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The effects of feeding β-glucan to *Pangasianodon hypophthalmus* on immune gene expression and resistance to *Edwardsiella ictaluri*

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ABSTRACT

Pangasianodon hypophthalmus (striped catfish) is an important aquaculture species and intensification of farming has increased disease problems, particularly *Edwardsiella ictaluri*. The effects of feeding β-glucans on immune gene expression and resistance to *E. ictaluri* in *P. hypophthalmus* were explored. Fish were fed 0.1% fungal-derived β-glucan or 0.1% commercial yeast-derived β-glucan or a basal control diet without glucan. After 14 days of feeding, the mRNA expression of immune genes (transferrin, C-reactive protein, precerebellin-like protein, Complement C3 and factor B, 2a MHC class II and interleukin-1beta) in liver, kidney and spleen were determined. Following this fish from each of the three diet treatment groups were infected with *E. ictaluri* and further gene expression measured 24 h post-infection (h.p.i.), while the remaining fish were monitored over 2 weeks for mortalities. Cumulative percentage mortality at 14 days post-infection (d.p.i.) was less in β-glucan fed fish compared to controls. There was no difference in gene expression between dietary groups after feeding for 14 days, but there was a clear difference between infected and uninfected fish at 24 h.p.i., and based on principal component analysis β-glucans stimulated the overall expression of immune genes in the liver, kidney and spleen at 24 h.p.i.

1. Introduction

The striped catfish, *Pangasianodon hypophthalmus*, is the most economically important species farmed in Vietnam, with a total export value of 1.6 billion USD in 2014 [1]. Intensive aquaculture can lead to problems with major outbreaks of disease and *Edwardsiella ictaluri* and *Aeromonas hydrophila* represent two important bacterial pathogens in *P. hypophthalmus* aquaculture [2,3]. Immunostimulants have proven to be a very useful food additive for the aquaculture industry, since they can be easily fed to fish to enhance their immune response at times of stress and to improve resistance to disease [4–6]. The immunostimulants most commonly used in aquaculture tend to be β-glucans, which are a heterogeneous group of glucose polymers containing β-(1,3)-linked β-D-glucopyranosyl units and β-(1,6)-linked side chains of varying lengths [7].

The immuno-stimulatory activity of β-glucans has been shown to be mediated through changes in the gene expression of pro-inflammatory cytokines and chemokines [8–10], with many examples of this in the literature where β-glucans have been administered to fish either orally or by injection [9,11–14]. There are only a few studies examining the expression of immune genes in *P. hypophthalmus*, for example, Huong-Giang et al. [15] examined serum amyloid P component (SAP) and C-reactive protein (CRP) genes in *P. hypophthalmus*.

A variety of innate immune parameters in *P. hypophthalmus* were investigated in an earlier study in which fish were fed either fungal-derived β-glucan or yeast-derived β-glucan [7], and differences were observed between immunostimulated fish and those

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fed with the control diet. Nevertheless, these differences were not reflected in the level mortalities obtained between the dietary groups when the fish were experimentally infected with *E. ictaluri*, possibly due to the high level of mortalities obtained in the experimental infection and the high variation among the replicate tanks within groups. The aim of the present study was investigate the influence of dietary β-glucans on survival and immune gene expression in *P. hypophthalmus* after challenge with *E ictaluri* using a less severe challenge than that used in a previous study.

2. Materials and methods

2.1. Experimental animals

P. hypophthalmus (initial weight 36 ± 0.34 g, mean ± SD) were purchased from a local fish farm and transported to the Novus Aqua Research Center (NARC), Linh Trung Ward, Thu Duc District, Ho Chi Minh City, Vietnam. The fish were quarantined and acclimated to laboratory conditions (dissolved oxygen 6.0 ± 0.5 mg L^{-1}; temperature 28 ± 1 °C; total ammonium 0.05 ± 0.01 mg $N{-}NH_4$ L^{-1}; Nitrite 0.1 ± 0.1 mg $N{-}NO_2$ L^{-1}; Nitrate 0.01 ± 0.01 mg $N{-}NO_3$ L^{-1}; pH 7.5 ± 0.25) for at least 2 weeks prior to the start of the experiment.

2.2. Experimental design

Three dietary groups were used in the study: a basal control group with no β-glucan added; 0.1% w/w fungal-derived β-glucan; and 0.1% w/w commercial yeast-derived β-glucan as previously described by Sirimanapong et al. [7]. Each treatment was conducted in four replicate 340 L fibre-glass tanks (75 fish per tank). Fish received the basal control diet at 3% body weight per day for 14 days during the acclimation period prior to starting the experiment, after which fish were fed with the experimental feeds at 3% body weight per day for 14 days.

Forty-eight fish (*i.e.* four fish per tank) were sampled at 14 days post-feeding (d.p.f.) and 24 h post-infection (h.p.i.) with *E. ictaluri* (as described below). Samples of spleen, head kidney and liver were placed in RNAonshore (0.5 M Na_2EDTA, 1 M sodium citrate, ammonium sulphate and nuclease free H_2O) and stored at −80 °C for subsequent immune gene analysis.

The twelve tanks of fish from the three dietary groups were experimentally infected with *E. ictaluri* after 14 d.p.f. and subsequently divided into twenty four 80 L tanks. The fish in twelve of the tanks (4 tanks per experimental group) were infected with a freshly prepared culture of *E. ictaluri* (NLF33) by immersion exposure for 30 min according to Sirimanapong et al. [7], using 1×10^6 cfu ml^{-1} *E. ictaluri* added to 10 L of water, while the fish in the remaining tanks were immersed in autoclaved culture medium (10 mL in 10 L). From each of the initial 24 tanks, 10 fish were randomly assigned to each 30 L fibre-glass tank to monitor immune gene expression in response to the infection. The 20 fish remaining in the 50 L fibre-glass tanks were used to monitor the level of cumulative mortalities over a 14 day infection period. The water temperature was maintained at 26 ± 1 °C over the course of the trial.

2.3. Analysis of immune genes

2.3.1. Identification of immune genes

ESTs (expressed sequence tags) were kindly provided by Novus Aqua Research Center (NARC), Linh Trung Ward, Thu Duc District, Ho Chi Minh City, Vietnam (unpublished data), obtained from a cDNA library prepared from 500 g *P. hypophthalmus* challenged with *E. ictaluri* for 24 h, representative of intestine, head kidney, liver and muscle samples prepared by pyrosequencing. The resulting sequences were processed to produce four EST data-sets (intestine 2532 ESTs; head kidney 3179 ESTs; liver 1314 ESTs and muscle 1070 ESTs). The EST sequences obtained were then compared to the NCBI RefSeq database using BLASTN and a list of immune-related *P. hypophthalmus* ESTs were generated (expectation-value (e-value) $< 5 \times 10^{-5}$, total coverage > 33% of the query sequence and >100 nucleotides in length).

2.3.2. Design of RT-qPCR primers

Specific RT-qPCR primers, shown in Table 1, were designed for the selected *P. hypophthalmus* immune genes identified from the ESTs using NCBI Primer-Blast software [http://www.ncbi.nlm.nih.gov/tools/primer-blast/] [16]. The criteria used for primer selection was 3′ self-complementarity less than 1, matched melting temperatures of 60 ± 0.5 °C, a predicted amplicon of less than 200 base pairs, 50–60% GC content and primer lengths of 18–24 nucleotides.

2.3.3. RNA isolation

Tissue samples (<100 mg) were incubated in 1 ml of TRIZOL reagent® (Sigma, UK) on ice for 45 min, before homogenisation with a bead beater (Mini-Beadbeater 24, USA) for 45 s or until the tissue was disrupted.

The homogenized samples were incubated at 20 °C for 5 min, then 50 μl of 1-bromo-3-chloropropane (Sigma, UK) was added and the tube shaken vigorously for 15 s. The samples were incubated at 20 °C for 15 min and centrifuged (Sigma® 4K15, UK) at 20,000 × g for 15 min at 4 °C. The aqueous (upper) phase *i.e.* 200 μl, was removed from the transparent phase using a wide-bore pipette tip and transferred into a new tube and 100 μl of RNA precipitation solution (*i.e.* 1.2 M NaCl, 0.8 M sodium citrate) added, together with 100 μl of isopropanol (Fluka, UK). The tubes were gently inverted 4–6 times. The samples were incubated for 10 min at 20 °C and centrifuged at 20,000 × g for 10 min at 4 °C. The supernatant was removed and the pellet washed with 1 ml of 75% ethanol, for 15 min at 20 °C. They were then flicked to detach the pellet from the bottom of the tube, inverted a few times and centrifuged at 20,000 × g for 5 min at 20 °C. Finally the RNA pellet was air dried at 20 °C for 3–5 min until all visible traces of ethanol were removed. The samples were suspended in 100 μl of RNAase free-water and incubated at 20 °C for 30–60 min with gentle flicking of the tubes every 10 min to aid re-suspension. The concentration of the samples was measured using a nanodrop spectrophotometer (ND-100; USA) and adjusted to a final concentration of 500 ng μl^{-1} for the liver and kidney tissue samples, and to 300 ng μl^{-1} for the spleen samples using RNAase-free water. The samples were finally stored at −70 °C.

The quality of RNA extracted was checked on a 1.5% agarose gel (Sigma, UK). Samples were prepared by using 2 μl of 2X loading dye (Biolabs; UK) and mixed with 2 μl of sample. The aliquots of RNA with the loading buffer were heated for 5 min at 75 °C, chilled on ice and run on a 1.5% agarose gel using a gel electrophoresis system (BIO-RAD® HU13; USA) at 75 V for 30 min.

2.3.4. cDNA synthesis

RNA from eight pools of two fish per treatment were used for cDNA synthesis using a high-capacity cDNA reverse transcription kit (Applied Biosystem, UK) according to manufacturer instructions. The synthesised cDNA samples were stored at −70 °C until used.

In addition, cDNA prepared from the liver and kidney of each group, was collected and pooled and a serial dilution of this pool was prepared (1 × , 5 × , 500 × and 5000 ×) by dilution in 5 ng μl^{-1} λ phage DNA, 2 mM Tris pH 8.0, and 50 μl aliquots of each dilution was placed in tubes and stored at −70 °C. This dilution series was used initially to test the efficiency of primer combinations, and was