Aquaculture 432 (2014) 106–113

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Effect of florfenicol on selected parameters of immune and antioxidant systems, and damage indexes of juvenile *Litopenaeus vannamei* following oral administration

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ARTICLE INFO

Article history:
Received 13 January 2014
Received in revised form 3 April 2014
Accepted 14 April 2014
Available online 29 April 2014

Keywords:
Florfenicol
Litopenaeus vannamei
Immune defense parameters
Antioxidant status
Biomolecule damage

ABSTRACT

Florfenicol (FLR) is a commonly used antibiotic for the treatment of bacterial diseases in white shrimp *Litopenaeus vannamei*. The aim of this study was to investigate the effects of florfenicol (FLR) on immune defense parameters, antioxidant status and biomolecule damage of the white shrimp *Litopenaeus vannamei*. In this study, juvenile shrimp (9.5 ± 0.5 cm) were fed coated with FLR 100 mg kg^{-1} and 200 mg kg^{-1} body weight for 6 days followed by a period of feeding without antibiotic for 10 days. Hemolymph and hepatopancreas were collected on the 3rd and 6th day of feeding the antibiotics and at 1, 3, 6 and 10 days after FLR withdrawal. Results showed there were no significant differences in proPO activity in hemocytes, α_2-macroglobulin (α_2-M) and antibacterial activities in plasma between 100 mg kg^{-1} FLR treatment and control group, while 200 mg kg^{-1} FLR treatment suppressed all of them ($P < 0.05$), and both treatment significantly decreased the total hemocyte count (THC) and phagocytic activity in a dose-dependent manner. Results also showed that 100 mg kg^{-1} FLR treatment significantly increased superoxide dismutase (SOD) activity, while 200 mg kg^{-1} FLR treatment suppressed it. Moreover, FLR had a suppressive effect on antioxidant status of T-AOC, GSH contents and GSH/GSSG ratio. Additionally, FLR can cause biomolecule damage, such as decreased the *F* value and increased the levels of malondialdehyde (MDA) and protein carbonyl (PC) contents. The present results suggest that FLR can affect the physiological health of *L. vannamei*, and these results will provide scientific datas for antibiotic safety evaluation.

1. Introduction

Recently, diseases caused by virus epidemics and vibriosis have become a serious problem causing economic losses for shrimp farming (Chiu et al., 2007; Jayasree et al., 2006). Therefore the development of solutions for improved resistance and survival of shrimp with pathogen infection is crucial to sustain the shrimp culture industry. Chemicals and antibiotics are widely used to prevent or treat such infections (Reboucas et al., 2011), and florfenicol (FLR) is the most commonly used antibacterial drugs in world aquaculture, which is a synthetic drug with chemical structure and spectrum of bactericidal activity similar to chloramphenicol, has been shown to have potent activity against several pathogenic bacteria (Caipang et al., 2009; Fodey et al., 2013).

The use of these antimicrobial drugs in aquaculture has its positive effects. However, several hazards and side effects have been associated with the excessive use of antibacterial drugs, including (1) they have been increasingly the cause of food safety for human consumers. The excessive use of antibiotics in aquaculture results in the presence of residual antibiotics in commercialized aquatic products, which may cause direct human health concerns (Zaki et al., 2011). Due to this reason, legislation in some countries had stipulated Maximum Residue Limits (MRL) for antibiotics and banned some antibiotics used in the treatment of cultured animals; (2) they have a high biological activity and some parental compounds, their metabolites and/or their degradation products damage ecological security, the unintentional consumption of antibiotics is leading to the development of resistant bacterial strains, drug residues in fish farm sediments (Saglam and Yonar, 2009); and (3) their use is expected to further affect the physical health of raised animals. The interaction of drugs with lymphoid tissues may alter the functions and balance of the immune system and induce undesirable effects, such as immunosuppression, uncontrolled cell proliferation, alterations of other host defense mechanisms against pathogens, and even neoplasia (Tu et al., 2010; Yonar et al., 2011). Drugs, such as oxytetracycline, oxolinic acid, and florfenicol, have been associated with immunosuppressive effects and oxidative stress in carp and rainbow trout (Caipang et al., 2009; Lundén et al., 2002; Yonar, 2012).

Although FLR was approved by the FDA for use in shrimp aquaculture, and it has potential use in the treatment of vibriosis and necrotizing hepatopancreatitis infections in farm-raised shrimp including the white shrimp *Litopenaeus vannamei* (Wang et al., 2013), little effort

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http://dx.doi.org/10.1016/j.aquaculture.2014.04.024
0044-8486/

was made to study the effects of FLR on the physiological health of cultured shrimp particularly concerning the immune and antioxidant defense systems. Most of the scientific studies are focusing on the development and validation of technologies and methodologies allowing straight forward traceability of individual aquaculture animals to ensure food safety for human consumption. However, there are very few articles reporting on the effects of antibiotics on aquatic farmed shrimp.

Like other invertebrates, shrimp lack adaptive immune system and only rely on various innate immune responses to fight against invading pathogens, including phagocytosis, encapsulation, prophenoloxidase activating system (proPO system), antimicrobial peptides (AMPs), lysozyme and other components such as proteinase inhibitors (Söderhäll and Cerenius, 1992). Due to its complexity, the shrimp immune system is extremely vulnerable to xenobiotic stress, and there is evidence that xenobiotic can impact immune function in *L. vannamei* (Tumburu et al., 2012). Growth, disease and survival of an organism are partly determined by the capability of the immune system (Blaise et al., 2002), therefore, immune function is important in assessing sublethal effects of contaminant exposure (Luengen et al., 2004). Immunosuppressive effects of FLR have been studied in a vast number of fish species (Saglam and Yonar, 2009; Yonar et al., 2011), and related studies on shrimp are much needed to fill the gaps.

Reactive oxygen species (ROS) are produced when an organism is subjected to xenobiotics in their aerobic metabolic pathways (Livingstone, 1991). Xenobiotic-enhanced oxyradical generation is one possible mechanism of pollution toxicity (Gómez-Mendikute and Cajaraville, 2003). Organisms can adapt to increasing ROS production by upregulating antioxidant defenses, such as the activities of antioxidant enzymes (Livingstone, 2003). But failure of antioxidant defenses to detoxify excess ROS production can lead to significant oxidative damage to tissue macromolecules including DNA, proteins and lipids (Halliwell and Gutteridge, 1999). Most cells have protective mechanisms to balance ROS production and avoid oxidative stress, namely antioxidants. Antioxidant defenses include a variety of non-enzymatic molecules and enzymes that scavenge ROS. There are several reports on the effects of different antibiotic drugs on oxidative stress in aquatic invertebrates, for example, Caipang et al. (2009) reported that oral administration of florfenicol modulates the immune response and antioxidant defense in Atlantic cod and these may, in turn, affect their ability to resist bacterial pathogens. With respect to shrimp, most of these studies have evaluated the effects of pollutant or environmental parameters on "oxidative stress" (Chang et al., 2009). However, very few have reported the effects of antibiotics on antioxidant defenses.

The aim of the present study was to investigate the possible effects of FLR on immune response parameters, antioxidant status of *L. vannamei* juveniles following oral administration. We also looked at oxidative damage to DNA, lipids and proteins to evaluate oxidative stress status by measuring respectively DNA damage (DNA strand breakage), which is one type of oxidative damage and has been used as a biomarker to assess the genotoxicity of pollutants (e.g. Benzo (a) pyrene, Aroclor 1254) to marine organisms (Ching et al., 2001), malondialdehyde (MDA), which is a commonly used indicator to evaluate lipid peroxydation, and carbonyl proteins, which are recently being used as biomarker of oxidative damage to protein in fish (Parvez and Raisuddin, 2005). In addition, the significance of the study was to offer a potential practical approach for health management and the potential use of biomarkers for assessing the physical health evaluation on shrimp.

2. Materials and methods

2.1. Experimental organisms

Healthy *L. vannamei*, averaging 9.5 ± 0.5 cm in body length, were obtained from a commercial farm in Nanshan, Qingdao, China. The shrimp were acclimated in tanks containing aerated seawater (salinity 31‰, pH 8.2) at 25 ± 0.5 °C for one week prior to the experiment. During the acclimation period, one third to half of the water in each tank was replaced twice daily and the shrimp were fed with a formulated shrimp diet daily. Only apparently healthy animals at the inter-molt stage were used for the study. The molt stage was decided by the examination of uropoda in which partial retraction of the epidermis could be distinguished.

2.2. Oral administration

The amounts of 20 and 40 g FLR for per kg shrimp weight were mixed with feed and given to the shrimp manually at a rate of approximately 0.5% shrimp body weight per day, and the FLR treatments were 100 mg kg^{-1} body weight (BW) (the manufacturer's instructions for treating with shrimp bacterial diseases) group and 200 mg kg^{-1} body weight (BW) group. The drugs were coated onto the surface of the pellets using capelin oil (Samuelsen and Bergh, 2004). After a week of conditioning, the shrimp were fed twice daily for 6 days with the medicated feeds.

2.3. Sample collection

Shrimp (n = 8) from both the antibiotics-fed and non-fed (control) groups were sampled at 3 and 6 days of feeding with the antibiotics and at 1, 3, 6 and 10 days post-withdrawal. Only shrimp in the intermolt phase were used. This was to minimize internal variations, and because changes in physiological functions are generally observed during the molting phase in crustaceans (Bonilla-Gómez et al., 2012). Hemolymph (200 μL) was withdrawn from the ventral sinus at the base of the first abdominal segment of each shrimp into a 1 mL sterile syringe (25 gage needle) containing an equal volume of anticoagulant solution (30 mM trisodium citrate, 0.34 M sodium chloride, and 10 mM EDTA-Na_2, at a pH of 7.55 with osmolality adjusted to 780 mOsm kg^{-1} using 0.115 M glucose) (Liu and Chen, 2004). The anticoagulant-hemolymph of 8 shrimp was pooled and gently mixed in a sterile Eppendorf tube immediately. Then a small part was used to count the hemocyte and to analyze the phagocytic activity of hemocyte immediately; the remainder was centrifuged at 800 ×*g* for 10 min at 4 °C, and the supernatant fluid was dispensed into 2.0 mL Eppendorf tubes as plasma samples and stored at −80 °C for analysis of other immune and antioxidant parameters. After the plasma sample was prepared, cells precipitated at the bottom of the tube were suspended and rinsed gently by 150 μL shrimp salt solution (SSS, 450 mmol L^{-1} NaCl, 10 mmol L^{-1} KCl, 10 mmol L^{-1} HEPES, pH 7.3) and centrifuged at 800 ×*g* for 10 min under 4 °C. The supernatant was then discarded and the wash process was repeated once. Afterwards, 150 μL SSS was added into the tube and then the cells was broken into pieces for 1 min by Ultrasonic Cell Disruption System with output power at 20 W and duty cycle at 30% in the ice-bath. Finally, the tube was centrifuged at 15,000 ×*g* for 20 min under 4 °C and the supernatant was pipetted into a new tube and stored at −80 °C as the HLS sample.

Meanwhile, the hepatopancreas of the same sampled shrimp was excised, washed in cold normal saline (0.8%, w/v), blotted dry, and flash frozen with liquid nitrogen in a mortar. Then the frozen hepatopancreas of 8 shrimp was pooled, ground, weighed, dispensed into 2.0 mL Eppendorf tubes and stored at −80 °C for antioxidant parameters and biomolecule damage determination. All assays of immune, antioxidant and damage parameters were conducted in triplicate.

2.4. Enzyme analysis

2.4.1. Determination of immune defense parameters

The immune defense parameters are measured by total hemocyte count (THC) in the hemolymph, prophenoloxidase (proPO) activity in the hemocytes, phenoloxidase (PO) activity, and α_2-macroglobulin (α_2-M) activity in plasma, phagocytic activity of hemocyte, antibacterial