Fish & Shellfish Immunology 34 (2013) 254–264

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Azadirachta indica (neem) leaf dietary effects on the immunity response and disease resistance of Asian seabass, *Lates calcarifer* challenged with *Vibrio harveyi*

Allah Dad Talpur [a,b,*], Mhd Ikhwanuddin [b]

[a] Department of Fisheries, Government of Sindh, Pakistan
[b] Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, Kuala Terengganu, Terengganu, Malaysia

ARTICLE INFO

Article history:
Received 28 June 2012
Received in revised form
26 September 2012
Accepted 2 November 2012
Available online 20 November 2012

Keywords:
Immune response
Intraperitoneally
Phagocytic activity
Dietary
Survival

ABSTRACT

The present study was aimed to address the possible evaluation of *Azadirachta indica* (neem) leaf-supplemented diets on innate immune response in Asian seabass, *Lates calcarifer* fingerlings against *Vibrio harveyi* infection. Fish were fed for two weeks diets containing six graded levels of neem leaf at 0 g, 1 g, 2 g, 3 g, 4 g and 5 g per kg feed. Fish fed neem leaf-supplemented diet displayed significant differences ($p < 0.05$) in weight gain, specific growth rate (SGR) and feed conversion ratio (FCR) compared to the control group fed without neem leaf-supplemented diet. Various innate immune parameters were examined pre-challenge and post-challenge. Fish was injected intraperitoneally with a lethal dose of *V. harveyi* containing 10^8 cells mL^{-1}. Supplementation of neem leaf diet significantly increased phagocytic activity, superoxide anion production, serum lysozyme, serum bactericidal activity, serum anti-protease activity throughout the experimental period when compared with the control group. Dietary doses of neem leaf diet significantly influenced the immune parameters, haematological parameters and blood biochemical indices of treated fish. The results suggested that fish fed neem leaf-supplemented diet improved the immune system and increased survival rate in *L. calcarifer* fingerlings against *V. harveyi* infection.

1. Introduction

The Asian seabass, *Lates calcarifer* is an eminent commercial fish species for aquaculture. The culture of *L. calcarifer* in marine net-cages is a popular aquaculture activity worldwide including in Malaysia. The culture of *L. calcarifer* in marine net-cages has suffered due to bacterial infections particularly by the occurrence of vibriosis caused by *Vibrio harveyi*, which results in heavy losses and causes economic loss to fish farmers [1]. Vibriosis due to *Vibrio* sp. is one of the main bacterial diseases in mariculture systems [2]. Vibriosis caused by *V. harveyi*, a halophilic Gram-negative bacterium that is known to cause disease to fish, shrimp and shellfish either in the culture systems or in the wild aquatic environments [3]. Vibriosis owing to infection of *V. harveyi* is responsible for anorexia, darkening of the whole body of the fish, haemorrhagic ulcers on the mouth or skin surface, tail and fin rot, muscle's focal necrotic lesions and swollen intestine, and eye opacity [4,5]. In general, young fish are more susceptible to infection followed by severe mortality [5]. Intraperitoneal injection of *V. harveyi* at 5.0×10^4 cfu mL^{-1} to *L. calcarifer* fingerlings demonstrated high virulence resulting in100% mortality within two days of post-challenge [1]. The control of infections due to *V. harveyi* could facilitate effective surveillance and prevention of the disease in aquaculture. Conventionally, the prevention of disease outbreaks in aquaculture systems are usually attempted by using antibiotics or disinfectant chemicals [6]. Antibiotics used in aquaculture have problems including toxicity, cost and governmental restrictions [7] and frequent use of antimicrobial drugs, pesticides, and disinfectants in aquaculture has led to drug-resistant microbes [8,9]. In fact, antibiotic resistance displayed by microbial pathogens has encouraged more environment-friendly approaches to screening of plants for their potential antimicrobial activity to control disease [10,11]. In particular, infections caused by *V. harveyi* in fish could be controlled through immunostimulant plants as feed additive [12]. Bricknell and Dalmo [13] defined the immunostimulant as "a naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens". *Azadirachta indica* known as Margosa or neem, is an evergreen tree of potential medicinal value found in most tropical countries [14]. Many authors have

* Corresponding author. Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia. Tel.: +60 16 950 2627; fax: +60 9 668 3390.
E-mail address: mrtalpur@yahoo.com (A.D. Talpur).

1050-4648/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fsi.2012.11.003

reported that the neem possesses significant antibacterial [15–17] antifungal [18] and antiviral properties [19]. Continuing with the beneficial effects of neem, it has been considered to have broad-spectrum prophylactic and therapeutic functions, as well as significant modulating effect on the humoral and cell-mediated immune system [20]. In addition, the neem has been reported to have anti-inflammatory, anti-oxidative activity, hepato-protective, cancer chemo-preventive potential [21–24] and to be effective as an anti-diabetic agent in animals [25,26].

Food plays a vital role in fish growth and health, therefore the pertinent feed additive, which has a potent effect against microbe pathogens, has become very important for feed formulations. The neem has been recognised for its potential broad-spectrum prophylactic and therapeutic role; however, there have been no reports on the neem-supplemented diet to control disease caused by Vibriosis or other opportunistic pathogens in *L. calcarifer*. Based on assumption that the neem may act as an immunostimulant against pathogenic infection in *L. calcarifer*. The present investigation was carried out to evaluate the effects of dietary supplementation of neem leaf on the innate immune responses, haematological and biochemical indices of blood/serum of *L. calcarifer* and disease resistance against *V. harveyi* infection.

2. Materials and methods

2.1. Seabass fingerlings

L. calcarifer fingerlings of average weight 18 ± 2 g were procured from a local hatchery in Malaysia. The health statuses of fish were examined instantaneously upon arrival. Fish were quarantine bathed in 10% formalin for 20 min and were acclimatised for 20 days in 1000 (L) disinfected chlorine-free sea water (salinity 20 ppt). Fish were fed with basal diet at 5% of body weight twice a day in two equal parts at 9.00 a.m and 4.00 p.m. Water exchange was done daily at a rate of 50% and water quality was monitored daily throughout the experiment. Temperature was maintained at 28 ± 1 °C, dissolved oxygen concentration >6.0 mg L^{-1}, pH 8.0 ± 0.2 and salinity 20 ± 1 ppt.

2.2. Neem leaves

Fresh neem leaves were collected from the neem in the surrounding area of the universiti Malaysia Terengganu, Gong Badak, Kuala Terengganu, Malaysia. Leaves were washed with fresh tap water and were dried under shade. Leaves were milled into powdery form and then kept in a dry, clean, airtight jar before being added to diet.

2.3. Preparation of experimental diets

Ingredients of basal diet are shown in Table 1. Proximate composition of the basal diet (local feed) comprised crude protein 41.6%, lipids 17.12%, ash 14.6%, moisture 9.7% and fibre 3% [12].

Six feed groups were made for experimental trials, neem leaf-supplemented (NLS) diet was obtained by incorporating the neem leaf powder at levels of 0 g, 1 g, 2 g, 3 g, 4 g and 5 g/kg feed for T0, T1, T2, T3, T4 and T5 respectively. Water was added and the feedstuffs of basal diet mixed mechanically in (Hobart D300T) for 20 min at a low speed to assure the homogeneity of the ingredients. Pellets were then prepared using a pellet machine (GZL pellet mill, China). The pellets were air dried at ambient temperature under flow hood for 24–48 h, and were stored in labelled screw-cap airtight containers at room temperature.

Table 1
Percentage addition of ingredients for formulated basal diet.

Ingredients	Percent incorporated
Fish meal	50
Wheat meal	25
Soybean meal	15
Fish oil	5
Vitamins and minerals (pre mixture)	2
Cornstarch	3

2.4. Experimental design

Healthy fingerlings of *L. calcarifer* ($n = 180$) were selected for the experimental use and equally distributed into six experimental groups based on feed application following a complete randomised selection using 300 L round tanks filled with disinfected sea water (20 ppt) up to 250 L and equipped with aeration. Control fed with basal diet without NLS diet, treated groups were fed NLS diet for two weeks (15 days) at 1 g, 2 g, 3 g, 4 g and 5 g/kg of feed during pre-challenge. After challenge fish were fed NLS diet for two weeks (15 days) at same ratio as above and control fed with basal diet without NLS diet. Experimental trials were carried out in duplicate. One group was used for challenge assay and other group for weight gain, specific growth rate (SGR) and feed conversion ratio (FCR). The growth performance including percentage weight gain, specific growth rate (SGR) and feed conversion ratio (FCR) for each group was determined as described by Choudhury et al. [29].

Wt. gain% = final wt - initial wt/initial wt × 100
SGR = log of final wt-log of initial wt/no. of days
FCR = feed given (dry wt)/body wt × gain (wet wt)

2.5. Pathogen bacteria

V. harveyi was isolated from *Portunus pelagicus* (Linnaeus, 1758) larviculture, tested for pathogenicity [27,28] and used in this study as according to Talpur and Ikhwanuddin [12]. Briefly, pathogen was grown in marine broth (Merck, Germany) prepared with sea water for 24 h at 37 °C. Culture broth was centrifuged at 13,000 × g for 10 min. The supernatants were discarded and the bacterial cells were washed twice in sterilised sea water, and the pellets were resuspended in sterilised sea water for experimental use. The concentration was adjusted by means of optical density to 0.9 at $OD_{630\ nm}$, which corresponded to 10^8 cells mL^{-1}.

2.6. Challenge assay

After feeding fish with NLS diets for two weeks (15 days), randomly 15 fish from subgroups including control were

Table 2
Growth parameters of *L. calcarifer* fingerlings fed on different levels of neem leaf-supplemented (NLS) diets for 15 days.

Gram of neem leaf diet/kg feed	Weight gain %	SGR	FCR
0	62.2 ± 4.47	1.4 ± 0.2	1.76 ± 0.34
1	69.9 ± 3.01[a]	1.6 ± 0.1[a]	1.75 ± 0.13[a]
2	78.7 ± 3.84[a]	1.8 ± 0.3[a]	1.74 ± 0.22[a]
3	89.2 ± 3.41[a]	1.9 ± 0.1[a]	1.65 ± 0.32[a]
4	92.3 ± 4.25[a]	2.0 ± 0.6[a]	1.65 ± 0.32[a]
5	94.3 ± 6.35[a]	2.1 ± 1.0[a]	1.67 ± 0.42[a]

Data expressed as mean ± SE, $p < 0.05$, $n = 15$. SGR, specific growth rate; FCR, feed conversion ratio.
[a] Values in columns were significantly different ($p < 0.05$) from control.