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Lactococcus lactis BFE920 activates the innate immune system of olive flounder (*Paralichthys olivaceus*), resulting in protection against *Streptococcus iniae* infection and enhancing feed efficiency and weight gain in large-scale field studies

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ABSTRACT

The protective effect of a food-grade lactic acid bacterium *Lactococcus lactis* BFE920 against disease of olive flounder (*Paralichthys olivaceus*) cultivated on a large scale was studied. Initially, antimicrobial activity of *L. lactis* against several fish pathogens was evaluated *in vitro*; the probiotic showed strong antibacterial activity against *Streptococcus iniae, Streptococcus parauberis* and *Enterococcus viikkiensis*, and moderate activity against *Lactococcus garviae*. When olive flounders were fed for two weeks with experimental diets containing varying concentrations of *L. lactis* (1×10^6, 5×10^6, 2.5×10^7 and 1.25×10^8 CFU/g feed), all the experimental feed groups showed 68–77% survival upon challenge with *S. iniae*. A field-scale feeding trial with *L. lactis* dietary supplement was conducted in a local fish farm ($n = 12{,}000$) for three months, and disease resistance, innate immune parameters and growth performance were evaluated. The average weight gain and feed efficiency were increased up to 6.8% and 8.5%, respectively. At the end of the feeding trial, the olive flounders were challenged with *S. iniae*. The *L. lactis*-fed group was protected from *S. iniae* challenge with a 66% survival rate. This disease protection is due to the flounder's innate immunity activated by the *L. lactis* administration: increased lysosomal activities and production of IL-12 and IFN-γ. These data clearly indicated that *L. lactis* BFE920 may be developed as a functional feed additive for protection against diseases, and for enhancement of feed efficiency and weight gain in olive flounder farming.

1. Introduction

Lactococcus lactis, a spherical-shaped, Gram-positive, catalase-negative lactic acid bacterium, is widely used as a starter culture in fermented dairy products [1]. As a probiotic microorganism, *L. lactis* is known for its diverse host-beneficial properties such as immune modulatory effects [2], improvement of digestion [3], and reduction of antibiotic-associated diarrhea [4] in animals. In addition, *L. lactis* has been approved to be used alive as the first genetically modified organism for clinical trials of human diseases such as inflammatory bowel diseases [5–8]. In the same strategy, recombinant *L. lactis* expressing an antigen was generated and the efficacy of the subunit vaccine was investigated in tilapia as a fish model [9].

L. lactis BFE920, the strain used in this study, was first isolated from bean sprouts [10] and showed exceptional antimicrobial activities against a broad spectrum of food-borne pathogens, such as *Listeria monocytogenes, Salmonella enteritidis, Bacillus cereus* and *Clostridium perfringens* [11]. *Streptococcus iniae*, a Gram-positive coccoid with beta–hemolytic activity, was first isolated and identified from Amazon freshwater dolphins in 1972 [12]. *S. iniae* has been reported to be responsible for numerous outbreaks of streptococcal infection in rainbow trout, tilapia, and olive flounder farms in Asia and the US [13–16]. *S. iniae* infection is highly lethal causing about 30–50% mortality in fish [17]. The global economic loss in aquaculture industries due to *S. iniae* outbreaks was estimated at around 100 million US dollars [17].

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Olive flounder (*Paralichthys olivaceus*) is one of the most favorite marine fish and is economically important in Korea, China and Japan. Since antibiotics are commonly and widely used to treat diseased fish in fish farms, many problems due to the overuse of antibiotics have been reported, including environmental damage, emergence of antibiotic-resistant pathogenic strains, and increased cost for fish farming [18,19]. Therefore, an alternative to antibiotics is urgently and even desperately required for prevention of and/or intervention in *S. iniae* outbreaks in fish farms. The prospective use of safe and disease-prevention probiotics offers exciting benefits [20,21].

In this study, we examined the effects of *L. lactis* BFE920 as a feed additive for flounders. The studies involved aspects of innate immune activity, protection from *S. iniae,* and feed conversion efficiency of flounders at both laboratory ($n = 20–25$) and large scale ($n = 12{,}000$). The *L. lactis* BFE920 feeding induced strong disease-protective effects against *S. iniae*, a major flounder pathogen, and increased weight gains and feed efficiencies significantly.

2. Materials and methods

2.1. Experimental animals and feed

2.1.1. Lab-scale feeding

Fries of olive flounder, weighing 40 ± 3 g, were kindly provided by Odo Aquaculture Inc., Pohang, Korea. The fries were acclimatized for two weeks in closed-circulatory 250 L tanks containing 20–25 fish per tank. About 50% of the tank seawater was replaced with fresh seawater twice a week. Commercially available extruded pellet (EP) feed was coated with varying concentrations of *L. lactis* at 1×10^6, 5×10^6, 2.5×10^7 and 1.25×10^8 CFU/g feed. Fish were fed twice a day at 3–5% of their body weight for either 2 weeks or 3 months.

2.1.2. Field-scale feeding

Large-scale field experiments were conducted at Chung Yang Aquaculture Inc. and Odo Aquaculture Inc., Pohang, Korea, from August to December 2012. Flounders weighing 55 ± 5 g were randomly selected and divided into four 6 m × 6 m × 0.50 m tanks at 3000 fish/tank ($n = 12{,}000$). Each experiment was performed in duplicate by feeding two tanks with the experimental feed coated with *L. lactis* (2.5×10^7 CFU/g) and the other two controls with regular diet. Experimental feed was prepared by mixing 20 kg of EP feed with 5 L of tap water containing 200 ml of *L. lactis* culture (2.5×10^9/ml) or with tap water only using a concrete mixer. They were kept at 4 °C until use. Fish were fed three times daily until satiation.

2.2. S. iniae challenge test

A cohabitational infection method was used for challenging the experimental olive flounders with *S. iniae* FP5228, obtained from Fish Pathology Division, National Fisheries Research & Development Institute, Pusan, Korea. To generate infectors, untreated healthy flounders were infected with *S. iniae* 1×10^5 CFU/g i.p., and marked by clipping the caudal fin. The infector flounders were then cohoused with experimental subjects in the same tanks at an infector to subject ratio of 1:5.

2.3. Cell isolation

Three fish from each experimental group were sacrificed for isolating cells from blood and spleen. Blood was collected from flounder's caudal veins using a 1 ml syringe with 26-gauge needle and was mixed with acid-citrate-dextrose (ACD) solution 1% v/v to prevent clotting. The spleens explanted from flounder peritonea were sheared by forcing through 70 μm nylon mesh in RPMI containing 1% penicillin/streptomycin and 20% FBS. The cell suspension was then centrifuged at 4000× *g* for 30 min at 4 °C in a discontinuous Percoll (Sigma) gradient of 1.084 g/ml, 1.072 g/ml, and 1.030 g/ml.

2.4. Myeloperoxidase assay

Myeloperoxidase is a key lysosomal enzyme present in azurophilic granules of neutrophil. 1×10^5 peripheral blood mononuclear cells (PBMC) or splenic mononuclear cells in a 15 μl volume were seeded onto a 96-well plate. Into each well, 135 μl Hank's Balanced Salt Solution (HBSS), 0.02% cetyl trimethylammonium bromide (CTAB), 50 nM phorbol 12-myristate 13-acetate (PMA) was added, and then 45 μl of tetramethyl benzidine HCl (TMB) solution (Affymetrix, USA) was included. After 2 min of incubation at room temperature, 50 μl 4 M H_2SO_4 stop solution were added to each well. Optical density (OD) was read at 450 nm.

2.5. Nitro blue tetrazolium (NBT) assay

1×10^5 PBMC or splenocytes in a 50 μl volume were mixed with 50 μl of 0.2% NBT and incubated at room temperature for 30 min. After 30 μl of the cell and NBT mixture were transferred to a new tube, 1 ml of dimethylformamide (Sigma) was added, thoroughly vortexed, and centrifuged at 2000 rpm at 4 °C for 5 min. The OD of the supernatant was measured at 620 nm. All assays were performed in duplicates.

2.6. Detection of cytokine expression via quantitative RT-PCR (qPCR)

Spleens were explanted from six fish from each experimental group and each specimen was instantly frozen in liquid nitrogen. 30 mg of each sample in 1 ml of Easy-BLUE™ Total RNA Extraction Kit solution (iNtRON Biotechnology, Korea) were homogenized using a rotor-stator homogenizer (Power Gen 125 and 7 × 95 MM Flat Generator, Fisher Scientific, USA). Total RNA was extracted according to the manufacturer's instruction and was treated with RNase-Free DNase I (Qiagen, Germany). First cDNA was synthesized using SuperScript® III Reverse Transcriptase (Invitrogen, USA), following the manufacturer's manual. Relative expressions of olive flounder IFN-γ and IL-12b to β-actin were determined by qPCR analyses using HotStart-IT SYBR Green qPCR Master Mix (Affymetrix, USA) and StepOnePlus™ Real-Time PCR System (Invitrogen, USA). The PCR program was 85 for 2 min, followed by 40 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 40 s, and elongation at 72 °C for 40 s. The annealing temperature for IL-12b was 62 °C. Melting curve analysis of the amplicon was performed at the end of each PCR. The primers were designed via Primer-BLAST with thermodynamic oligo and template alignment algorithms. The sequences of primers are as the following: b-actin_forward 5′-TGCAGAAGGAGATCACAGCC-3′, b-actin_reverse 5′- ACTCCTGCTTGCTGATCCAC-3′, il12_forward 5′-CTCTCCCTAC GCC-GAGGAAA-3′, il12_reverse 5′-GCTAATCTGGGGACTGTCGG-3′. ifng_forward 5′-CTGCCGAACACGACTCCC-3′, and ifng_reverse 5′-GCTCTGCTCCTTGAAGCGAT-3′.

2.7. Growth and feed conversion efficiency

In the field experiment, the body weights of experiment fish were measured every 30 days for 3 months. Fifty fish were randomly selected and put in a basket, and their body weights measured 3 times and averaged. At each tank containing 3,000