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Feeding *Glycyrrhiza glabra* (liquorice) and *Astragalus membranaceus* (AM) alters innate immune and physiological responses in yellow perch (*Perca flavescens*)

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ABSTRACT

The current work assessed the potential immunomodulatory and growth-promoting effects of *Astragalus membranaceus* (AM) and *Glycyrrhiza glabra* (liquorice) in Yellow perch (*Perca flavescens*). In this regard, fish with an average weight of 31 ± 1.0 g were divided into five groups, and fed daily with an additive-free basal diet (control); 1, 2, and 3% (w/w) *Glycyrrhiza glabra,* and the fifth diet was incorporated with a combination of 1% *G. glabra*-AM for a four-week period. Immunological, biochemical and growth parameters were measured; and sub-groups of fish were exposed to 1-week starvation. The results showed that incorporating AM and liquorice in the diet significantly improved Immunological [superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), Lipid peroxidase (LPx) and lysozyme activities], biochemical [Aspartate Aminotransferase (AST) and Alanine Transaminase (ALT) activities; and glucose and cortisol concentrations] and growth performance parameters [body mass gain (BMG), specific growth rate (SGR), length, condition factor (K) and feed conversion ratio (FCR)]. In addition, markedly up-regulated the expression of related genes [Insulin-Like Growth Factor-1 (IGF-1), Serum amyloid A (SAA), Complement Component C3 (CCC3), Alpha 2 Macroglobulin (A2M), SOD and GPx] in treated fish groups compared to the control. Conclusively, feeding AM and liquorice diets significantly increased ($P < 0.05$) growth performance, antioxidant and immune response profiles throughout the entire experiment, suggesting their beneficial rule as natural anti-stress agents.

1. Introduction

Yellow perch (*Perca flavescens*) is one of the most valuable fish species in North America, particularly in the Midwest [1]. Various husbandry practices can result in physiological stress response such as elevated rearing densities, starvation, handling, anesthesia, and transportation stressors [2–5]. Starvation enhances reactive oxygen species generation that will lead to oxidative stress [6]. Oxidative stress results when the antioxidant defenses are overcome by pro-oxidant forces and reactive oxygen species are not adequately removed [7].

Most phytochemicals are considered natural anti-oxidants and redox active molecules similar to that of superoxide dismutase (SOD) and xanthine oxidase inhibitors. Their activity can be attributed to many types of active components that they contain [8,9]. In this study, attention focused on *Glycyrrhiza glabra* (liquorice) and *Astragalus membranaceus* (AM). *Glycyrrhiza glabra* is one of the oldest and most frequently used herbal medicinal plants [10]; it belongs to family *Fabaceae* [11]. A large number of active components have been isolated from Liquorice, with glycyrrhizic acid normally being considered to be the main biologically active component [12,13]. It has been reported to possess immunomodulatory, hepatoprotective and antioxidative attributes [14] and growth-promoting effects in fish [15]. Dried root of *Astragalus membranaceus* is a traditional Chinese medicinal herb [16] that contains more than 126 different components [17], such as: *Astragalus* polysaccharides (APS), organic acids, alkaloids, glucosides, and volatile oil as major components [18,19].

To the best of our knowledge, no studies for the effect of the two medicinal plants on the expression of immune related genes (SAA, CCC3, A2M, SOD and GPx) and growth related gene (IGF-1) have

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been reported in fish. The purpose of the present study was to evaluate the effects of dietary supplementation of *G. glabra* and a mixture of *G. glabra* -AM on the immune response and growth performance of yellow perch in response to starvation stress exposure.

2. Materials and methods

2.1. Fish

P. flavescens of average weight 31 ± 1.0 g were procured from the Aquaculture Research Center Hatchery at Ohio State University South Centers, Piketon, Ohio, USA and kept in a 2200 l fiberglass aerated tank filled with disinfected de-chlorinated well water. The health status was examined for evidence of diseases according to the methods of Austin et al. [20]. Fish were acclimated for ten days by feeding a basal diet. Temperature was maintained at 15 ± 0.36 °C and dissolved oxygen concentration at 5 ± 0.89 mg l^{-1}. Water quality parameters were monitored daily throughout the experiment.

This study and all experimental procedures involving animals were performed according to the protocol approved by the Ohio State University Institutional Animal Care and Use Committee.

2.2. Herbal plants

Both *G. glabra* and *A. membranaceus* were commercial products in powder form provided by Oregon's wild harvest, Sandy, Oregon, USA.

2.3. Experimental diets

Five experimental diets were prepared. Briefly, fine commercial powdered basal diet *Aquamax® Fingerling starter 300, PMI Nutritional International, LLC., Brentwood, MO, USA; Lot # 5D03* (Crude Protein Minimum 50.0%, Crude Fat Minimum 16.0%, Crude Fiber Maximum 3.0%, Calcium Minimum 2.0%, Phosphorous (P) Minimum 1.3%, Sodium (NA) Minimum 0.1% and Ash Maximum 12.0%) was divided into five portions, the first three portions were incorporated with 10, 20, and 30 g *G. glabra*/kg feed to obtain the desired concentrations of 1, 2, and 3%/kg, and the fourth portion was incorporated with a combination of 10 *G. glabra* (Liquorice): 10 *A. membranaceus* (AM) g/kg feed to obtain the desired concentration of 1%/kg and the last portion was kept free of any additives as a control. All components were then combined and mixed for 15 min. After that, each blend was adjusted to a desired pre-extrusion moisture content of ~45% by adding adequate amounts of water, then mixed again for 15 min. After extrusion, the pelletized feed blends were dried in a laboratory oven (Thelco Precision, Jovan, Winchester, Virginia, USA) at 50 °C for 24 h. After drying, the diets were broken up and sieved into proper pellet size (1.5 × 3.0 mm and 2.5 × 5.0 mm), then were packed in clean dry plastic containers and stored at −15 °C until use.

2.4. Experimental design

The experiment was designed to include two phases (I and II).

2.4.1. Phase I (pre-exposure)

The 450 fish were evenly assigned into five experimental groups in five rectangle fiberglass tanks (240 × 60 × 35 cm), each having three champers or replicates (30 fish per replicate), in a flow-through system. The control group was fed a basal diet without any herbal plants incorporated, and the four treated groups received *G. glabra* at 1, 2, 3%/kg, and a mixture of 1% *G. glabra*-AM (mix diet). Fish were fed to satiation in two equal parts twice a day at 9:00 a.m. and 4:00 p.m. for four weeks. Water quality was monitored throughout the experiment and adjusted at 17 ± 1.2 °C [21–24] and dissolved oxygen (DO) at 5 ± 0.89 mg/L. Temperature and DO were measured and recorded in the morning and afternoon daily for each tank/treatment. All chambers were siphoned daily to remove excess feed and fecal matter to maintain good water quality. Water flow was adjusted across all tanks to maintain the targeted temperature for each tank. The number of dead fish was recorded daily in all groups during the experimental period.

2.4.2. Phase II (starvation exposure)

Experimental diets were withheld from all experimental tanks, except for the control group that received the control basal diet. Three fish were sampled from each replicate after one week of food deprivation.

2.5. Sampling

2.5.1. Blood sampling

Sampling included two sampling points: (1) after feeding on experimental diets for four weeks and before exposure to starvation stress (pre-exposure; at the end of phase I) and (2) after exposure to 1-week starvation stress (post-exposure; at the end of phase II). Three fish per replicate (9 fish per group) were carefully netted, and were euthanized by tricaine methanesulfonate (MS222) at 250 ppm in water (Syndel Laboratories Ltd.,Vancouver, British Columbia) for blood and tissue sampling. Blood was drawn from caudal vessels using 1 cc U-100 heparinized syringes (Becton Dickinson, Franklin Lakes, NJ, USA) in heparinized tubes and kept on crushed ice until centrifugation at 3600 rpm for 5 min at 4 °C to obtain plasma samples. Separated plasma was stored in clean centrifuge tubes at −80 °C until used for assaying Glucose, cortisol, ALT, and Lysozyme activities values at both sampling points. Sedimented RBCs from the same blood samples were lysed in four times its volume of ice-cold HPLC grade water (Sigma, USA), centrifuged at 5000 g for 15 min at 4 °C, then the supernatant (erythrocyte lysate) was collected and stored at −80 °C for determination of SOD, GPx, and CAT activities at both sampling points.

2.5.2. Tissue sampling

After blood sampling, the fish were carefully dissected to isolate liver samples, which were then divided into two parts. The first part was used for assaying different antioxidant indices and the second part was stored in RNAlater (Ambion, USA) and kept at −20 °C for studying gene expression. The first part was rinsed with phosphate buffered saline (PBS), pH 7.4, to remove any red blood cells and clots and transported on ice and then each sample was divided into 3 portions, one portion was homogenized in AST kit's assay buffer for Assaying AST, the second portion was homogenized in 50 mM PBS containing 1 Mm EDTA (pH 7.4) for assaying SOD, CAT, GPx, and ALT, and the final portion was homogenized on ice in Malondialdehyde (MDA) Lysis Buffer for assaying LPx. Then, it was centrifuged at 15,000 × g for 10 min at 4 °C to remove insoluble materials and supernatant was separated and stored in clean centrifuge tubes at −80 °C until assayed.

2.6. Immunological and biochemical parameters

2.6.1. Immunological parameters

2.6.1.1. Lysozyme activity. Lysozyme activity was measured using a Microplate Spectrophotometer (BioTek's Epoch™, USA), according to Lysozyme Detection kit's manufacture's protocol (Sigma-Aldrich, USA) at 450 nm and using the following formula:

Lysozyme activity (U/ml) = [(ΔA_{450}/min Test − ΔA_{450}/min