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IMMUNOSTIMULATORY EFFECT OF BENGUET PINE (*Pinus kesiya*) POLLENON NILE TILAPIA (*Oreochromis niloticus* L.)

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ABSTRACT

There is limited information on the immunostimulatory effect of pine pollens on finfishes. This study was conducted to evaluate the possible immunostimulatory effect of Benguet pine (*Pinus kesiya*) pollen-supplemented diet to vaccinated and non-vaccinated Nile tilapia (*Oreochromis niloticus*).Briefly, vaccinated or non-vaccinated fish weighing 30-40g each were fed with either pure or pollen-supplemented commercial diet. After 3-week period of feeding, parameters examined includes hematology, cell-mediated immune response and antibody production. Hematological examinations revealed that there was only significant difference on the hematocrit value which was high in non-vaccinated fish fed with pure commercial diet (T2). The cell-mediated immune responses measured in terms of the somatic index and differential counts of the leukocytes and neutrophils in the spleen were significantly different among all treatment groups. On the other hand, despite no significant differences were observed on the antibody production between vaccinated fish fed with either pure (T4) or pollen-supplemented diet (T5), there was remarkable presence of antibody production on non-vaccinated fish fed with pollen-supplemented diet. Hence, the study shows a promising potential of Benguet pine pollen as an effective immunostimulant that can be used to aquaculture industry in reducing mortality and control of fish diseases.

Key words: Immunostimulant, Benguet pine pollen, *Pinus kesiya*, *Oreochromis niloticus*

INTRODUCTION

Benguet pine (*Pinus kesiya)* is a conifer species considered as one of the most economically and culturally important species in Benguet province, Philippines [1]. *Pinus kesiya* of the family Pinaceae are common in highland regions of northern Philippines that provide optimum conditions for its growth [2]. It is commonly found in elevated areas of Baguio City, the mountain province, Zambales and Mindoro [3] and a renowned source of turpentine and timber in the Philippines. However, scientific studies and investigations on its nutritional and medicinal properties were limited.

One of the constraints in tilapia aquaculture is the inevitable occurrence of diseases due to water quality problem. This results to the deterioration of fish immunity during the culture period. Different approaches have been conducted to control diseases including sanitary prophylaxis, disinfection and chemotherapy [4], with particular emphasis on the use of antibiotics that can result in the development of a drug-resistant bacteria, environmental pollution and harmful residues in fish [5].

The use of expensive chemo-therapeutants and antibiotics resulted to accumulations in the tissues as residues and development of drug resistant organisms. Hence, attention is focused on the use of immunostimulants for disease control measures in aquaculture. Interest in the use of immunostimulants is currently increasing to control fish diseases [6,7].With this, many have focused on the use of medicinal plant products for modulating the immune response, as well as potential immunostimulants fortifying the immune response of cultured species to effectively prevent and control occurrence of diseases caused by different bacterial and viral pathogens [8,9].

The study was conducted to determine the potential use of Benguet pine pollen (*P. kesiya*) as possible immunostimulant in Nile tilapia (*Oreochromis niloticus*).

MATERIALS AND METHODS

Collection of Pine Pollen

A total of 518 pieces of Benguet pine catkins were collected in Baguio City, Philippines, followed by segregating, air-drying and transporting

to Central Luzon State University laboratory for an additional week of air drying. The granules from each pollen cone were manually removed, sieved and placed in a tightly sealed container until further use.

Preparation of Experimental Fish

A total of 225 pieces male Nile tilapia, with body weight ranging from 30-40 g, were sourced out from the GenoMar Supreme Philippines Inc. The pre-maxillae were removed and the fish were conditioned for 3-week period in 3 ppt saline environment prior to the commencement of the study. The fish were randomly divided into 5 treatment groups (Table 1) replicated thrice. Fish were kept in aerated glass aquaria measuring 30 x 60 x 30 cm. Two set-ups were used, one for hematology and cell mediated immune response study (i.e., with a stocking rate of 7 pieces of fish per replicate) and the other for microtiter agglutination test (i.e., with a stocking rate of 8 pieces of fish per replicate).

Formalin-killed Vaccine Production and Fish Vaccination

Formalin- killed *Aeromonas hydrophila* vaccine was produced following previous report [10]. Briefly, *A. hydrophila* was mass produced on Tryptic Soy Broth (TSB) at 30°C. After 48h incubation, bacterial cells were collected by centrifugation at 6,500 rpm for 15 minutes, washed thrice with physiological saline solution (PSS), followed by centrifugation to collect the washed bacteria. Formalin (0.4%) was added to the bacterial cells, allowed to stay overnight, and finally washed with PSS thrice after centrifugation.

The formalin-killed *A. hydrophila* vaccine was adjusted to a concentration of 1 x 10^8 cells/mL following McFarland scale. Any remaining live *A. hydrophila* on the prepared formalin-killed vaccine were tested prior to use. Absence of any microbial growth in nutrient agar indicates that all bacterial cells are already formalin-killed and can be used for vaccination. The prepared formalin-killed *A. hydrophila* vaccine was injected intramuscularly (at 0.3 mL/fish) in each respective individual fish based on the treatment groups (see Table 1).

Preparation of Pine Pollen-Supplemented Diet and Feeding of Experimental Fish

A commercially available fish diet (i.e., grower pellet with 31% CP) was

used in the preparation of non-pollen-supplemented and pollen-supplemented diets. In the preparation of pollen-supplemented diet, 10g of pine pollens were dissolved in 95% ethanol for 7 days at room temperature with daily stirring. The use of 10g pine pollen in the treatment was based from the earlier studies on the potential use of this pollen as phytoandrogen [3]. The pine pollen extract was blended in osterizer for a minute and mixed thoroughly with 2 kg finely grinded commercial fish diets. Then, the mixed diet was moistened with water and pelletized before final air-drying. A total of 4 kg of feeds were prepared for the entire 21-day experimental period.

All treatment groups were fed to satiation twice daily between 9:00 to 10:00 AM and 3:00 to 4:00 PM for a period of 21 days. The pure and pollen-supplemented commercial diets were given to the respective treatment groups (see Table 1).

Maintenance of Water Quality

Optimal water quality was maintained through supplemental aeration. Salinity level was raised from 3 ppt (during the 3-week conditioning period) to 5 ppt throughout the experiment to ensure mucus production and avoid further bacterial and parasitic infestation. Feed wastes were removed daily through siphoning with 70% water exchange every other day to ensure good water quality. The dissolved oxygen, pH and temperature were maintained to deal levels and recorded thrice weekly, one in the morning and in the afternoon.

Hematological Analyses

Two hundred microliter (200 µL) blood samples were drawn-out from each experimental fish (i.e., 5 samples from each replicate) through cardiac puncture using a sterile syringe. The blood samples were placed in test tubes with anticoagulant (EDTA) and sent to a diagnostic laboratory (AC Medlinks Specialty Clinics & Diagnostic Center, Science City of Muñoz, Nueva Ecija, Philippines) for hematological examination.

Cell-mediated Immune Response

The cell-mediated immune responses measured in the spleen include the spleen somatic index and differential counts of the leukocytes and neutrophils. The spleen somatic index was examined on three fish per replicate using standard

protocol [11,12]. Spleen indices were calculated by dividing spleen weight by the total fish body weight. For the differential count of the leukocytes and neutrophils, fish were sedated in chloroform and disinfected with 70% ethanol. The fish abdomen was surgically cut and the spleen was collected aseptically and weighed. Lympho-proliferative assay of spleen cells was done by cutting the spleen into pieces with scissors and squashed. The spleen cells were allowed to pass through a sterile stainless mesh and the cells were collected in a petri dish. The viable count of spleen cells was determined similar to the counting of total white blood cells. The smears of spleen cell suspension were then prepared on slides in replicates. Smears were stained with a commercially available stain set (Diff Quik) and the relative values of each cell in the spleen samples were computed.

Microtiter Agglutination Test

Microtiter agglutination test was done using standard protocol to determine the level of antibody produced against *A. hydrophila*. Briefly, collected blood samples were allowed to stand overnight in collecting tubes without anti-coagulants in order to separate the blood cells from the blood serum. Then, the first lane of a 96-well microtiter plate was filled with 100 µL of fish serum, while 50 µL of PSS was filled to the second lane up to the last lane. Mixing of solution was done before carrying out two-fold serial dilution by transferring 50 µL solution starting from the first lane down to the last lane. Following this, 50 µL of live *A. hydrophila* suspension (1×10^8 CFU/mL) was added and mixed to each well. Then, microplates were mixed by gently tapping on the side of the microtiter tray, covered and then left incubated for 16-18 h overnight at 37°C for agglutination reaction to proceed. The agglutination end point was established as the last serum dilution where agglutination was visible. Presence of visible spot or sharp point button (i.e., accumulated bacteria at the bottom of the wells) indicates negative agglutination, while positive agglutination can be viewed with visible mat or absence of visible spot at the bottom (Figure 1).

Statistical Analysis

Statistical analyses were performed using one-way Analysis of

Variance and Tukey's Post Hoc Test for the comparison among means.

RESULTS AND DISCUSSION

Hematological Analyses

Result of the hematological examination shows that only hematocrit values have significant differences observed among treatment groups, whereas other hematological parameters(erythrocyte count, hemoglobin, leukocyte count, segmented neutrophils, eosinophil count, lymphocyte count, monocyte count and platelet count) were not significant (Table 2). Despite the presence and absence of significant differences, the hematological parameters were found to be within the reference values reported [13], except for the segmented neutrophil, lymphocyte and monocyte counts.

Monitoring for hematological parameters have been undertaken by other researchers [14], but this is an initial report on possible influence of feeding with pine pollen to the hematological parameters. Given that the present study was conducted for only 21-day period, a significant change in the hematological parameters could possibly be observed when feeding of Benguet pine pollen is prolonged as similarly observed from previous studies treated with plant-based immunostimulants [15-17]

Cell-mediated Immune Response

Results revealed that the spleen index were significantly different among treatment groups (Table 3). Spleen weight has a direct relationship with fish immunity [12]. Spleen enlargement (splenomegaly) has been reported to occur in response to natural and experimental infections [18]. The present study indicates no spleen enlargement within the 21-day feeding trial with pine pollen-supplemented diets. For the leukocyte and neutrophil counts, no significant differences were observed among the treatment groups (Table 3). This indicates that vaccination of *A. hydrophila* and feeding of pine pollen (*P. kesiya*) had no effect on the white blood cell count in the spleen within a 21-day experimental period.

Microtiter Agglutination Test

Microtiter agglutination test showed no antibody production against*A. hyrophila*, as indicated by

absence of agglutination, in the control group injected only with PSS and fed with pure commercial diet (T1), as well in the non-vaccinated fish fed with pure commercial diet (T2). On the other hand, presence of agglutination was observed in both treatment groups injected with *A. hydrophila* vaccine (T4 and T5) indicating antibody production against *A. hydrophila.* Surprisingly, antibody production against *A. hydrophila* was observed in non-vaccinated fish that were fed with pine pollen-supplemented diet (T3) (Figures 1 and 2). This indicates that as *A. hyrophila* is known to be present in the body of a healthy fish, feeding with pine pollen-supplemented diet had induced the immunity of the fish producing specific antibody against naturally infecting *A. hydrophila* within the body system of the fish.

There were studies showing that plant extracts enriched-diets have beneficial effects on fish health and immune system that could be an important role in preventing disease outbreaks in aquaculture systems. Nile tilapia fed with diets containing different doses of mistletoe (*Phoradendrons erotinum*) for a period of 80 days showed 42% more survivability in a challenge with *A. hydrophila* than the control group [19]. Another study also showed that non-specific immune response in juvenile olive flounder fish (*Paralichthyso livaceus*) was enhanced (i.e., increased phagocyte respiratory burst, serum lysozyme and myeloperoxide activities) with dietary supplementation of kelp (*Ecklonia cava*) [20]. Such enhancement of immune response was probably due to its high antioxidant and polyphenolic content, thereby indicating that diets enriched with plant extracts have beneficial effects on fish health and immune system that could be an important role in preventing disease outbreaks in aquaculture systems [20].

The present study clearly indicates that pine pollen can induce the natural production of antibodies against *A. hydrophila*, which has a positive effect on the innate immune response, and has been proven to be effective immunostimulantin Nile tilapia.

CONCLUSION

Benguet pine pollens have shown to increase fish innate immune response even in non-vaccinated fish. The study

has shown that pine pollens can be a possible substitute as cheap, easy to prepare and eco-friendly immunostimulants that can help improve fish health status particularly in controlling and combating pathogenic bacterial diseases in Nile tilapia.

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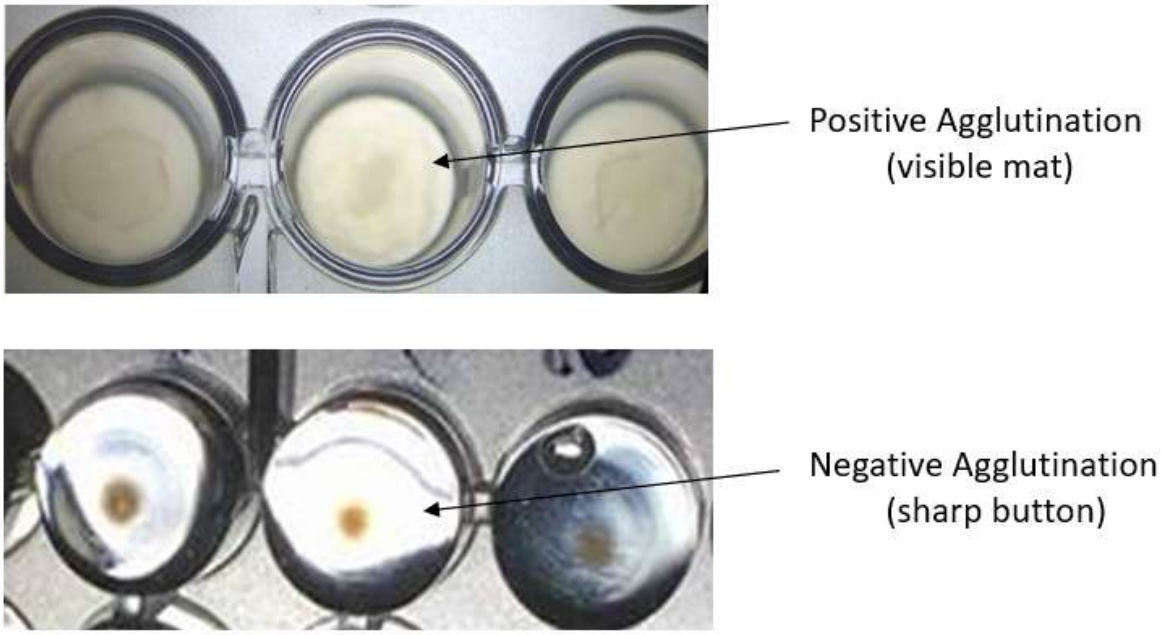

Figure 1: Presence of agglutination as shown by a visible mat (A) at the bottom of the well, while a sharp pointed button indicates absence of antibody production.

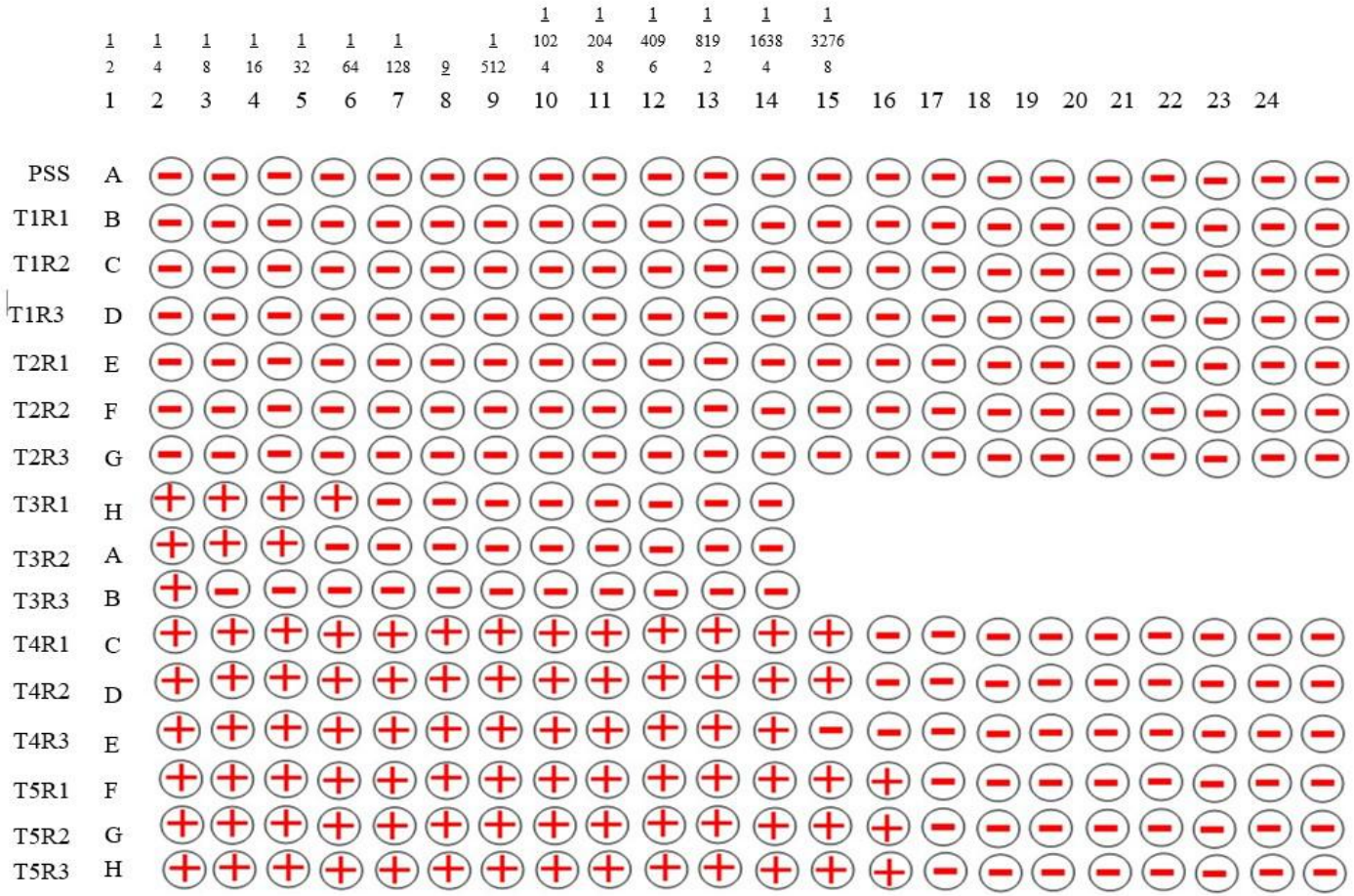

Figure 2: Result of the microtiter agglutination test among the different treatment groups. Note: (+) positive agglutination; (-) no agglutination

Table 1: Description of the different treatment groups used in the study

Treatment Groups	Description
1	Injected with physiological saline solution + fed with pure commercial diet
2	Non-vaccinated + fed with purecommercial diet
3	Non-vaccinated + fed with pine pollen-supplemented commercial diet
4	Vaccinatedwith *A. hydrophila*vaccine + fed with pine pollen-supplemented commercial diet
5	Vaccinatedwith *A. hydrophila* vaccine + fed with pure commercial diet

Table 2: Results on hematological tests of vaccinated and non-vaccinated treatment groups

Hematological Parameters	Refererence Values [13]	Treatment Groups				
		T1	T2	T3	T4	T5
Erythrocyte count (10^6/µL)	1.91-2.83	1.94±0.04^a	2.18±0.07^a	1.74±0.19^a	2.02±0.10^a	2.38±0.18^a
Hematocrit (%)	27-37	30.33±0.33^{ab}	38.67±2.60^a	26.33±3.93^b	33.0±1.15^{ab}	33.33±2.19^{ab}
Hemoglobin (g/dL)	7.0-9.8	8.13±0.09^a	9.23±0.15^a	7.57±0.84^a	8.83±0.50^a	8.53±0.30^a
Leukocyte count (10^3/µL)	21.5-154.7	137.47±3.32^a	134.27±2.86^a	133.83±6.32^a	140.47±4.44^a	140.5±5.63^a
Seg. Neutrophils (10^3/µL)	0.5-9.87	0.03±0.00^a	0.04±0.01^a	0.03±0.01^a	0.05±0.01^a	
Eosinophils (10^3/µL)	0.035-1.65	0.05±0.00^a	0.07±0.01^a	0.05±0.01^a	0.06±0.00^a	
Lymphocytes (10^3/µL)	6.8-13.64	0.86±0.01^a	0.82±0.02^a	0.88±0.01^a	0.83±0.02^a	
Monocytes (10^3/µL)	0.4-4.3	0.05±0.00^a	0.07±0.01^a	0.04±0.02^a	0.06±0.01^a	
Platelet count (10^3/µL)	25-85.2	50.67±6.64^a	77.00±3.51^a	56.67±15.39^a	48.00±16.37^a	

Values represent mean (± Standard Deviation) of the different parameters on hematological results. Different superscript letters in rows significantdifferences(P<0.05) among treatment groups

Table 3: Spleen index, leukocyte count and neutrophil countof fish spleen amongtreatment groups

Treatment Groups	Fish wt (g)	Spleen weight (mg)	Spleen length (mm)	Spleenwidth (mm)	Spleen index	Leukocyte count	Neutrophil count
T1	112.51 ±9.27^a	208. ± 35.29^a	20.61 ± 3.05^a	7.33 ± 0.37^a	1.85^a	31.00 ± 0.58^a	69.00 ± 0.58^a
T2	110.98 ± 57^a	229 ± 48.17^a	22.36 ± 3.09^a	7.16 ±0.09^a	2.07^a	36.67 ± 3.33^a	63.33 ± 3.33^a
T 3	102.12 ± 5.13^a	223 ±14.99^a	22.67 ± 1.67^a	7.58 ± 0.05^a	2.20^a	40.00 ± 0.00^a	60.00 ± 0.00^a
T4	91.87 ± 5.75^a	193 ± 39.29^a	19.08 ± 1.74^a	6.58 ± 0.42^a	2.11^a	30.00 ± 5.00^a	70.00 ± 5.00^a
T5	111.28 ±7.06^a	234 ± 18.90^a	23.74 ± 1.05^a	6.90 ± 0.20^a	2.10^a	50.00 ± 10.00^a	50.00 ± 10.0^a

Values represent mean (± Standard Deviation) data on fish weight, spleen weight, spleen length, spleenwidth, spleen index, leukocyte count and neutrophil count. Different superscript letters in columns represent significant differences (P<0.05) among treatment groups

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