a), and red blood cell counts (b) of Nile tilapia fed with broccoli sprout-supplemented diets for one and two weeks. Error bars show mean standard deviations. No significant differences were found among treatments within the same week.

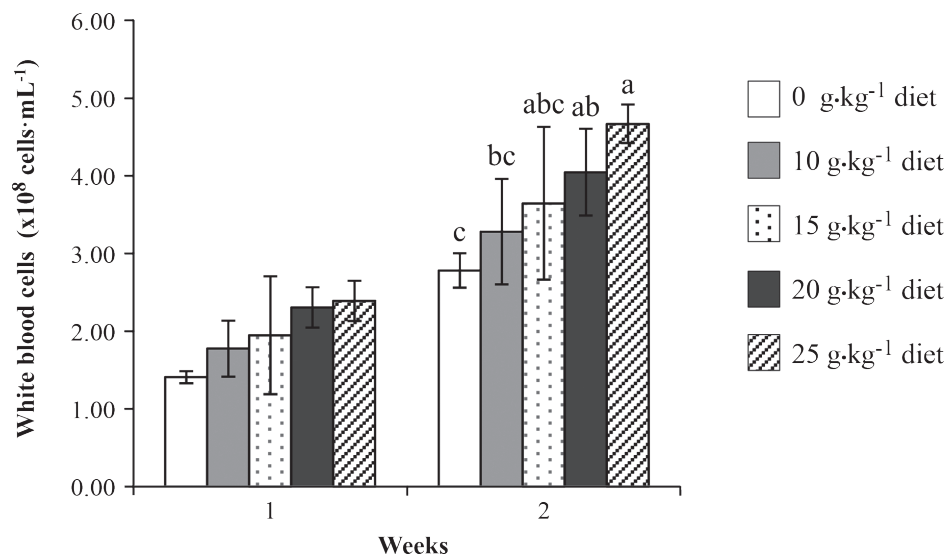


Figure 2. White blood cell counts of Nile tilapia fed with broccoli sprout-supplemented diets for one and two weeks. Means (\pm SD) within the same week with different letters are significantly different ($p < 0.05$). The first week did not show any significant differences among treatments ($p > 0.05$).

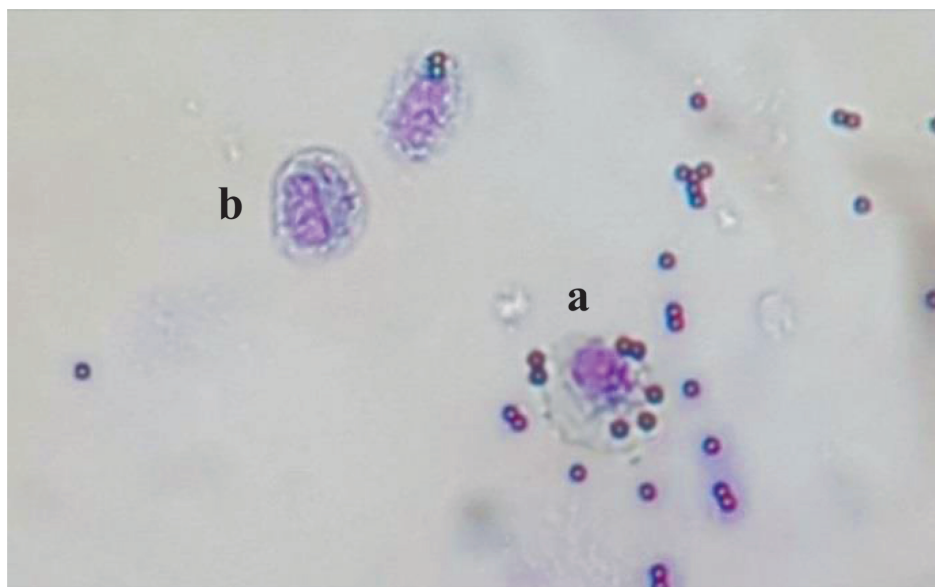


Figure 3. Leukocyte phagocytizing an invasive agent (latex bead) (a), and leukocyte lacking phagocytic activity (b).

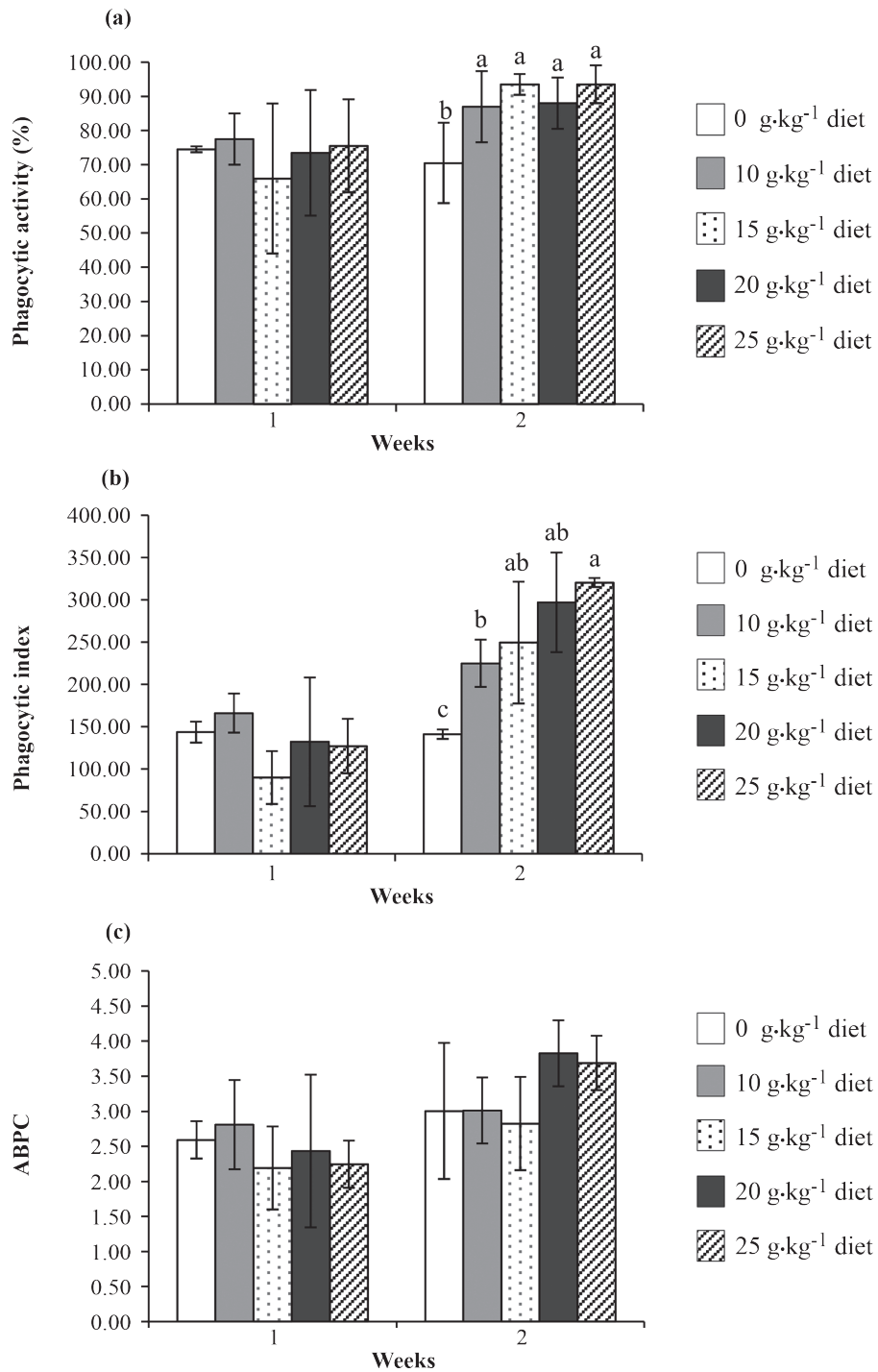


Figure 4. Phagocytic activity (a), phagocytic index (b), and average number of beads ingested/cell (ABPC) (c) of Nile tilapia fed with broccoli sprout-supplemented diets for one and two weeks. Error bars represent standard deviation. No significant different among means in the same week ($p > 0.05$).

DISCUSSION

Analysis of hematological parameters can be used to monitor health status, detect illnesses, and to follow the progress of disease and response to therapy (Clauss *et al.*, 2008). The results of this study showed that addition of broccoli sprouts to fish diet did not provide any significant changes in total red blood cells. Similarly, Pasko *et al.* (2018) observed that adding broccoli sprouts to the diet did not alter the red blood cell parameters in rats. Furthermore, Woyengo *et al.* (2011) reported that increasing dietary levels of canola (*Brassica napus*) in the feed showed no effect on the blood hematocrit and hemoglobin levels in broiler chickens. In contrast, feeding Nile tilapia with broccoli sprouts at 20 and 25 g·kg⁻¹ diet for two weeks showed significant enhancement in total white blood cell counts. Sulforaphane from broccoli has been shown to enhance bone marrow cellularity and total white blood cell counts on the 9th day in BALB/c mice treated with sulforaphane at 500 µg·dose⁻¹·animal⁻¹·day⁻¹ (Thejass and Kuttan, 2007). Furthermore, Oke *et al.* (2017) reported that feeding Nile tilapia fingerlings with a diet supplemented with broccoli leaf powder at 0.50 g·100 g⁻¹ of feed for 56 days increased white blood cell counts. White blood cells, or leukocytes, are the backbone of the immune response in both innate and adaptive immunity. Innate immunity largely involves granulocytes and macrophages. The latter are considered effector cells of the innate immunity and function in the phagocytosis of pathogens (Hirayama *et al.*, 2017). Phagocytosis is widely considered to be a primitive defense mechanism and plays an important role in the innate immune response (Seeley *et al.*, 1990; Esteban *et al.*, 2015). Here, we showed that supplementation of broccoli sprouts at 10, 15, 20 and 25 g·kg⁻¹ concentrations for two weeks significantly improved the phagocytic activity of leukocytes in Nile tilapia. This result agrees with the previous observations in BALB/c mice (Thejass and Kuttan, 2007) and RAW 264.7 cells (Suganuma *et al.*, 2011), in which phagocytic activity was enhanced by sulforaphane. Broccoli sprouts, therefore, which are rich in sulforaphane, could improve cellular immunity in Nile tilapia.

The humoral immune system relates to blood-related immunity, in which serum proteins, otherwise known as antibodies, are released in response to pathogens and effect the elimination of these pathogens. In the present study, broccoli sprout-supplemented diets stimulated a humoral immune response as evidenced by the increase in percentage inhibition of *A. hydrophila* and *S. agalactiae* in serum isolated from Nile tilapia. Furthermore, an increase in percentage inhibition was correlated with the increase in concentration of broccoli sprouts in the diet. In particular, over 60 % inhibition of both bacteria was observed for 20 and 25 g·kg⁻¹ treatments. Our results support an earlier study showing that sulforaphane can enhance the production of specific antibodies (Thejass and Kuttan, 2007). The extreme antibody titer value (1024) in sulforaphane-treated BALB/c mice was detected on the 12th day, while the control showed the extreme titer of only 128 on that day (Thejass and Kuttan, 2007). Moreover, Liu *et al.* (2018) reported that broiler chickens fed dietary inclusion of broccoli residues fermented with probiotics showed increased levels of immunoglobulins composed of IgA, IgG and IgM. In addition, sulforaphane was also found to exhibit antibacterial activity against *Helicobacter pylori*, which causes gastritis and peptic ulcers (Fahey *et al.*, 2002).

CONCLUSION

Financial losses due to the impact of diseases in farmed tilapia have prompted countless studies on how to enhance the immune system of the fish. Particular emphasis has been given to finding natural or organic sources of feed supplements as possible solutions to disease problems. Here, we demonstrated that diet supplementation with broccoli sprouts in Nile tilapia resulted in improved fish immunity. We also showed that a diet with at least 20 g·kg⁻¹ concentration of broccoli sprouts given for at least two weeks could significantly increase total white blood cells, which are important in both cellular and humoral immunity in Nile tilapia. Taken together, our results showed that broccoli sprouts can be a viable feed supplement for tilapia.

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