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Effects of dietary supplementation of *Lactobacillus rhamnosus* or/and *Lactococcus lactis* on the growth, gut microbiota and immune responses of red sea bream, *Pagrus major*

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ABSTRACT

Pagrus major fingerlings (3·29 ± 0·02 g) were fed with basal diet (control) supplemented with *Lactobacillus rhamnosus* (LR), *Lactococcus lactis* (LL), and *L. rhamnosus* + *L. lactis* (LR + LL) at 10^6 cell g^{-1} feed for 56 days. Feeding a mixture of LR and LL significantly increased feed utilization (FER and PER), intestine lactic acid bacteria (LAB) count, plasma total protein, alternative complement pathway (ACP), peroxidase, and mucus secretion compared with the other groups ($P < 0.05$). Serum lysozyme activity (LZY) significantly increased in LR + LL when compared with the control group. Additionally, fish fed the LR + LL diet showed a higher growth performance (Fn wt, WG, and SGR) and protein digestibility than the groups fed an individual LR or the control diet. Superoxide dismutase (SOD) significantly increased in LR and LR + LL groups when compared with the other groups. Moreover, the fish fed LR or LL had better improvement ($P < 0.05$) in growth, feed utilization, body protein and lipid contents, digestibility coefficients (dry matter, protein, and lipid), protease activity, total intestine and LAB counts, hematocrit, total plasma protein, biological antioxidant potential, ACP, serum and mucus LZY and bactericidal activities, peroxidase, SOD, and mucus secretion than the control group. Interestingly, fish fed diets with LR + LL showed significantly lower total cholesterol and triglycerides when compared with the other groups ($P < 0.05$). These data strongly suggest that a mixture of LR and LL probiotics may serve as a healthy immunostimulating feed additive in red sea bream aquaculture.

1. Introduction

Red sea bream is one of the main marine finfish species cultured in Japan. The demand for red sea bream has grown tremendously for the last decade since it is a high-quality sashimi grade fish with high market value [1,2]. Recently, intensive aquaculture system has been expanded and is emerging as one of the most practical and promising tools to meet the requirements of red sea bream. However, in intensive fish farming, animals are subjected to stress conditions that weaken fish immune systems, leading to increased susceptibility to pathogens [3]. These diseases have resulted in production losses and remain as one of the major cause of concern in fish farms [4]. In recent years, one of the major limiting factors in intensive fish culture, an environmental friendly alternative approach is the use of probiotics. These natural ingredients enhance the immune response of fish, confer tolerance against different stressors, and minimize the risk associated with the use of chemical products such as: vaccines, antibiotics, and chemotherapeutics [4,5].

Probiotics are live microorganisms that could improve digestive functions and promote growth and welfare of fish when consumed in adequate amounts [6]. Several reports suggested that probiotics supplementation can improve the growth, feed utilization, immune

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response, and stress resistance of fish [7,8]. Lactic acid bacteria (LAB) as probiotic supplements have been widely applied to enhance the immunity and disease resistance of fish, most notably *Lactobacillus* spp., *Bacillus* spp. and other Gram-negative bacteria [9–11]. LAB plays a beneficial role in the host gut environment by producing antibacterial substances such as lactic acid, acetic acid, hydrogen peroxide, bacteriocin and to inhibit the increment of harmful intestinal bacteria that suppress growth of competing bacteria [12–14]. At present, LAB as dietary supplements has been widely applied to enhance the immunity and disease resistance of fish [15–18].

Dietary administration of *L.* spp. enhanced the growth and immunity of *Epinephelus coioides* [19], *Epinephelus bruneus* [20], *Rachycentron canadum* [18], *Oreochromis niloticus* [21], *Labeo rohita* [22] and more recently *Pagrus major* [15]. *Lactococcus lactis* has demonstrated growth or health benefit to grouper *E. coioides* [23] and olive flounder *Paralichthys olivaceus* [24,25], however, there remains a distinct lack of literature regarding *L. lactis* for red sea bream. Although use of probiotics has greater potential for higher fish production, in most of the cases, probiotic microorganisms were used as a single species at one or more doses [15,16,26]. Limited literature are available on the use of two or more microorganisms as a probiotic mixture and their effects on growth performances and immunity of fish [3,18,24,27]. Some authors have reported that multi-species probiotic supplementation could be more effective than mono-species or mono-strain ones [28,29], mainly due to the higher probability of a microorganism *consortium* to survive in a changing environment like the gastrointestinal tract (GIT) and to dominate the associate microbiota [30,31], a potential stimulation of the immune system, given the diverse immune stimulation properties of strain specific properties [28,32,33] and a greater variety of antimicrobial properties associated with mixed formulations (*i.e.* production of organic acids, bacteriocins, hydrogen peroxide, biosurfactants, etc) preventing pathogen colonization and prosper in GIT [29].

The literature on the use of two probiotic species at the same time in the diet and their effect on growth, nutrient utilization, gut microbial population and immune response of fish are very limited. Therefore, the present investigation was carried out to evaluate the effects of dietary supplementation of *Lactobacillus rhamnosus* or/and *L. lactis* on the growth, immunity and gut microbiota of red sea bream, *P. major*.

2. Materials and methods

2.1. Probiotic bacteria

L. rhamnosus (LR) used in this study was kindly provided by Morinaga Milk Industry CO., LTD., Kanagawa, Japan, and the concentration of LR in the dry product is 1×10^9 cells g^{-1}. *Lactococcus lactis* (D1813) (LL) was made by Kyushu Medical Company, Fukuoka, Japan, and the concentration of LL in the dry product is 1×10^8 cells g^{-1}. α-Cellulose powder used to adjust to the required concentrations of LR and LL. The bacteria was stored at −20 °C until use.

2.2. Experimental diets and design

All the dietary components were obtained commercially, using brown fish meal and casein as main protein sources, soybean lecithin and pollack liver oil as main lipid sources, dextrin and α-Starch were supplied as the carbohydrate sources, activated gluten was used as a binder to produce pellet diet, and cellulose powder was used to adjust to 100% total proportion. Four experimental diets were formulated to be isonitrogenous (51.8% crude protein) and isolipidic (11.4% crude lipid), containing 0 (control), 1×10^6 cells g^{-1} *L. rhamnosus* (LR), 1×10^6 cells g^{-1} *L. lactis* (LL), or both 0.5×10^6 cells g^{-1} *L. rhamnosus* and 0.5×10^6 cells g^{-1} *L. lactis* (LR + LL) (Table 1). A lyophilized probiotics in powder form of LR or/and LL were added to the basal diet at the expense of α-Cellulose to obtain the levels required. Powdered dietary ingredients were thoroughly mixed for 15 min in a food mixer, then blended oil and water were added to form a soft dough. The dough was then pelleted (without steam injection) using a Pillet Mill with a (1.6–2.1 mm) diameter die. The experiment feed was dried at room temperature and stored in sealed plastic bags at −20 °C until use. New batches of feed were produced every two weeks to keep up LR and LL viability. The viability of the incorporated bacterial cells into feed was assessed by spreading onto triplicate plates of DeMan, Rogosa and Sharpe agar (MRS, MERCK, Darmastadt, Germany). Diet samples were first powdered well and serially diluted with sterile saline [phosphate-buffered saline (PBS, pH = 7.4)]. The agar plate inoculated with each dilution was incubated for 3–5 days at 25 °C. Colony forming unit (CFU g^{-1}) were determined for viable bacterial populations.

2.3. Fish and feeding trial

A total of 240 red sea bream (3·29 ± 0·02 g) were obtained from Akahoshi farm, Kumamoto Prefecture, Japan and transferred to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The fish were acclimatized for 1 week 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. Thereafter, fish were randomly allocated to twelve 100-L tanks (twenty fish per tank and triplicate tanks per treatment) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained under natural light/dark regime. During the experimental period, the monitored water quality parameters (mean ± S.D.) were: water temperature 21.4 ± 1.6 °C, pH 8 ± 0.5, salinity 33.3 ± 0.5 ppt and dissolved oxygen 6.1 ± 0.5 mg L^{-1}. Fish were hand fed to apparent satiation twice a day (09.00 and 16.00 h) for 56 days. Any uneaten feed left was removed after feeding and dried using a freeze drier then subtracted from total feed intake.

At the end of the feeding trial, all experimental fish were fasted for 24 h. The total number, individual body weight and length of fish from each tank were measured. Survival rate and growth parameters were calculated using the following equations:

$$\text{Body weight gain}(\%) = (\text{final weight} - \text{initial weight}) \times 100/\text{initial weight}$$

$$\text{Specific growth rate}\left(\text{SGR}\%, \text{day}^{-1}\right) = \{(\text{Ln}(\text{final weight}) - \text{Ln}(\text{initial weight})) \times /\text{duration}(56\ \text{days})\} \times 100$$

$$\text{Survival}(\%) = 100 \times (\text{final no. of fish}/\text{initial no. of fish})$$

$$\text{Feed intake}\left(\text{FI}, \text{g fish}^{-1} 56\ \text{days}^{-1}\right) = (\text{dry diet given} - \text{dry remaining diet recovered}) \times /\text{no. of fish}$$