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Immune responses and stress resistance in red sea bream, *Pagrus major*, after oral administration of heat-killed *Lactobacillus plantarum* and vitamin C

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ABSTRACT

The present study evaluated the interactive benefits of dietary administration of heat-killed *Lactobacillus plantarum* (LP) and vitamin C (VC) on the growth, oxidative status and immune response of red sea bream (*Pagrus major*). A diet without LP and VC supplements was employed as a control diet. Four other test diets with 0 or 1 g LP kg^{-1} combined with 0.5 or 1 g VC kg^{-1} (2×2 factorial design) were fed to red sea bream (2 ± 0.01 g) for 56 days. A significant interaction was found between LP and VC on final body weight (FNW), weight gain (WG), hematocrit (HCT), serum bactericidal (BA) and lysozyme (LZY) activities, mucus LZY and peroxidase (PA) activities, nitro blue tetrazolium (NBT), catalase, mucus secretion and tolerance against low salinity stress test (LT_{50}) ($P < 0.05$). In addition, FNW, WG, specific growth rate, feed and protein efficiency ratio, serum (BA, LZY, PA and NBT), mucus (LZY and PA), superoxide dismutase, malondialdehyde and mucus secretion were significantly affected by either LP or VC ($P < 0.05$). Furthermore, only LP was a significant factor on survival, plasma total cholesterol, mucus BA and alternative complement pathway ($P < 0.05$). However, VC supplementation affected on HCT and LT_{50}. Interestingly, fish fed with both LP at 1 g kg^{-1} diet with VC at 0.5 or 1 g kg^{-1} diet showed higher growth, humoral and mucosal immune responses, anti-oxidative status, mucus secretion and LT_{50} as well as decreased plasma, triglyceride and total cholesterol levels than the fish fed control diet ($P < 0.05$). These results demonstrated that dietary LP and VC had a significant interaction for red sea bream with the capability of improving growth performance and enhancing stress resistance by immunomodulation.

1. Introduction

In intensive aquaculture systems the farmed fish often subjected to stresses that are above and beyond their capacity of endurance, such as high temperature, crowding, water quality deterioration and the invasion of bacteria and viruses. All these adverse environmental factors might disturb the balance and harmony between fish and the environment, causing stress response in fish and, consequently, affecting production negatively [1]. As a result, fish may develop depressed immune systems and compromised non-specific barrier (e.g. skin), enhancing their susceptibility to different stressors [2]. To reduce stress, the fish industry has been using antibiotics and hormones trying to offer an adequate diet to maintain the health of cultured fish [3]. However, the use of antibiotics represents environmental hazards, spreads pathogen-resistant genes, immunosuppression and limits the efficiency of this treatment [4]. Diverse types of feed additives using dietary supplements of probiotics, prebiotics, β-glucans, vitamin C and immunostimulants may help to reduce the susceptibility of fish to stress and diseases [1,5–8].

Heat-killed *Lactobacillus plantarum* (LP), namely, Immuno-LP20™, is known as probiotics for their health-promoting effects, including enhancement of the growth performance, immune system, stress resistance and reduction of blood lipid concentrations of red sea bream, *Pagrus major* [5,6]. Although probiotics were

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originally defined as live microbial components, non-viable microbes have also been shown to exhibit beneficial effects that are equivalent to, or even greater than, those of live microbes [7,9–11]. The use of probiotics, benefit the host by producing inhibitory compounds, competing for chemicals and adhesion sites, modulating and stimulating the immune function, and improving the microbial balance [9–11]. Several studies have demonstrated that killed probiotics can improve growth performance, feed utilization, disease resistance and immunostimulation of cultured fish species [1,5–8,12–19].

Vitamin C (VC) is one of the best known feed additives and plays an essential role in normal physiological functions and stimulation of the immune response of fish [20]. A number of investigations confirmed the fact that VC plays an important role as water-soluble antioxidant for red sea bream [21–23]. The major line of antioxidative defense system against radicals by VC is to prevent lipid peroxidation in fish plasma [21]. Moreover, VC also promotes beneficial effects on the growth performance, serum bactericidal activity, phagocytic activity, serum complement activity, antibody levels, lysozyme activity, and mucosal parameters of several fish species [20,24–26]. Additionally, previous research findings indicate that dietary supplementation with immunomodulatory vitamins such as VC can improve immune response and disease resistance of a variety of fish species [24,25,27,28]. Until now, the interactive effects of probiotics and VC on the health status, immune response and stress resistance of fish are not documented yet.

The red sea bream, *Pagrus major* is a very important cultured species in East Asia countries, particularly Japan due to its high market value, desirable taste and recent supply shortage. Recently, diseases of cultured red sea bream showed an increasing trend, especially in intensive aquaculture system causes disease outbreak. Therefore, the present study was conducted to clarify the possible interactions between dietary LP and VC on growth performance, blood chemistry profile, immune response and tolerances against stress of red sea bream, and the result would suggest new avenues for the alleviation of stress and prevention of fish diseases.

2. Materials and methods

2.1. Preparation of the experimental diets

Beside the control diet, four diets contained 2 levels (0 and 1 g kg^{-1} diet) of heat-killed *Lactobacillus plantarum* (LP) (House Wellness Foods Corp., Itami, Japan [5,6]) combined with 2 levels (0.5 and 1 g kg^{-1} diet) of vitamin C (VC) (L-Ascrobil-2-phosphate-Mg) were formulated (2 × 2 factorial design) to evaluate combined effects of dietary LP and VC. Brown fish meal and casein were used as protein sources, and soybean lecithin and pollack liver oil were the main lipid sources. All ingredients were thoroughly mixed in a food mixer and dry pelleted in a laboratory pellet mill, through a (1.6–2.1 mm) diameter die. Pellets were dried in an oven at 50 °C for 2 h, and then stored in a freezer in airtight bags until use. Chemical analyses of the diets were performed following the Association of Official Analytical Chemists methods [29]. VC contents of test diets were determined based on the method of Gao et al. [21]. Ingredients and proximate composition of the experimental diets are presented in Table 1.

2.2. Growth trial

The experiment was performed at the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan, with red sea bream (*P. major*) obtained from Tawaki farm, Kumamoto, Japan. The fish were acclimatized for 2 weeks in the laboratory condition and reared in a 500 L tank. During this period, a commercial diet (50% crude protein; Higashimaru, Japan) was supplied to the fish. The trial was run in 15 polycarbonate tanks of 100 L water capacity (26.3 ± 1.2 °C water temperature) in a flow through sea water system. Tanks were equipped with an inlet, outlet, and continuous aeration (1.5–2 L min^{-1}) of filtered seawater (33.3 ± 0.5 g L^{-1} salinity) and dissolved oxygen was kept near saturation (6.1 ± 0.5 mg L^{-1}). Thereafter, 20 fish with an initial mean body weight of 2 ± 0.01 g were distributed to each tank and the experimental diets randomly assigned to triplicate groups. The trial lasted 56 days and during that period fish were fed by hand, twice daily, 7 days a week, until apparent visual satiation. Utmost care was taken to avoid feed losses. At the termination of the experiment, the fish were fasted for 24 h, and then, the total number, individual body weight and length of fish in each tank were measured.

2.3. Calculation of growth, survival and feed utilization

Body weight gain (WG), specific growth rate (SGR), survival (SUR), feed efficiency ratio (FER), protein efficiency ratio (PER) and condition factor (CF) parameters were calculated using the following equations:

$$WG\ (\%) = (W_0 - W_f) \times 100/W_0;\ SGR\ (\%\ day^{-1}) = \{(Ln\ (W_f) - Ln\ (W_0))/t\ (56\ days)\} \times 100;\ SUR\ (\%) = 100 \times (N_f/N_0)$$

Where W_0 and W_f were initial and final body weight (g) of fish, respectively; t was duration of experiment in days; N_f and N_0 were initial and final number of fish, respectively.

$$FER = \text{live weight gain (g)/dry feed intake (g)}$$
$$PER = \text{live weight gain (g)/dry protein intake (g)}$$
$$CF\ (\%) = \text{weight of fish (g)/(length of fish)}^3\ (\text{cm})^3 \times 100$$

2.4. Plasma, serum and skin mucus collection

Heparinized (1600 UI ml^{-1}, Nacalai Tesque, Kyoto, Japan) syringes were used to collect blood from the caudal vein of 5 fish in each replicate tank (15 fish per treatment) and pooled. Partial heparinized whole blood was used to analyze the hematocrit and nitro blue tetrazolium (NBT) activity while plasma was obtained by centrifugation at 3000 × g for 15 min under 4 °C. The resulting plasma was frozen at −80 °C for the blood chemistry profile analysis. Serum samples were obtained from the caudal vein of another 5 fish in each replicate tank (15 fish per treatment) with non-heparinized disposable syringes. After clotting at 4 °C during 3–4 h, each sample was centrifuged and the serum removed and frozen at −80 °C until use.

Skin mucus samples were collected from red sea bream specimens using the method of Dawood et al. [5,6]. Briefly, skin mucus were collected from dorso-lateral body surface of 5 fish per each replicate tank (15 fish per treatment) by using a small piece of sterilized cotton and immediately suspended in 1 ml of phosphate-buffered saline (PBS, pH = 7.4). Sample was then put into a hand-made set of two centrifugal tubes, the upper tube had a small filter, with which the mucus in the cotton will be collected in the lower tube while centrifuged. The sets of the double-tube (1.5 ml centrifugal tube) were centrifuged at 3000 × g for 5 min under 4 °C (MX-160, Tomy Seiko Co., LTD, Tokyo, Japan), and the supernatant in the under tube was transferred into another centrifugal tube (510-GRD, QSP, San Diego, CA, USA) and kept at −80 °C until the analysis.