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Dietary supplementation of *Pediococcus pentosaceus* enhances innate immunity, physiological health and resistance to *Vibrio anguillarum* in orange-spotted grouper (*Epinephelus coioides*)☆

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ABSTRACT

Groupers (*Epinephelus* spp.) are economically important fish species in Southeast Asian aquaculture. Vibriosis caused by *Vibro* spp. is one of the severe bacterial diseases that devastate the grouper aquaculture industry. Probiotics have been reported to show the potential to enhance fish immunity and to antagonize pathogens. In our previous study, a lactic acid bacterium *Pediococcus pentosaceus* strain 4012 (LAB4012), isolated from cobia intestine, protects cobia from photobacteriosis after a 2-week feeding. In this study, we examined the potential of LAB4012 to be a probiotic for the orange-spotted grouper through feeding, thus to guard against vibriosis. *In vitro*, LAB4012 culture supernatant with low pH suppressed the growth of *Vibrio anguillarum*, and lactic acid in the metabolite of LAB4012 appeared to be the major factor to the growth inhibition of *V. anguillarum*. *In vivo*, the challenge test showed that the cumulative mortality of the LAB4012-fed groupers was significantly lower than that of the control fish after *V. anguillarum* infection. Supplementation of LAB4012 in commercial diet not only enhanced the growth rate and erythrocyte numbers of the groupers, but also regulated the gene expression of the pro-/anti-inflammatory cytokines. One day post-infection of *V. anguillarum*, the leukocyte numbers in the peripheral blood and the phagocytic activity of the head-kidney phagocytes in the LAB4012-fed groupers were found significantly increased, when compared with those without LAB4012-feeding. These results suggested that LAB4012 can be a dietary probiotic for groupers in modulating the immunity and protecting the groupers from *V. anguillarum* infection.

1. Introduction

Grouper is an economically important fish species for aquaculture in Southeast Asian countries. Due to the intensive culture, groupers have been suffering from not only viral but also bacterial diseases, such as vibriosis, resulting in serious economic loss. Vibriosis is also known as fatal hemorrhagic septicemia, and the causative agents of vibriosis belong to the genus *Vibrio*, such as *Vibrio alginolyticus, Vibrio harveyi,* and *Vibrio anguillarum*. The *Vibrio* spp. infect fish through the skin or oral intake. An outbreak of vibriosis usually occurs when fish are immunocompromised, under stress, and overcrowded at water temperature over 15 °C [1,2]. The typical clinical signs are lethargy and ulceration, and high numbers of *Vibrio* spp. can be found in the blood and the hematopoietic tissues. Moreover, the intestine of the infected fish was filled with yellow liquid and associated with gastroenteritis [3]. However, in acute epizootics, the infected fish always die without showing any clinical signs [1,4,5]. *V. anguillarum* is used for challenge test in this study, and is a gram-negative, curved rod, non-spore forming, and facultative anaerobic bacterium with flagella. In aquaculture, *V. anguillarum* can infect more than 50 marine and brackish economic fish species, and has caused high morbidity and mortality rates [5,6].

For many years, the control strategies on vibriosis mostly employ chemotherapeutants, such as antibiotics and disinfectants. However, widespread use of medicines has resulted in antibiotic contamination of the environment and fish [7], and led to increasing the frequencies of antibiotic resistant bacteria in aquaculture systems [8–17]. Therefore, alternative ways for the control of vibriosis must be developed, such as the use of antimicrobial

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peptides, vaccines, and probiotics. Probiotic-feeding is a good strategy to control the disease because it improves host health and immunity, rendering the host more pathogen-resistant.

In our previous studies, *Pediococcus pentosaceus* strain 4012 (LAB4012), a lactic acid bacterium, was isolated from the intestine of cobia (*Rachycentron canadum*). In cobia, feeding with LAB4012 can enhance the growth rate and respiratory burst activity and significantly decrease the cumulative mortality after challenge with *Photobacterium damselae* subsp. *piscicida* (*Pdp*) [18]. However, the potential of dietary LAB4012 to protect groupers from vibriosis is still unknown. Therefore, we examined the inhibitory activity of LAB4012 supernatant against *V. anguillarum in vitro* and the protection efficacy of LAB4012-feeding on groupers against *V. anguillarum* challenge. Furthermore, we analyzed the impact of LAB4012-feeding on the growth rate, gene expression levels of pro-/anti-inflammatory cytokines, peripheral blood cell counts, and the phagocytic activity and respiratory burst activity of head-kidney phagocytes in groupers. This study provides the information about the influence of LAB4012-feeding on grouper innate immunity and the effect of LAB4012-feeding on protecting groupers from *V. anguillarum* infection.

2. Materials and methods

2.1. Orange-spotted groupers and bacteria

Orange-spotted groupers obtained from Merit Ocean Biotech INC (Taiwan) were reared in aerated seawater with 25‰ salinity at 26 °C. Fish health was evaluated by observation of fish morphology, behavior, mortality, and appetite. In addition, five fish were randomly sampled and confirmed healthy by pathology examination and microbiology tests which were conducted by plating the homogenate of head-kidney, spleen, and liver on tryptic soy agar (TSA, Difco) and thiosulfate citrate bile salts sucrose (TCBS) agar (Difco), the *Vibrio* selective medium agar. Besides, the fish were confirmed to be betanodavirus- and fish iridovirus-free by real-time PCR (Section 2.6).

Pediococcus pentosaceus strain 4012 (LAB4012) was a gift from Dr. Hung-Hsi Hu (National Penghu University, Taiwan), and was cultured in MRS broth (Merck) at 37 °C [18]. *V. anguillarum* was a gift from Dr. Chen-Chun Ku (National Penghu University, Taiwan) and was cultured in TCBS agar (Difco) or tryptic soy broth (TSB) (Becton, Dickinson and Company) with 15‰ salinity at 30 °C. The identity of *V. anguillarum* used in the present study was confirmed by specific primers reported in Hong et al. (2007) [19]. For preparation of challenge test, *V. anguillarum* was cultured on tryptic soy agar (TSA) (Difco) for overnight, and harvested by flushing with PBS. After 3 cycles of PBS washing-and-pelleting by centrifuging at 6230 × *g* for 3 min, the pellet of *V. anguillarum* was resuspended in PBS for the challenge test.

2.2. The inhibition activity of LAB4012 culture supernatant on the growth of V. anguillarum

A colony of LAB4012 was inoculated in 3 ml MRS and cultured for overnight. The overnight-cultured LAB4012 was transferred to 100-fold volume of MRS broth, and 15 ml of culture suspension was collected every hour from 1 to 18 h by centrifuging at 1600 × *g* for 15 min at 4 °C and filtrating through the filter with pore size of 0.45 μm (Sartorius stedim biotech). The pH value and the concentration of lactic acid in each LAB4012 supernatant was respectively determined by using pH meter (JENCO) and Lactate Assay Kit (Biovision).

To examine the impact of LAB4012 culture supernatant, lactic acid, and the pH value on the growth of *V. anguillarum*, four kinds of tested medium and one control medium were prepared, including (1) LAB4012 culture supernatants, separately collected at 1–18 h post-culture, (2) MRS broth, pH adjusted to 4.2 with HCl, (3) 18h-cultured supernatant of LAB4012, pH adjusted to 6.3 with NaOH, (4) MRS broth containing 73 mM lactic acid, and (5) the control medium, i.e., MRS broth with pH 6.3. To examine the influence of each tested medium on the growth of *V. anguillarum*, the 0.1 ml *V. anguillarum* with the optical density value at 595 nm (OD_{595}) of 1 was inoculated into 4 ml TSB supplemented with 1 ml tested medium. After incubation at 30 °C for 1–8 h, the biomass (measured at OD_{595}) of *V. anguillarum* in each tested and control medium were determined by MRX II ELISA reader (DYNEX technologies).

2.3. Preparation of LAB4012-contained diet

The diet supplemented with LAB4012 was prepared as described below. LAB4012 was cultured in MRS broth until the OD_{600} value of liquid culture reached 2 (approx. 10^9 CFU ml^{-1}). LAB4012 was harvested by centrifugation at 1600 × *g* for 15 min at 4 °C, resuspended in PBS, and then sprayed on the commercial diet at a dose of 10^9 CFU g^{-1}. The prepared diet was preserved at 4 °C for 3 days at most. The 3-day-preserved diet was dissolved in PBS, and the numbers of alive LAB4012 were calculated to be 6×10^8 CFU g^{-1} at least.

2.4. V. anguillarum challenge test

To evaluate the protection efficacy of LAB4012-feeding against *V. anguillarum* infection, groupers with average body weight of 2 g were divided into 4 groups. Two groups ($N = 20$) were fed with 4% body weight of LAB4012-contained diet, and the others fed without LAB4012 were served as negative control. After 3-week feeding, the groupers were IP-challenged with *V. anguillarum* at doses of 5×10^5 and 6×10^5 CFU per fish. Groupers were no more fed with LAB4012 after *V. anguillarum* challenge, and the cumulative mortality of groupers was recorded for 7 days.

2.5. Growth rate and immune gene expression

Groupers with average body weight of 1.5 g were daily fed with LAB4012-contained diet at a rate of 4% body weight for 4 weeks. The average body weight of 50 groupers was determined at 1–4 weeks post-feeding (wpf). The percent weight gain was calculated as: [(final body weight − initial body weight)/(initial body weight)] × 100%. Five groupers of each group were randomly sampled at 1 to 4 wpf, and the immune gene expression of head-kidney and spleen was analyzed by real-time PCR (Section 2.6).

Groupers with average body weight of 8.3 g were daily fed with LAB4012-contained diet at a rate of 2% body weight for 1 week, and then intraperitoneally (IP) challenged with a non-lethal dose of *V. anguillarum* (6.3×10^4 CFU per fish). At 1–3 days post-infection (dpi), three fish of the fed and non-fed groupers were randomly sampled, and the immune gene expression of head-kidney and spleen was analyzed by real-time PCR (Section 2.6).

2.6. Reverse transcription and real-time PCR

The acid guanidinium thiocyanate-phenol-chloroform extraction method [20] was used to extract the total RNA from organs. Reverse transcription was carried out by incubating 6 μl total RNA at 42 °C for 1 h in 30 μl of 1 × reaction buffer containing 0.3 μM oligo dT_{20}, 0.4 mM dNTP, 11.7 mM DTT, 40 U ribonuclease inhibitor rRNasin (Promega), and 60 U MMLV reverse transcriptase (Promega). Real-time PCR was conducted to determine the expression level of immune gene. The sequences of primer sets for immune