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Improved growth rate and disease resistance in olive flounder, *Paralichthys olivaceus*, by probiotic *Lactococcus lactis* WFLU12 isolated from wild marine fish

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ABSTRACT

The use of probiotics is a strategy employed to improve host health status and to prevent infectious diseases. The current study was aimed at investigating the diversity of lactic acid bacteria (LAB) and *Bacillus* species in the gastrointestinal tracts of wild marine fishes, as well as the beneficial effects of *Lactococcus lactis* WFLU12 as a host-derived probiotics in olive flounder. In marine fishes, wild olive flounder and rock bream were shown to be good sources of LAB and *Bacillus* isolation, respectively. Some isolates, including the strain WFLU12, have shown stronger inhibitory activity against various aquatic bacterial pathogens and more tolerance to low pH and bile acids compared to some strains isolated from sources other than marine. *Lc. lactis* WFLU12 was found to confer to olive flounder protection against streptococcosis caused by *Streptococcus parauberis* through competitive exclusion and increased innate immune responses. Interestingly, the natural infection rate in the probiotic fed group (33% = 10/30) was significantly lower than that in the control group (60% = 18/30). None of the nisin Z and colicin V-producing probiotic-fed fish were naturally infected by *S. parauberis* during the feeding period. In addition, more importantly, this promising probiotic strain significantly promoted fish growth along with better feed conversion and specific growth rate. This study demonstrates that the use of host-derived probiotics can offer a significant advantage in terms of optimum survival and function in the gastrointestinal tract of the intended host.
Statement of relevance: In this study, host-derived probiotic strain outperforms elimination of pathogen through competitive exclusion in the gastrointestinal tract and increased innate immune responses. More importantly, this promising probiotic strain significantly promoted fish growth along with better feed conversion. This study will provide insight into how optimal probiotics should be selected and developed. It might facilitate the replacement of commercial fish probiotic products originated from terrestrial sources with host-derived probiotics in the near future.

1. Introduction

In recent years, vaccination, immunostimulants, and probiotics have received considerable attention as alternatives to chemotherapeutic agents. A number of studies have demonstrated that the use of live microorganisms as probiotics can provide health benefits to hosts when ingested in adequate amounts. Similar to the mammalian digestive system, fish guts also provide a niche for the adherence, colonization, and proliferation of many microbial species that affect various physiological processes of the host (Cahill, 1990; Gómez and Balcázar, 2008). Certain bacterial species preferentially colonize the digestive tracts of fish through water and food in the aquatic environment, which differs greatly from the terrestrial environment. Many researchers have used lactic acid bacteria (LAB) and various members of the *Bacillus* genus as probiotic candidates in aquaculture, including *Lactobacillus* spp. (Nikoskelainen et al., 2001; Aly et al., 2008), *Lactococcus lactis* (Balcázar et al., 2008), *Pediococcus acidilactici* (Castex et al., 2008), and *B. subtilis* (Moriarty, 1998; Balcázar and Rojas-Luna, 2007; Aly et al., 2008). However, most of the bacteria used as probiotics in fish were originally isolated from milk, cheese or other terrestrial sources.

Recently, the use of host-associated microorganisms as probiotics has gained much attention, though it remains unclear whether host-derived microorganisms are better as probiotics than those with non-host origins (Lazado et al., 2015). However, because host-associated microorganisms are in their natural habitat, it is reasonable to suspect that they would have optimal benefits in similar environments. The exploration of fish gut microbiota with tolerance to a variety of environmental conditions such as temperature, low pH, osmotic pressure, bile salts, and antimicrobial activity might uncover valuable and beneficial strains for

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use in aquaculture. *In vivo* screening for the ability of probiotics to inhibit pathogens is a very important process, as it can exclude false positives from *in vitro* screening (Vine et al., 2006). Also, probiotic cells in the gastrointestinal tract may play an important role in defense against invading pathogenic microbes (Ringø et al., 1998) as well as in the stimulation of the host immune system (Schiffrin and Blum, 1999).

Although the gut microbiota of marine fish has been studied for different purposes, little information is available on LAB and the genus *Bacillus* isolated from wild fish (notably marine) and their effects on the host. Therefore, the objectives of this study were to understand the diversity of LAB and bacillus species in wild marine fish, and to assess the beneficial effects of the selected strain (*Lactococcus lactis* WFLU12) on olive flounder as a host-derived probiotic.

2. Materials and methods

2.1. Bacteria and culture conditions

A total of 20 pure cultures of LAB and *bacillus* species were isolated from the gut mucus of wild fish as previously described (Kim and Kim, 2013). These fish included red seabream (*Pagrus major*) ($n = 12$), largescale blackfish (*Girella punctata*) ($n = 9$), and rock bream (*Oplegnathus fasciatus*) ($n = 4$). All fish were obtained from Jeju Island. Briefly, the guts were aseptically removed in their entirety from healthy fish and slit open lengthways. The gut mucus was collected. Dilutions (10^{-2}, and 10^{-1} in saline) of homogenous intestinal mucus were spread over duplicate plates of de Man, Rogosa, and Sharpe agar (MRS; BD, Difco, USA) and incubated at 25 °C for up to 7 days. Cultures were routinely grown on tryptic soy agar (TSA) (BD, Difco, USA) supplemented with 1% NaCl at 25 °C for 5 days. Nine strains purchased from KCTC (Korean Collection for Type Culture, Daejeon, South Korea) and KCCM (Korean Culture Center of Microorganisms, Seoul, South Korea) were used for comparisons.

2.2. Bacterial identification

Pure cultures grown overnight on brain heart infusion (BHI) (BD, Difco, USA) or MRS agar were used to test for Gram staining as well as catalase and oxidase production. Biochemical identification tests for isolates were carried out using BioMerieux API 50 CHB and API 50 CHL test kits (Biomerieux, Marcy L'Etoile, France) following the manufacturer's instructions. Amplification and sequencing of 16S rRNA were conducted as previously described (Kim and Kim, 2013). The closest type strain was determined using the RDP SeqMatch tool (https://rdp.cme.msu.edu/).

2.3. Phenotypic characterization of bacterial cultures

An overnight culture of each isolate in 10 mL of BHI medium at 28 °C was used as the inoculum. Cells were centrifuged and re-suspended in 0.85% sterile saline. Afterward, 100 μL of the suspension was inoculated in 10 mL of BHI medium in each test tube to get 10^7 CFU mL^{-1}. The temperatures tested were 10 °C and 45 °C. Together, the BHI medium supplemented with NaCl at concentrations (4%, 6.5%, and 9% (*w*/*v*)) or adjusted with different pH values (2, 4.4, and 9.6) were used to test for their ability to survive in different level of salinity, or acid condition. The tubes were incubated with shaking at the tested temperature or at 28 °C for tests on the pH and concentration of NaCl. Each sample was streaked onto an agar plate to evaluate the presence or absence of cell viability after 24 h, and visual examination for its growth was carried for up to 5 days. The turbidity of each tube was also noted as an indication of growth. Each treatment was tested in triplicates.

Bile salt tolerance was determined using the method described by Gilliland et al. (1984) with some modifications. Briefly, BHI or MRS broth (10 mL) containing 0, 0.05, 0.1, 0.2, 0.3, 0.4, or 0.5% bile salts (Sigma-Aldrich Chemie GmbH, Switzerland) was inoculated with 10^7 CFU/mL of each isolate or strain from the respective overnight growth cultures after centrifugation at 3000*g* for 15 min and washing three times. Samples were incubated at 28 °C for 24 h with shaking (100 rpm). Control (no bile salts) and test cultures were evaluated at 2, 4, and 24 h for the presence or absence of growth by streaking the samples onto BHI or MRS agar plates.

The bacterial supernatant of *Lc. lactis* WFLU12 was collected after an overnight culture in 10 mL of MRS was centrifuged at 3000*g* for 15 min and then filtered with membrane filter (0.22 μm). The supernatant was used for the agar well diffusion test to determine the inhibitory activity against bacterial fish pathogens. The supernatant treated with heat at 65 °C or 100 °C for 30 min was also used to understand the nature of antagonistic substances. The following bacterial fish pathogens were tested: *Aeromonas salmonicida*, *Edwardsiella tarda*, *Vibrio anguillarum*, *V. ichthyoenteri*, *Streptococcus iniae*, and *S. parauberis.* They were harvested from an overnight culture on TSA (supplemented with 1% NaCl) and then suspended with 0.85% sterile saline to get the final density at 10^8 CFU mL^{-1}. Fifty microliters of the supernatant of the probiotics was immediately inoculated into wells (6 mm in diameter) of BHI agar plate (supplemented with 1% NaCl) on which the suspension of each target bacteria was already spread. Antagonism was noted based on the presence of an inhibitory zone after incubation at 28 °C for 48 h.

2.4. Examination of feeding behavior and natural infection in pilot-scale system

Lc. lactis WFLU12 was found to be potentially useful as it lacked harmful effects (all fish fed very well, showed no inflammatory responses in the injection sites, and were not infected with the probiotic strain) after intramuscular and intraperitoneal injection and demonstrated antagonistic activity against some bacterial fish pathogens. Therefore, it was selected to determine the ability for competitive exclusion in the intestines of olive flounder. Commercial extruded feed (DongA One Corporation, Korea) consisting of crude protein (52%), crude ash (15%), crude fat (10%), phosphorus (2.7%), crude fiber (2%), and calcium (1.2%) was used as the basal diet with or without supplementation of the probiotic strain. The suspended probiotic candidate (*Lc. lactis* WFLU12) or saline as control (50 mL) was added drop wise to the basal feed (250 g), and let the pellets dry properly for several hours in clean bench. The probiotic concentration in the feed (~10^9 CFU g^{-1}) was confirmed by spreading 100 μL of dilution of the diets suspended in saline (1 g per 10 mL) on MRS agar and incubating at 28 °C for 2 days. All diets were maintained at 4 °C until use.

One hundred fish (average weight 80.84 ± 9.37 g) were randomly assigned to two 800-liter flow-through tanks containing pumped-up ambient seawater filtered through a sand filter system at the Institute of Fisheries Science of Pukyong National University, Gijang, Busan, Korea. Fish were fed 3% of their total body weight per day. We routinely and thoroughly examined whether or not they consumed all feed provided, and any anomalies in feeding and swimming behavior. Fish body weight and length were measured every two weeks and the feed ration for each group was adjusted accordingly. Ten fish were euthanized and aseptically dissected at 2, 4, and 8 weeks post feeding. All fish were examined externally and internally for gross signs of disease and parasites. Swabbed materials derived from the kidney and the spleen were placed on TSA (BD, Difco) supplemented with 1% NaCl and incubated at 28 °C for 72 h. Representative colonies were collected and identified by PCR using pathogen specific-primers and/or sequencing of the 16S rRNA gene fragments (primers are listed in supplementary Table S1) following the procedure described by Kim and Kim (2013). Immunological assays were conducted following the methods described in the supplementary method section.