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Immunostimulatory effects of artificial feed supplemented with a Chinese herbal mixture on *Oreochromis niloticus* against *Aeromonas hydrophila*

Jufen Tang [a, b, c, 1], Jia Cai [a, b, c, 1], Ran Liu [a, b, c], Jiamin Wang [a, b, c], Yishan Lu [a, b, c], Zaohe Wu [b, c, d], Jichang Jian [a, b, c, *]

[a] College of Fishery, Guangdong Ocean University, Zhanjiang 524088, China
[b] Guangdong Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Economic Animals, Zhanjiang 524088, China
[c] Guangdong Key Laboratory of Control for Diseases of Aquatic Economic Animals, Zhanjiang 524088, China
[d] Zhongkai University of Agriculture and Engineering, Guangzhou 510225, China

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ABSTRACT

The effects of a Chinese herbal mixture (CHM) composed of *astragalus*, *angelica*, *hawthorn*, *Licorice root* and *honeysuckle* on immune responses and disease resistant of Nile tilapia (*Oreochromis niloticus* GIFT strain) were investigated in present study. Fish were fed diets containing 0 (control), 0.5%, 1.0%, 1.5% or 2.0% CHM (w/w) for 4 weeks. And series of immune parameters including lysozyme, cytokine genes TNF-α and IL-1β, superoxide dismutase (SOD), peroxidase (POD), malondialdehyde (MDA) were measured during test period. After four weeks of feeding, fish were infected with *Aeromonas hydrophila* and mortalities were recorded. Results of this study showed that feeding Nile tilapia with CHM-supplementation diet stimulated lysozyme activity, SOD activity and POD activity in serum, induced TNF-α and IL-1β mRNA expression in head kidney and spleen, but decreased serum MDA content. All CHM-supplemental groups showed reduced mortalities following *A. hydrophila* infection compared with the group fed the control diet. These results suggested that this CHM can be applied as a tilapia feed supplement to elevate fish immunity and disease resistance against *A. hydrophila*.

1. Introduction

Tilapia (*Oreochromis* sp.) is one of the widely cultured fish species around the world. However, the diseases caused by bacterial pathogens in tilapia culture were becoming severe and resulted in significant morbidity and mortality [1,2]. In order to control the proliferation of these bacteria, antibiotics were used widely in intensive aquaculture. But prolonged use of antibiotics could lead to many negative side effects such as antibiotic resistant in bacteria, antibiotics residues in environment and fish products [3,4]. Therefore, exploring new methods for preventing infectious diseases have become very urgent in tilapia culture.

Immunostimulant can enhance non-specific immunity of fish and protect fish against infectious pathogens [5]. In general, using immunostimulant in combination with fish vaccine is a promising method for prevention of fish diseases [6]. Up to date, many substances including polysaccharide [7–9], polypeptide and protein [10–12] and various kinds of probiotics [13–15] have been used for fish immunostimulants. Herbs, which have been used as medicine and human immunity intensifier for thousand years in China [16], have attracted the attention of researchers in last decade. To date, it has been proved that administration of herbs can improved the innate and adaptive immune response of different freshwater or marine fish and shellfish against bacterial, viral, and parasitic diseases [17]. In tilapia, herbal extracts or their products such as *inospora cordifolia* [2], *Camellia sinensis L* [18], *Nyctanthes arbortristis* [19], *Toona sinensis* [20] and *Sophora flavescens* [1] have been documented to enhance the immune response and disease resistance of tilapia against bacterial pathogen [1,18,19]. However, to our knowledge, the immunostimulatory roles of Chinese herbal mixture in fish remain largely unknown.

* Corresponding author. Fisheries College, Guangdong Ocean University, Huguang Yan East, Zhanjiang 524088, Guangdong Province, China. Tel./fax: +86 759 2383507.
E-mail addresses: jianjc@gmail.com, matrix924@foxmail.com (J. Jian).
[1] These authors contributed to the work equally and should be regarded as co-first author.

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In this study, a Chinese herbal mixture (CHM) consisted of *astragalus*, *angelica*, *hawthorn*, *Licorice root* and *honeysuckle* was prepared in our laboratory. And the effects of supplemental dietary CHM on immunity and disease resistance of *Oreochromis niloticus* against *Aeromonas hydrophila* were investigated.

2. Materials and methods

2.1. Fish and management

Tilapia (*O. niloticus* GIFT strain) weighing approximately (20 ± 2) g, were obtained from a commercial aquaculture farm in Lianjiang, Guangdong province, China. The fish were acclimatized in tanks at (28 ± 2) °C aerated water for 2 weeks. The fish were fed twice daily with control diet prepared in our laboratory. The control diet contained 5% fish meal, 9% soybean meal, 14% cottonseed meal, 25.5% rapeseed meal, 1.5% calcium hydrogen phosphate, 0.18% salt, 1.5% soybean oil. No other immunostimulant was added in the control diet. The water quality was maintained by canister external water filters during the experiment.

2.2. Immunostimulant diets preparation

The herbs used in this study were purchased from a local traditional Chinese medicine shop. These herbs were dried in an oven at 60 °C then smash sift out through 80 mesh sieves. The dried, powdered herbs were mixed with a certain proportion. The herbal mixture was incorporated into a control diet at different rates of 0.5%, 1.0%, 1.5%, 2% (w/w) then made into pellet feed. The experimental pellets were packed and stored in a freezer at 4 °C until further use.

2.3. Experimental design

To study the immune-related index, the tilapia were assigned into five groups of 24 each, in triplicate. The respective groups were fed CHM-supplemented diets at 0% (control group), 0.5%, 1%, 1.5%, 2.0% for 4 weeks. Each replicate consisted of six randomly sampled fish from four CHM-supplemented groups and the control group.

A. hydrophil used for challenge experiment was isolated from diseased *O. niloticus* and kept in our laboratory. To study the resistance of the tilapia to *A. hydrophil*, experimental and control groups consisting of 30 fish with three replicates were tested. All groups were fed the CHM containing at 0% (control group), 0.5%, 1.0%, 1.5%, 2.0% for 4 weeks. After fourth week of feeding, the fish were injected intraperitoneally (i.p) with 0.2 ml of a 1.0×10^8 CFU/ml *A. hydrophil* suspended in phosphate-buffered saline (PBS), whereas the control group were injected intraperitoneally (i.p) with 0.2 ml PBS. Preliminary work determined that this bacterial concentration causes death in approximately 75% of the fish in the control group. Mortalities were recorded daily for 2 weeks. *A. hydrophil* was re-isolated from the livers of the dead fish to confirm that the cause of mortality.

2.4. Sample collection

Blood samples were collected from the caudal vein of tilapia in each group every week. For serum separation, the blood was clotted 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 supernatants were collected and stored at −80 °C until further use.

Tissue samples including head kidney and spleen for Real-time PCR (qRT-PCR) analysis were collected on 1st week and store at −80 °C before RNA isolation.

2.5. Lysozyme activity assay

Serum lysozyme activity was determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). Briefly, 200 μl fish serum were added to 1.8 ml of *Micrococcus lysodeikticus* suspension in phosphate buffer and followed by 15 min reaction at 37 °C. And 200 μl reacted suspension was removed into a 96-well plate and measured at 530 nm in a microplate reader (Bio-TEK, USA). Lysozyme activity was defined as μg per ml serum.

2.6. Real-time PCR (qRT-PCR) analysis of cytokine genes

The mRNA expression of genes tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) were examined in the head kidney and spleen. Total RNA of examine tissues were using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol, the quality of total RNA was detected by electrophoresis on 1% agarose gel. The first-strand cDNA was synthesized using EasyScript First-Strand cDNA Synthesis SuperMix (Transgen, China) following DNaseI(-NEB, USA) treatment. The specific primers used for qRT-PCR were listed in Table 1. Amplification of β-actin mRNA was used as internal controls. Primers used for β-actin amplification were listed in Table 1.

The qRT-PCR assay was carried out through IQ5 Real-time PCR System (Bio-Rad laboratories). Dissociation curve analysis of amplification products was performed at the end of each PCR reaction. The amplification was carried out in a 25 μl reaction volume, containing 12.5 μl of 2× SYBR Premix Ex Taq (TaKara, Japan), 1 μl sense primer and 1 μl anti-sense primer (10 μM), 2 μl of 1:5 diluted cDNA and 8.5 μl of PCR-grade water. The thermal profile for qRT-PCR was 94 °C for 5 min followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. After the PCR program, qRT-PCR data were analyzed with IQ5 Software. The baseline was set automatically by the software. The relative expression levels of target genes were analyzed by $2^{-\triangle\triangle CT}$ method [21]. PCR efficiency was calculated according to the protocol in Ref. [22].

2.7. Superoxide dismutase (SOD) activity assay

Serum SOD activity was assayed through commercial test kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). One unit of SOD activity was defined as the amount of enzyme necessary to produce a 50% inhibition of the nitroblue tetrazolium reduction rate measured at 550 nm. SOD activity was expressed as SOD units per ml serum.

2.8. Malondialdehyde (MDA) content assay

The MDA content of serum was detected using commercially available kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) at 532 nm in microplate reader. MDA content was expressed as nmol/ml.

Table 1
Sequences of primers used in this study.

Primers	Sequences (5′–3′)
TNFα-S	CCTGGCTGTAGACGAAGT
TNFα-A	TAGAAGGCAGCGACTCAA
IL1β-S	GACAGCCAA AAG AGG AGC
IL1β-A	TCTCAGCGATGG GTGTAG
Actin-S	CCAGGCAGCTCGTAACTCT
Actin-A	GAAATCGTGCGTGACATCAA