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Effects of dietary probiotic, *Lactococcus lactis* subsp. *lactis* I2, supplementation on the growth and immune response of olive flounder (*Paralichthys olivaceus*)

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ABSTRACT

We investigated the effects of a potential probiotic strain *Lactococcus lactis* subsp. *lactis* I2 on the immune response and growth of olive flounder (*Paralichthys olivaceus*), and their capacity to prevent streptococcosis after *Streptococcus iniae* challenge. The *L. lactis* subsp. *lactis* I2 strain, isolated from olive flounder intestine, was supplemented orally as a feed additive (~10^8 CFU g^{-1}) to fish for 5 weeks. Compared with the untreated group, the rate of growth was increased in the I2-diet group. The administration of I2 to olive flounder enhanced non-specific immune parameters, such as lysozyme, antiprotease, serum peroxidase and blood respiratory burst activities. At 9 days after challenge with *S. iniae* (10^8 CFU), the untreated control group experienced a 90% mortality rate, whereas all of the I2-cell-supplemented fish survived. These results show that *L. lactis* subsp. *lactis* I2 exerted beneficial effects as a probiotic and has potential as an alternative to antibiotics for the prevention of streptococcosis in aquaculture.

1. Introduction

The olive flounder (*Paralichthys olivaceus*) is a farmed fish frequently consumed in Korea, for which demand is increasing. In 2011, the output of olive flounder was more than 50% (~40,805 tons) of the total output of fish by Fisheries Information Service (http://www.fips.go.kr/). The increased productivity, high-density culture and poor water quality have weakened disease-resistance, resulting in frequent outbreaks of fish diseases, and ultimately causes economic losses (MIFAFF, 2003).

Fish diseases are caused by a number of pathogens, such as parasites, bacteria and viruses. Of these, bacteria are the leading pathogen in commercial aquaculture. Antibiotics or formalin-inactivated antigen vaccines have been used to treat bacterial infections in aquaculture systems. However, the development of antibiotic-resistant strains due to overuse of antibiotics and the reduced efficacy of the remaining antibiotics in fish have given rise to important problems (Karunasagar et al., 1994; Smith et al., 1994). The stress associated with vaccine administration to fish places a limitation on vaccination. To overcome these problems and to protect fish from bacterial diseases, many studies have evaluated the application of lactic acid bacteria (LAB) as feed additives instead of antibiotics or vaccine treatments (Brunt and Austin, 2005; Choi and Yoon, 2008; Gildberg and Mikkelsen, 1998; Pirarat et al., 2006; Sakai et al., 1995; Sharifuzzaman and Austin, 2009; Vendrell et al., 2008). LAB are recognized as an alternative to antibiotics or vaccines because they are safe and induce an immune response against pathogenic bacteria (Gatesoupe, 1999).

Because LAB produce antimicrobial compounds as metabolites (e.g., lactic acid, diacetyl, carbon dioxide, hydrogen peroxide and bacteriocins) and to inhibit the increment of harmful intestinal bacteria (Calo-Mata et al., 2008; Gatesoupe, 1999; Perdigon et al., 1990), traditional fermented foods, such as soy sauce, and dairy products containing LAB have been used in the food industry. In addition, LAB cell wall components (capsular polysaccharides, peptidoglycans, lipoteichoic acids) induce cytokine production by macrophagocytes (Haza et al., 2004) and complement activation (Kim et al., 2002). The method of attachment and metabolite-producing stages of probiotic bacteria in host bodies are not well-understood (Gatesoupe, 1999). A probiotic strain suitable for treatment in marine aquaculture should be identified.

According to a report by the Fisheries Information Service of Korea, 32% of total fish infections are caused by bacteria, of which 19% are due to *Streptococcus* spp. (http://www.fips.go.kr/). *Streptococcus iniae*, which is a marine pathogen and the causal agent of streptococcosis, is a gram-positive bacterium that infects at least 27 cultured fish species, including olive flounder, causing a greater than 50% mortality rate and a marked economic burden (Agnew and Barnes, 2007; Kim et al., 2011). Probiotic bacteria isolated from marine sources may be effective against pathogenic bacteria in fish (Kesarcodi-Warson et al., 2008). Previously, we isolated *Lactococcus lactis* subsp. *lactis* I2, a nisin Z-producing LAB, from the intestine of healthy olive flounder; this bacterium inhibited the growth of pathogenic bacteria, including *S. iniae* (Heo et al., 2012). The nisin Z produced by *L. lactis* subsp. *lactis*

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has been approved as a safe food additive by both the Food and Drug Administration (FDA) and the World Health Organization (WHO).

In this study, to determine the applicability of the nisin Z-producing strain in aquaculture, we investigated the effect of *L. lactis* subsp. *lactis* I2 as a food additive in terms of growth of flounder and induction of an immune response against *S. iniae* infection.

2. Materials and methods

2.1. Bacterial cultivation

Nisin-Z-producing *L. lactis* subsp. *lactis* I2, isolated in our laboratory from flounder intestine, was cultured in MRS (Difco, Detroit, MI, USA) (Heo et al., 2012). *S. iniae* (KCTC 3657) was cultured in brain–heart infusion (BHI; Becton Dickinson, Heidelberg, Germany) broth at 37 °C up to 10^8 CFU/ml and used in a challenge test (Heo et al., 2012). Bacterial stocks were stored at −80 °C in their respective broths with 20% glycerol until use.

2.2. Fish

A total of 300 juvenile olive flounder (*P. olivaceus*; average weight = 5.72 ± 0.17 g) were obtained from Geojedo, Korea. The fish were divided into two equal groups, an I2 group and a control group, and reared in continuously aerated free-flowing seawater, which was maintained at ~18 °C, for at least 1 week. Of the fish in each group, 10 were used to investigate the effects of the food additive on growth, 12 were used in a challenge test and 40 were used to investigate the immune response. Each experiment was repeated three times. All fish were fed the appropriate diet at 1.5% of their body weight daily. The health of the fish was checked immediately upon arrival in the laboratory and at 14-day intervals thereafter (Kim and Austin, 2006).

2.3. Experimental diet

A pure culture of *L. lactis* subsp. *lactis* I2 was incubated at 37 °C for 16 h and used to prepare the experimental diet. The diet of the I2 group was mixed with *L. lactis* subsp. *lactis* I2 to ~10^8 cells per gram commercial diet (Su-Hyup No.4, Korea); this dose was determined in preliminary experiments. The viability of *L. lactis* subsp. *lactis* I2 in the feed was assessed by plate counts on MRS medium. Fish in the control group were supplied with commercial feed only.

2.4. Sample collection

lood was collected from all fish from the caudal vein and Alserver's solution was used to perform a nitroblue tetrazolium (NBT; Sigma, St. Louis, MO, USA) reduction activity test. Serum was separated and subjected to lysozyme, antiprotease, peroxidase and bacterial killing assays.

2.5. Growth effects of experimental diets on fish populations

Ten fish from each sub-group were fed the experimental diet for 5 weeks; body weights and lengths were measured during this period. Growth effects were determined by calculating weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER), condition factor (CF), hepatosomatic index (HSI), and visceral somatic index (VSI).

2.6. Immunological assays

Lysozyme and antiprotease activities were determined using the ethods of Ellis (1990). To determine lysozyme activity, 50 μl of serum was added to 1 ml lyophilized *Micrococcus lysodeikticus* (Sigma, St. Louis, MO, USA) at a concentration of 0.2 mg ml^{-1} (w/v) in phosphate-buffered saline (PBS, pH 6.2). The optical density (OD) was recorded at 530 nm after 1 and 20 min at 22 °C. One unit of lysozyme activity was defined as the amount of serum that caused a decrease in the OD of 0.001 units min^{-1}. For antiprotease activity, 10 μl of serum was incubated with 10 μl of 5 mg/ml trypsin solution (Sigma, St. Louis, MO, USA) for 10 min at 22 °C. Then, 100 μl of 0.1 M PBS (pH 7.0) and 125 μl of 2% (w/v) azocasein (Sigma, St. Louis, MO, USA) were added and the solution was incubated for 1 h at 22 °C. The reaction was stopped by addition of 500 μl of 10% (v/v) trichloroacetic acid (TCA) and then centrifuged at 6000 rpm for 5 min. The supernatants (400 μl) were transferred to new tubes containing 1 N sodium hydroxide (400 μl) and the OD at 450 nm was determined using a spectrophotometer (Ultrospec 3000, Pharmacia Biotech, USA). PBS was used as the control. The inhibitory activity of antiprotease was expressed in terms of the percentage of trypsin inhibition [(Control OD − Sample OD)/Control OD × 100] (Zuo and Woo, 1997).

The peroxidase content of serum was measured using the method described by Sitjà-Bobadilla et al. (2008), with some modifications. In brief, 15 μl of serum was mixed with 85 μl of Hank's Buffered salt solution (HBSS) and then added to peroxidase substrate [100 μl of 5 mM tetramethylbenzidine hydrochloride (TMB; Sigma, St. Louis, MO, USA) and 100 μl of 2.5 mM H_2O_2]. The serum mixture (300 μl) was incubated for 2 min. The reaction was stopped with 100 μl of 2 M H_2SO_4 and the OD at 450 nm was determined. PBS was used as a blank instead of serum.

The production of oxygen radicals by leukocytes was assessed by the reduction of NBT according to Taoka et al. (2006). Blood was added to an identical volume of NBT solution (2 mg ml^{-1}) and incubated for 30 min. Then, 100 μl of *N,N*-dimethylformamide was added. The solution was centrifuged at 3000 rpm for 10 min. Supernatant (100 μl) was diluted with 900-μl PBS (pH 7.0) and then the OD at 550 nm was determined using a spectrophotometer.

2.7. Challenge test

Juvenile olive flounders from each group were fed twice per day for up to 5 weeks with each experimental diet. After two weeks, 12 fish from each sub-group were intraperitoneally challenged with 100-μl *S. iniae* suspension (1×10^8 CFU), *S. iniae* was injected. Mortality was recorded daily for 2 weeks post-challenge; the results are presented as cumulative survival rates.

2.8. Statistical analysis

All experimental data were analyzed by one-way analysis of variance (ANOVA), least significant difference (LSD), and Duncan's comparison tests as appropriate. All data were analyzed using the software Statistical Package for the Social Sciences (SPSS ver. 17.0, SPSS, Chicago, IL, USA). Differences were considered to be significant at values of $P<0.05$.

3. Results

3.1. Effects of experimental diets on the growth of flounder

After 5 weeks of feeding, the body weights of olive flounder fed probiotic-containing feed was higher than that of the control group (Table 1). Growth performance parameters for fish in the I2-diet and control groups were 0.97 ± 0.03% and 0.89 ± 0.01% for CF, and 1.80 ± 0.20% and 1.28 ± 0.15% for HIS, respectively. Especially, CF and HIS were significantly higher ($P<0.05$) in the I2-diet group compared to the control group. Other parameter were 46.94 ± 0.70 % and 44.52 ± 0.34 % for WG, 78.62 ± 0.81% and 77.97 ± 0.32% for FE, 1.12 ± 0.01% and 1.06 ± 0.01% for SGR, 0.94 ± 0.01% and 0.77 ± 0.01% for FER,