Fish & Shellfish Immunology 46 (2015) 678–685

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Assessment of the immune-modulatory and antimicrobial effects of dietary chitosan on Nile tilapia (*Oreochrmis niloticus*) with special emphasis to its bio-remediating impacts

Nermeen M. Abu-Elala [a, *], Samah H. Mohamed [b], Manal M. Zaki [c], Alaa Eldin Eissa [a]

[a] Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Cairo University, Giza 11221, Egypt
[b] Department of Immunology, Animal Health Research Institute, Dokki, Giza, Egypt
[c] Department of Animal and Poultry Hygiene, Faculty of Veterinary Medicine, Cairo University, Giza 11221, Egypt

ARTICLE INFO

Article history:
Received 20 May 2015
Received in revised form
2 August 2015
Accepted 5 August 2015
Available online 7 August 2015

Keywords:
Chitosan
Immunostimulant
Innate immunity
Bio-remediation
Nile tilapia

ABSTRACT

Fish, pathogen and environment are three counterparts who are sharing the same circle of life. To keep fish up to their optimal health, environment should be competently improved and pathogen count/virulence should be seized. Using of bioactive immunostimulants to achieve these objectives is the hypothesis under assessment. Thus, the present study was performed to evaluate the use of shrimp shells derived chitosan as an immunostimulant as well as preventive regime against *Aeromonas hydrophila* infection of Nile tilapia and to assess its antibacterial/aquatic bio-remediating effects. Results achieved by feeding 1% chitosan as preventive/therapeutic regimes have revealed a remarkably enhanced several innate immunological parameters (e.g., Phagocytic activity/index, NBT, Lysozyme activity and ACH50), increased resistance against *A. hydrophila* and strikingly improved water quality compared to the 0.5 and 2% chitosan containing diets. Conclusively, experimental results suggest the commercial usage of chitosan as an efficient immunostimulant and bio-remediating agent in aquaculture.

1. Introduction

Nile tilapia (*Oreochromis niloticus*) is one of the most important commercial freshwater fish worldwide due to high growth, desirable taste and high market value [1]. The intensive fish production has been seriously impacted by the intrusion of numerous bacterial pathogens which represent the larger sector of infectious agents jeopardizing both fish as well as their consumers [2]. The rising global ban of antibiotic usage and lack of an efficient vaccine strategy have forced the fish aquaculturists to seek alternative secured methods for disease management. Probiotic, prebiotic, synbiotic, herbal extracts, acidifiers and immunostimulants have been widely applied with reasonable degrees of success [3].

Immunostimulants are a group of biological/synthetic substances that stimulate the cellular and humoral non-specific defense mechanisms [4]. Chitosan is a natural non-toxic immunostimulant derived from the process of de-acetylation of chitin, a major shell component of crustaceans such as crab, shrimp and, crawfish [5]. Chitin and chitosan have received considerable attention for their commercial applications in the biomedical, food, and chemical industries [6]. Currently, chitosan has attracted interest of the aquaculture sector. It possesses unique properties, including low-toxicity, biocompatibility, and low-cost and good handling properties [7]. It demonstrates marked anti-bacterial activities against wide range of bacteria [8]. Anas et al. [9] demonstrated the antibacterial activity of chitosan against 48 isolates of Vibrio species from prawn larval rearing systems. It has been used as immunostimulant to protect salmonids against bacterial disease [10,11], common carp against *Aeromonas hydrophila (A. hydrophila)* [12], rainbow trout against *Aeromonas salmonicida* and *V. anguillarun*, brook trout against *A. salmonicida*, yellowtail against *Pasteurella piscicida*, white shrimp against *Vibrio alginolyticus* and Kelp Grouper against *V. alginolyticus* [5,13].

Chitosan improves the cellular and humoral immunity of different fish species through enhancing the respiratory burst, phagocytosis, alternative complement, lysozyme activity and serum antibacterial peptides activity in different fish species like, gilthead sea bream, *Sparus aurata*, white shrimp, *Litopenaeus vannamei*, olive flounder, *Paralichthys olivaceus*, cobia, *Rachycentron canadum* and *Cyprinus carpio* [13–18]. It also regulates antioxidant

* Corresponding author.
E-mail address: Nermeen_abuelala@cu.edu.eg (N.M. Abu-Elala).

http://dx.doi.org/10.1016/j.fsi.2015.08.004
1050-4648/

enzyme activities and reduces lipid peroxidation [19]. Chitosan-coated diets are thought to reduce water pollution by increasing floating time and reducing the rate of pellet collapse [16].

Motile Aeromonas Septicemia (MAS) caused by *A. hydrophila* is the most common bacterial disease affecting cultured tilapia. The utilization of antimicrobials applies additional pressure on the microbial world and triggers the natural emergence of bacterial resistance [20]. Moreover, the heterogencity of the Aeromonas strains aborts the production of commercial vaccine. Thus, the present study was performed to evaluate the use of chitosan as an immunostimulant and a possible alternative preventive measure against *A. hydrophila* and to assess its beneficial antibacterial, bioremediation and immunomodulatory effects during the disease management.

2. Material and methods

2.1. Fish samples

One hundred and eighty apparently healthy *O. niloticus* (mean individual weight of 40.12 ± 4.25 g) were obtained from a private fish farm. Fish were acclimated to the laboratory conditions for two weeks before starting the experiment. The experimental fish were fed to satiation twice daily. All institutional and national guidelines for the care and use of aquatic animals were followed. The experimental fish were kept in well aerated 12 glass aquaria (80 cm × 30 cm × 40 cm) supplied with de-chlorinated tap water. The photoperiod was 12 h light/12 h dark. The water temperature was maintained at (24 ± 1 °C).

2.2. Preparation of chitosan

The shrimp shells were deproteinized with 3.5% (w/w) NaOH solution for 2 h at 65 °C, and demineralized with 1N HCl for 1 day at ambient temperature and subsequently decolorized with acetone for 2 h at 50 °C and dried for 2 h at ambient temperature. The removal of acetyl groups from the prepared chitin was achieved by mixing with NaOH (50%) with stirring for 2 h at 115 °C in a solid to solvent ratio of 1:10 (w/v). The resulting chitosan was washed till neutrality in running tap water, rinsed with distilled water, filtered, and then dried at 60 °C for 24 h [21].

2.3. Fish diet and experimental design

Commercial fish pelletized food (28% protein) was purchased from a private company for animal feed production. The dry pellets were covered with coating solution (1% tamarind gum and 1% gelatin) mixed with different chitosan concentrations in treated groups and 0% chitosan in control group [16,22]. In this study, two trials were conducted Table 1. First trial, fish were divided into four groups (forty five fish/treatment, three replicates/tank). One served as control group fed on basal diet, and the other three groups fed on chitosan at concentrations 0.5%, 1% and 2%. The fish groups were fed to satiation twice a day for three weeks. Second trial, the control group was divided into two groups (fifteen fish/group), one serve as control positive (G1) *I.P.* inoculated with *A. hydrophila* and the second was challenged with *A. hydrophila* and fed on 1% chitosan (G2) (as therapeutic regime). In the same time fifteen fish pre-treated with 1% chitosan obtained from the first trial (G3) was challenged with *A. hydrophila* (as preventive regime). The fish groups; G2 and G3 were continuously fed on 1% chitosan for one week post challenge.

2.4. Immunological assays

2.4.1. Sample collection

At the last day of the first trial, blood samples were collected from the caudal vessels onto 100 IU/ml sodium heparin to estimate the cellular non-specific immunological parameters. The other humoral parameters were assessed depending on the serum samples. Second set of blood and serum samples were collected again after the second trial.

2.5. Cellular innate immune response

2.5.1. Phagocytic assay

Five heparinized 3 ml volume blood samples/replicate were carefully overlaid onto an equal volume of a histo-paque medium (1.077 g/ml, Sigma–Aldrich, MO, USA) on a polystyrene tube. The sample was centrifuged at 1500 × g for 20 min at 4 °C for separation of viable leucocytes from the peripheral blood. The leukocytes at the interface were collected and washed twice with RPMI-1640 medium supplemented with 100 IU/ml penicillin and 1 mg/ml streptomycin and adjusted to 4×10^7 ml-1 using the culture medium. The phagocytic activity was adapted from the method described by Esteban et al. [14] with slight modification. One ml of the cell suspension was placed onto a 1 ml volume of a 1×10^6 *S. serevisae* suspension and incubated at 37 °C for one hour. Ten μl of the mixture were spread onto the clean slide and stained with Giemsa stain. Under the oil immersion lens of an Olympus CX22 bright-field biological microscope, approximately 200 phagocytic cells were counted.

Phagocytosis percentage = no. of ingesting phagocytes/total no. of phagocytes

Phagocytic index = no. of ingested yeast cells/no. of ingesting phagocytes

2.5.2. Respiratory burst activity (NBT reduction test)

To measure the NBT, peripheral blood leucocytes (1×10^6 cells per well) were incubated with an equal volume of nitroblue tetrazolium 0.2% for 2 h at 28 °C [14]. The supernatants were discarded, and the cells were fixed with 100% (v/v) methanol for 5 min. Each well was washed twice with 125 ml of 70% (v/v) methanol. The fixed cells were allowed to air-dry. The reduced NBT (in the form of the blue precipitate formazan) was dissolved using 120 ml of 2 N potassium hydroxide (KOH) and 140 ml of dimethyl sulphoxide (DMSO, Sigma–Aldrich, St. Louis, MO, USA) per well. The turquoise-blue solution was measured with the enzyme-linked immunosorbent assay, ELISA reader at the wavelength 630 nm.

2.6. Humoral innate immune response

2.6.1. Lysozyme activity

Serum samples were measured using the turbidometric method described by Abu-Elala et al. [23]. A twenty five μl serum was added