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Modulation of cytokine expression by dietary levan in the pathogen aggravated rohu, *Labeo rohita* fingerlings

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ABSTRACT

A novel prebiotic, levan, has shown a positive beneficial role as a dietary immune-nutrient in fishes. However, the research investigations on the underlying mechanism of immune protection is unavailable, therefore in the present study we have attempted to reveal the effects of dietary microbial levan on the expression of cytokine genes in the pathogen challenged *Labeo rohita* fingerlings. Fish were randomly distributed in two treatment group in triplicates, control group fed without levan and treated group fed with 1.25% (w/w%) of microbial levan in the diet. After 60 days of feeding trial, fish from each group were challenged with virulent *A. hydrophila* strain and tissue samples were collected on different time points, 3 h, 6 h, 12 h, 24 h, 48 h, and 96 h. Dietary administration of microbial levan, significantly upregulated the m-RNA levels of pro-inflammatory cytokines like IL-1β, TNF-α and IL-12p40 in the intestine, gill, kidney and liver in a time dependent manner. An increase of 2.13-fold in the expression of IL-1β at 96 h post challenge in the kidney cells was detected, however at early time points (3 h & 6 h) expression of the same was down regulated. Upregulation of the TNF-α gene in the gill, liver and kidney at 6 h and in the intestine at 12 h was noticed. IL-12p40 m-RNA expressed highest upregulation of 3.65-fold at 3 h in the intestine and 2.0-fold at 12 h in the gill. IL-10 m-RNA expression of intestine in the infected L. *rohita* displayed 7.57-fold downregulation. Furthermore, increased respiratory burst activity and serum lysozyme activity was observed in a time dependent manner in infected rohu fed with levan supplementation. Overall, the current study revealed the upregulation of immune responsive cytokine genes (IL-1β, TNF-α and IL-12p40) with dietary microbial levan against the pathogen infected stress in *Labeo rohita* fingerlings and thus supports use of levan as food additive in aquaculture.

1. Introduction

Augmenting the immune system by means of the ecofriendly approach is one of the ideal strategies for intensive aquaculture where fishes are exposed to the several biotic and abiotic stressors (Kumar et al., 2018). Stressful condition induced by stressors leads to outbreak of infectious diseases and eventually huge economic implications to the aqua-farmers are inevitable. Over and above, increased consumer awareness in human health, welfare, and food safety concerns is forcing the aqua industry to find alternatives to antibiotics, growth promoting drugs, vaccines, and so forth which is unsustainable and impart negative impact on the ecological system (Wang et al., 2015). Hence, the research trends on the use of immunostimulating prebiotics have gained significant attention in the last twenty years due to its cost effectiveness and ecofriendly use (Douxfils et al., 2017). The role of prebiotics to boost non-specific defense mechanism, enhance growth performance and disease resistance were well documented in various aqua culture (Hoseinifar et al., 2015). The efficiency and efficacy of these natural prebiotics is dependent on the fish size, dose, duration and mode of administration (Hoseinifar et al., 2015). It has been proposed that prebiotics activate the innate immune system by interacting with pattern recognition receptor (PRRs) in the form of microbe-associated molecular patterns (MAMPs) (Hoseinifar et al., 2015; Brown et al., 2002). This ligand-receptor interaction activates signal transduction molecules such as NF-kB, that stimulate the expression of immune cells (Yadav and Schorey, 2002).

The common prebiotic evaluated for beneficial health promoting impact in fish so far is mannan-oligosaccharides (MOS) and fructo-

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oligosaccharides (FOS) (Hoseinifar et al., 2014). Out of common fructans such as inulin and levan, levan is very promising prebiotic consisting of β-d-fructofuranosyl residues and linked predominantly through β-(2,6), with branching through β-(2,1) linkages (Kang et al., 2009). Levan is produced extracellularly from sucrose-based substrates by a variety of bacteria, including the genera *Acetobacter, Bacillus, Erwinia, Gluconobacter, Halomonas, Microbacterium, Pseudomonas, Streptococcus, Zymomonas* and shown to have multifaceted properties for its use in multiple industries (Oner et al., 2016). Application of levan in aquaculture has been studied to augment the growth performance and enhanced non-specific immune response in terms of various parameters (Rairakhwada et al., 2007; Gupta et al., 2013; Huang et al., 2015). The capacities to modify gene expression and modulate immune system with incorporation of dietary prebiotics have been demonstrated in fishes (Zduńczyk and Pareek, 2009). Furthermore, expression profile of few immune responsive cytokine genes after prebiotic feeding have been documented in few species (Lokesh et al., 2012; Biswas et al., 2012; Cerezuela et al., 2013). But so far, none of the study has revealed the molecular mechanism behind the immune-protection of prebiotics especially levan and so research efforts in this direction is still under progress.

Cytokines are small specialized proteins molecules that are secreted to deliver enhanced immune response by multiple signaling pathways, which profusely contribute to the elimination of invading pathogens at the site of entry (Reyes-Cerpa et al., 2013). Therefore data generated on expression profile of cytokines genes in infected rohu post dietary levan feeding can be of great significance for the development of potential adjuvant for aquaculture (Sahoo and Sakai, 2010). Out of various cytokines, expression of pro and anti-inflammatory interleukin (IL) and tumor necrosis factors (TNFs) needs to be dealt with insight for its use as potential immunological biomarkers in infected fish (Biswas et al., 2012). IL-1β and TNF-α are the earliest expressed pro-inflammatory cytokines, produced mainly by monocytes and macrophages and regulate multiple aspects of the immune response (Denis and Archambault, 2001). IL-1β gets activated upon microbial invasion and tissue injury while TNF-α is involved in systemic inflammation and regulation of immune cells (Low et al., 2003; Subramanian et al., 2007). IL12p40 also known as IL-12β acts as critical regulator of Th1 type immunity (Pérez-Cordón et al., 2014). An anti-inflammatory cytokine interleukin-10 (IL-10) plays key suppressive role for the inhibition of many pro-inflammatory cytokines (Zou and Secombes, 2016).

Majority of the dietary immunostimulants or prebiotics effects on the expression of cytokines are mainly concentrated in spleen and head kidney following bacterial challenge (Douxfils et al., 2017). However, study pertaining to response of other lymphoid organs like intestine, gill and liver is very limited, which is absolutely essential to discovery organ/tissue dependent modulation with specificity of time points. Moreover, the *Aeromonas hydrophila*, persists in the visceral organs like kidney, liver and so forth even after several days' post infection (Geay et al., 2015). Because of this consistent persistency of pathogen, the modulation in the cytokine expression can be observed. Nevertheless, in the intestine, incorporation of prebiotic as feed additive modulates the expression of different cytokines due to presence of gut associated lymphoid tissues (GALT). Upon exposure of infection various immune cells of GALT work in unison to impart key role in elicitation of immune response (Inami et al., 2009; Vallejos-Vidal et al., 2016).

Labeo rohita (rohu) is one of the widely cultivated freshwater fish and highly relished in India. *A. hydrophila* is a common fish bacterial pathogen which is reported to cause a mass mortality in the Indian subcontinent (Gupta et al., 2008, 2015). Use of antibiotics like terramycin, oxytetracycline, sulphonamide etc. to eradicate this pathogen have been in vogue but, not found to be ecologically safe and environmentally sound. Therefore, boosting the innate immune responses through dietary levan incorporation could be an effective and sustainable approach in this direction. In view of the above information, further experimentation with standardised dose of levan could elucidate the molecular mechanisms of the expression of inflammatory cytokine genes at different time points in lymphoid organs. Expression of immune responsive genes will serve as health biomarkers of fish immune response. Therefore in the present study the pro-inflammatory (IL-1β, TNF-α andIL-12p40) and anti-inflammatory (IL-10) cytokines in the intestine, gill, kidney and liver were evaluated using Real-time PCR at different time intervals in a levan fed *A. hydrophila* infected *L.rohita*.

2. Materials and methods

2.1. Levan production, isolation and characterization

The bacterium *Acetobacter xylinum* (NCIM 2526) was obtained from National Chemical laboratory (NCL), Pune, India. The microbial levan was produced, recovered and characterized from the *A. xylinum* according to the optimized conditions of Srikanth et al. (2015a),b). Briefly, 5% v/v (1.2×10^3 no. of cells mL^{-1}) of seed culture was inoculated into the sucrose, 60.71 gL^{-1}containing medium at pH 6.77 and incubated at 28 °C for 122 h at 120 rpm. The cell-free supernatant obtained was heated to 80 °C for 5 min., pH was adjusted to 10 using 1 M KOH and 1% $CaCl_2$ was added to precipitate and remove the proteins. This was followed by the addition of cold ethanol in the ratio of 1:1 to induce the levan precipitation. The precipitated levan was recovered by centrifugation and washed with cold ethanol, dried, weighed and stored in vacuum dryer (Srikanth et al., 2015a,b).

The isolated levan was purified and the molecular weight was determined using high pressure size exclusion chromatography equipped with refractive index detector. Briefly, the column was equilibrated by 100 mM NaOH with a flow rate of 0.5 mL min^{-1} at 25 °C. The sample was eluted with 0.1 M $NaNO_3$. Commercial dextrans of 1–200 kDa were used as calibration standards. Potassium ferricyanide (PF) test and HPLC of hydrolyzed levan were conducted to confirm the presence of monomer in levan. Further, FTIR spectroscopy, ^1H and ^{13}C NMR were performed to characterize the functional groups, proton carbon finger print (Ahmed et al., 2014; Srikanth et al., 2015a, 2015b).

2.2. Experimental animals and design

L. rohita fingerlings were procured locally from state fisheries department, (Doranda, Ranchi, Jharkhand, India) with an average weight of 4.45 ± 0.48 g. The fish were quarantine with prophylactic dip in salt solution (1%) for 5 min and then acclimatized in the fiber reinforced plastic (FRP) tanks (Circular, 1000 L) for a period of 20 days prior to experiment. Aeration was provided throughout the experiment with compressed air pump. The fish were fed with practical diet (30% crude protein) at the rate of 3% of their body weight twice daily. One hundred fifty fingerlings of L. *rohita* were randomly distributed in two treatment groups each with three replicates following a completely randomized design. The fingerlings were fed to satiation and the feeding trial was conducted for 60 days. The uneaten feed and faecal matters were removed by siphoning out about 50% of the tank water on alternate days with care to avoid stress to the test organisms. All water quality parameters (dissolved oxygen: 6.18–6.82 mg L^{-1}; ammonia: 0.03–0.06 mg L^{-1}, pH: 7.26–7.62; temperature: 23.8–26.8 °C) were maintained in the normal range for rearing of L. *rohita*.

2.3. Feed and feeding

Two experimental diets were prepared with microbial levan and without microbial levan (control) (Table 1). Dry ingredients such as soyabean meal, fish meal (protein source) wheat flour, (carbohydrate source) were weighed and mixed with water to make dough followed by cooking in an autoclave at 15 psi for 20 min. After cooling, vitamins and minerals were added. Required quantities of microbial levan, dissolved in oil (sunflower oil and cod liver oil as lipid sources) were also added. Finally, the dough was pressed through a hand pelletizer to gain