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Lactobacillus sakei BK19 enriched diet enhances the immunity status and disease resistance to streptococcosis infection in kelp grouper, *Epinephelus bruneus*

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ABSTRACT

The effect of *Lactobacillus sakei* BK19 (10^8 cells g^{-1}) supplemented diet fed to kelp grouper, *Epinephelus bruneus* against streptococcosis caused by *Streptococcus iniae* and *Streptococcus parauberis* with reference to the innate immune response and disease resistance was evaluated at 1, 2, and 4 weeks. Maximum reduction in mortalities was observed in kelper feeding the probiotic diet for two weeks after challenged with the pathogens when compared to the infected group fed with basal diet; similarly the cellular and humoral immune responses such as head kidney macrophage phagocytic and peroxidase activities, serum lysozyme activity, and total protein levels increased significantly. The results reveal that, in streptococcosis infected kelp grouper feeding *L. sakei* BK19 enriched diet affords a higher level of disease protection due to stimulation of immune system.

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1. Introduction

The subfamily Epinephelinae under family Serranidae comprises groupers which include 159 species in 15 genera; groupers are distributed in the tropical and sub-tropical waters of Southeast Asian countries, including Korea, China, and Japan. Global grouper production has increased dramatically in recent years from 60,774, 99,378, 163,093 to 198,690 metric tons in 1990, 2000, 2005, and 2007, respectively [1]. Groupers fetch a premium wholesale price of up to US$100/kg in the Chinese live-fish markets of Hong Kong and Southern China [2]. Due to an insatiable market demand grouper culture is increasing rapidly; for instance in Southern China the farmed production of orange-spotted grouper has reached 10,000 mt annually [3]. Among the groupers, 20 species are cultured commercially, mainly in Hong Kong, Taiwan and the Southeast Asian region, usually by farming wild juveniles. Recently, culture of kelp grouper *Epinephelus bruneus* started in Korea and Japan with controlled larval production. However, uncertainties in production due to mass mortality in the larval and juveniles stages due to stress associated with environmental factors, nutrition, pathogens, etc impose a serious threat to the sustainability of culture.

In aquaculture, traditionally antimicrobial agents were incorporated in feed to stimulate growth and afford protection from diseases; however due to their extensive use the evolution of antimicrobial-resistance among pathogens [4] and the associated environmental problems have been well documented [5]. Therefore, the need for alternative techniques is increasing; in this regard probiotics may a play a considerable role as immunostimulants and antimicrobial agents. The advantages of probiotics as feed supplement include improved feed value, enzymatic contribution to digestion, inhibition of pathogenic microbes, antimutagenic and anticarcinogenic activity, growth promoting factors, increased immune response [6–14], improved water quality, survival and growth rates [15]. Hence with the increasing demand for environment friendly aquaculture and restrictions imposed against the use of various chemicals and antibiotics the use of probiotics in aquatic animals is increasing [16]. Furthermore, it has been proved that microflora of the digestive tract as partial hydrobionts play an important role in enhancing resistance to infectious diseases [17,18]. The application of microbial probiotics in aquaculture is

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now widely accepted in many countries [6–9,13,14,16,19–21]. Nowadays, a number of preparations with probiotics are commercially available for fish, shrimp, and mollusc farming as feed additives or for incorporation in water [12]. Though the use of different feeding regimes from 1 to 8 weeks is reported to improve growth performance and afford disease resistance in farmed fish [20,22–27], the basis for choosing these periods is often unclear.

In modern intensive aquaculture facilities the Streptococcal infections in fish have become an increasingly important health problem [28]. The main pathogenic species responsible for these infections include *Streptococcus iniae*, *Streptococcus agalactiae* [29], *Streptococcus parauberis*, and *S. dysgalactiae* [28,30,31]. In South Korea also the rapidly expanding aquaculture industry incurs a substantial economic loss due to streptococcosis caused by *S. iniae*, *S. parauberis*, and *Lactococcus garvieae* resulting in more than 50% mortality over a period of 3–7 days [32–37]. This study is the first report in *E. bruneus* fed with *Lactobacillus sakei* BK19 supplemented diet to protect and enhance the immune response from streptococcosis caused by *S. iniae* and *S. parauberis*.

2. Material and methods

2.1. Fish

Kelp grouper, *Ephinephelus bruneus* (weight 8.7 ± 1.3 g, $n = 250$) obtained from Dongbok fish farm located in Eastern Jeju Island, Republic of Korea were transported and acclimatized for 2 weeks in a recirculating culture system in circular cement tanks (capacity: 500-L) in the Marine and Environmental Research Institute, Jeju National University. Culture water was partially replaced with sand-filtered water once in a week. Continuous aeration was also provided to maintain dissolved oxygen levels at 8.4 ± 0.6 mg l^{-1}. The photoperiod of 10:14 h (light/dark) was provided by fluorescent light. During the experimental period ambient conditions were water temperature: 21.5 ± 1.4 °C, salinity: 33.1 ± 0.8 ppt, and pH: 7.5 ± 0.6. Fish were fed with a basal diet *ad libitum* twice a day at 09:00 and 15:00 h at a rate of 3% of their body weight (Table 1).

Table 1
Composition of experimental diets for kelp grouper.

Diet	(%)
Ingredients	
Fish meal	52
Krill meal	10
Fish oil[a]	6
Vitamin mixture[b]	5
Mineral mixture[c]	5
Guar gum	2
CMC Na[d]	4
Cellulose	16
Proximate compositions (g/kg)	
Crude protein	48.2
Crude fat	13.2
Crude Moisture	13.2
Crude ash	8.7
Lactobacillus sakei BK19	1

[a] Riken feed oil Ω (Eiken Shoji Co. Ltd, Tokyo, Japan).
[b] Vitamins (mg/100 g dry diet): thiamine HCl, 1.5; pyridoxine HCl, 1.5; nicotinic acid, 4.5; inositol, 125; folic acid, 1.5; choline chloride, 750; calcium ascorbate, 120; menadione-$NaHSO_4$, 5.15; riboflavin, 2.90; calcium pantothenate, 9.25; biotin, 4.70; cyanocobalamin, 1.00; vitamin A palmitate, 2.70; α-tocopherol, 120; α-cellulose, 825.
[c] Minerals (mg/100 g dry diet): KH_2PO_4, 210; $Ca(H_2PO_4)_4 \cdot H_4O$, 250; calcium lactate, 115; iron citrate 65; $ZnSO_4 \cdot H_2O$, 8; $CuSO_4 \cdot 4H_2O$, 5.00; $CoCl_2 \cdot 6H_2O$, 0.04; KIO_3, 0.12; a-cellulose, 443.0; dextrin, 365.
[d] Carboxymethyl cellulose sodium salt.

2.2. Probiotics, bacterial strains and growth conditions

L. sakei BK19 were obtain from flounder fish [38] and grown in Mann, Rogosa and Sharpe broth (MRS, Difco, Detroit, USA) supplemented with 1% (w/v) $CaCO_3$ at 32 °C and incubated for 48 h. Stock cultures were stored in MRS broth containing sterile (121 °C for 15 min) 20% (v/v) glycerol at −70 °C. Subculture was taken from the seed of MRS broth, incubated for 24 h at 32 °C and then centrifuged at 4 °C at 7000 g for 25 min (Sorvall RC Plus–Du Pont, USA).

2.3. Experimental diet

Supplemented diet was prepared with a pure culture of *L. sakei* BK19 from overnight growth on an MRS plate which was inoculated into 10-ml of MRS broth with incubation at 32 °C for 24 h. The culture was centrifuged at 5000 g for 20 min at 4 °C; the cell pellet washed twice and resuspended in 0.9% (w/v) saline and the concentration adjusted to 10^8 cells ml^{-1} using a haemocytometer slide (Improved Neubauer type, Merck, Lutterworth, Great Britain) at a magnification of ×400. Then, 100 g quantities of basal pellet feed were mixed with the suspension of *L. sakei* BK19 to achieve a dose of 10^8 cells g^{-1} as determined during the preliminary experiments (Table 1). The viability of *L. sakei* in the feed was assessed by plate counts on MRS plate following storage of the diets at 4 °C and 25 °C for four weeks. The final concentration of line *L. sakei* BK19 in the probiotics feed pellets before the feeding trial was 10^8 cells g^{-1}. The control (basal) diet contained an appropriate volume of saline instead of the bacterial suspension.

2.4. S. iniae

S. iniae isolated from infected olive flounder was grown in 10 ml with 1.5% sodium chloride (NaCl) in Brain Heart Infusion broth (BHIB; Difco) in a rotary shaker overnight at 250 rpm at 37 °C [36,37]. The subculture was prepared from the seed. A volume of 1-ml of the seed solution was taken into a flask with 100-ml of NaCl in BHIB and incubated at 37 °C for 24 h. The subcultures were grown twice under the same conditions for the experiment. Growth was measured by optical density at 700 nm and then through plate counting in BHI-NaCl.

2.5. S. parauberis

S. parauberis isolated from infected olive flounder was identified based on the phenotypic, biochemical, molecular characteristics and their culture were stored at −70 °C in tryptic soy broth (TSB) containing 10% glycerol until further use [37].

2.6. Experimental design

Two groups of 100 each kelp grouper were fed with diet enriched with and without probiotics twice daily. From each group a sample of 20 fish were randomly collected at 1, 2, and 4 weeks of interval and were challenge intraperitoneally (i.p.) with 0.1 ml phosphate buffer saline (PBS, Sigma–Aldrich) suspensions containing *S. iniae* or *S. parauberis* separately or mixed approximately 4.3×10^7 cells ml^{-1}. Another 50 fish were used as control was fed basal diet without probiotics received the same volume of PBS alone. All groups were maintained in above mentioned conditions. In our previous work we determined that these cell numbers led to the death of >70% or > 60% of the kelp grouper either bacteria. After two week challenge, the fish were studied for immunological assay and mortalities; all dead fish and the survivors were examined bacteriologically to determine the presence of the pathogens