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Effect of *Sophora flavescens* on non-specific immune response of tilapia (GIFT *Oreochromis niloticus*) and disease resistance against *Streptococcus agalactiae*

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ABSTRACT

The paper describes the effect of a diet supplemented with the Chinese traditional herbal medicine *Sophora flavescens* on the immunity and disease resistance of an *Oreochromis niloticus* GIFT strain. Experimental diets containing 0.025%, 0.050%, 0.100%, 0.200%, and 0.400% *S. flavescens*, as well as a control group without *S. flavescens* were used. We tested the non-specific humoral immune responses (lysozyme, antiprotease, and complement) and cellular immune responses (reactive oxygen species and nitrogen species production and myeloperoxidase), as well as disease resistance against *Streptococcus agalactiae*. *S. flavescens* supplementation at all dose significantly enhanced serum lysozyme, antiprotease, and natural hemolytic complement activity. Similarly, all *S. flavescens* doses enhanced cellular myeloperoxidase activity. The increased production of reactive oxygen species and reactive nitrogen intermediates by peripheral blood leucocytes was observed in most of the treatment groups throughout the test period. The fish fed 0.100% *S. flavescens* had a percent mortality of 21.1% and a relative percent survival of 73.3% compared with the group fed the basal diet during the *S. agalactiae* challenge. The results suggest that *S. flavescens* can be recommended as a tilapia feed supplement to enhance fish immunity and disease resistance against *S. agalactiae*.

1. Introduction

Diseases continue to threaten tilapia production because of intensive culture practices. Unfavorable environmental conditions such as temperature [1], dissolved oxygen level [2], unionized ammonia [3], and insecticides [4] as well as poor management practices (inadequate nutrition and rearing density [5]) may stress tilapia, causing immune suppression and increased susceptibility to streptococcal infections. In 2009, outbreaks of streptococcal infections in Southern China caused significant morbidity and mortality [6,7]. High economic losses seriously hindered the development of tilapia aquaculture.

Antibiotics could be used as an effective strategy for combating the proliferation of opportunistic bacteria in intensive aquaculture. However, continuous intensive use of antibiotics leads to antibiotic resistance in bacteria. Furthermore, antibiotic resistance can be transferred to the aquaculture environment and to human pathogens. On the other hand, antibiotic residues in aquaculture environments and in the subsequent fish products could cause adverse ecological and public health effects [8,9]. Although vaccinations effectively prevent fish disease [10], problems regarding inoculation and pathogen specificity limit their effectiveness. No commercial vaccines against streptococcal infections are available [11]. Therefore, more environment-friendly prophylactic strategies are urgently needed to promote sustainable tilapia production.

Herbal immunostimulants increase resistance to infectious disease by enhancing non-specific and specific immune mechanisms [12]. Although the exact mechanism of action of herbal extracts on the immune systems of fish and shellfish is poorly understood, immunostimulants from these extracts are believed to modulate the non-specific humoral and cellular defense mechanisms. These mechanisms are considered the first and most primitive line of defense against invading pathogens, which are more important in fish than in mammals. Herbs such as *Camellia sinensis* L. [13], *Nyctanthes arbortristis* [14], *Tinospora cordifolia* [15], *Toona sinensis* [16], *Eclipta alba* [17], *Solanum trilobatum* [18], *Astragalus membranaceus*, and *Lonicera japonica* [19] reportedly enhance the immune response and disease resistance of tilapia against *Aeromonas hydrophila* [13,14]. However, to the best of our knowledge,

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few herbal immunostimulants have been documented to prevent streptococcal infections.

In China, the dry root of *Sophora flavescens* has been widely used in the clinical treatment of eczema, skin disease, bacillary dysentery, jaundice, and fever [20]. Pharmacologic studies have revealed the antipyretic [21,22], antiulcer, anti-inflammatory [23,24], antimicrobial [25,26] antitumor [27,28], and antinociceptive activity [29] of the crude extracts and isolated constituents of *S. flavescens* and other *Sophora* plants. A few studies have reported the immunomodulatory effects of *S. flavescens* in mammalian species [30,31]. However, to the best of our knowledge, the present study is the first to assess the effect of dietary *S. flavescens* administration on the immunity and disease resistance of *Oreochromis niloticus* GIFT strain challenged with *Streptococcus agalactiae*.

2. Materials and methods

2.1. Fish and management

Tilapia (*O. niloticus* GIFT strain) weighing approximately (45 ± 5) g, were obtained from a commercial aquaculture farm in Yulin, Guangxi Zhuang Autonomous Region, China. The fish were acclimatized for 2 weeks in concrete tanks (capacity, 5000 l) at (28 ± 2) °C with a natural photoperiod. The fish were fed twice daily with a commercial pellet diet (extruded formula feed for tilapia, Diet No. 8992; Haid Feed Co., Ltd., Guangzhou, China) at 2% of their biomass. Continuous aeration was provided to maintain the dissolved oxygen levels at (8.5 ± 0.5) mg/ml. The tank was cleaned daily by siphoning off two-thirds of the water and replacing it with fresh water to maintain water quality during the experiment.

2.2. Preparation of immunostimulant diets

S. flavescens was purchased from the local medicinal plant market, and a voucher specimen (Specimen No. Ks-Ti1) was deposited in the Guangxi Institute of Botany. The powdered, air-dried root of *S. flavescens* was extracted three times in 95% ethanol at reflux for a total of 6 h. After removing the solvent in vacuo, the residue was dissolved in 80% ethanol and slowly sprayed onto the feed pellets to the required concentration. The pellets were then dried in an oven at 35 °C until the moisture content was less than 10%. The diet pellets were coated with 4% aqueous gelatin in distilled water as a binder at a ratio of 5:40 (v/w) to immobilize the herbal extract. The experimental pellets were packed and stored in a freezer at 4 °C until further use.

2.3. Experimental design

The non-specific immune parameters of tilapia examined using 18 fiber-reinforced plastic (FRP) tanks containing 350 l of aerated fresh water. The tilapia were assigned into six groups of 30 each, in triplicate. The respective groups were fed *S. flavescens* extract − supplemented diets at 0% (control group), 0.025%, 0.050%, 0.100%, 0.200%, or 0.400% (g kg^{-1}) for 30 d. Each replicate consisted of six randomly sampled fish from the tanks after 5, 10, 15, 20, and 25 days of feeding with the *S. flavescens* extract − containing and the control diets.

To study the resistance of the tilapia to *S. agalactiae*, experimental and control groups consisting of 30 fish with three replicates were tested. All groups were fed the *S. flavescens* extract − containing at 0% (control group), 0.025%, 0.050%, 0.100%, 0.200%, or 0.400% (g kg^{-1}) diet for 30 d. On day 10 of feeding, the fish were injected intraperitoneally with 0.2 ml of a 1.0 × 10^8 CFU ml^{-1} bacterial suspension of *S. agalactiae*, whereas the negative controls were injected intraperitoneally with 0.2 ml of phosphate-buffered saline (PBS; Beyotime, China). Preliminary work determined that this bacterial concentration causes death in approximately 75% of the fish in the control group. Mortalities were recorded daily for 30 days. *S. agalactiae* was isolated from the brains of 10% of the dead fish to confirm that the mortality was due to the bacterial infection. The relative percent survival (RPS) was calculated according to the method by Amend [32] as [(1 − percent mortality in the treatment group)/percent mortality in control group] × 100%.

2.4. Sample collection

Blood was collected with 27-gauge needles and 1 ml disposable syringes from the caudal vein of tilapia one of the triplicate tanks in each treatment [33] on days 5, 10, 15, and 25. For serum separation, 500 μl of blood was drawn and the whole bleeding procedure was completed within 1 min. The blood was allowed to clot for 30 min at room temperature and kept overnight at 4 °C. The blood clots were then centrifuged at 500 × *g* for 10 min. The resulting supernatant was collected and stored at −50 °C until further use.

Peripheral blood leucocytes were isolated by density gradient centrifugation as described by Misra et al. [34] with some slight modifications. The fish were bled using a 5 ml syringes filled with 2 ml of blood collecting medium [RPMI-1640 (Hyclone) supplemented with 50,000 IU l^{-1} of sodium heparin (Sigma–Aldrich), 1,00,000 IU l^{-1} of penicillin, and 100 mg l^{-1} of streptomycin (Beyotime, China)]. The diluted blood was slowly layered onto an equal volume of continuous gradients of 37% and 51% Percoll (Pharmacia; in 0.85% NaCl) and centrifuged at 500 × *g* for 30 min at 4 °C. The band of cells at the interface of the blood plasma and the Percoll layer was carefully harvested with a Pasteur pipette and washed twice with the same medium to remove the residual Percoll. The leucocytes were resuspended in culture medium [RPMI-1640 supplemented with 3% (v/v) of pooled tilapia serum, 1,000,000 IU l^{-1} penicillin, 100 mg l^{-1} streptomycin, and 4 mM L-glutamine (Amresco)]. Viable cells were counted using the tryphan blue (Sigma–Aldrich) exclusion method and the cell density was adjusted to 4 × 10^7 cell ml^{-1} using the same culture medium used to resuspend the cells prior to the assay.

2.5. Lysozyme activity assay

Serum lysozyme activity was modified from the methods described by Alexander et al. [15] and Yeh et al. [35]. Briefly, 10 μl of individual serum was mixed with 200 μl of 0.2 mg ml^{-1} lyophilized *Micrococcus lysodeikicus* suspension (Sigma) in 0.05 M sodium phosphate buffer (pH, 6.2). The mixture was incubated at 37 °C, and its optical density was detected at 530 nm after 1 min and after 6 min using a microplate reader (BioRad, USA). One unit of lysozyme activity was defined as the amount of enzyme that decreases the absorbance by 0.001 min^{-1} ml^{-1} serum.

2.6. Antiprotease activity assay

Serum antiprotease activity was assayed according to the methods of Bowden et al. [36] and Harikrishnan et al. [37] with minor modifications. Briefly, 2 mM BAPNA (sodium-benzoyl-DL-arginine-*p*-nitroanilide HCl; Himedia) was used as the substrate. First, 10 μl of the serum was incubated with 20 μl of trypsin solution (0.25% bovine pancreatic trypsin in 0.02% EDTA; Beyotime, China). Then, 500 μl of substrate was added. Tris–HCl (0.1 M, pH 8.2; Beyotime, China) was added to a final volume of 1 ml. The mixture was incubated at 22 °C for 25 min. The reaction was stopped with 150 μl of 30% acetic acid, and the optical density was read at 415 nm using a microplate reader (BioRad, USA) against a blank. The inhibitory activity of the antiprotease was expressed as the