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Diet supplementation of *Pediococcus pentosaceus* in cobia (*Rachycentron canadum*) enhances growth rate, respiratory burst and resistance against photobacteriosis

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ABSTRACT

Cobia (*Rachycentron canadum*) is an economically important fish species for aquaculture in tropical and sub-tropical areas. Cobia aquaculture industry has severely damaged due to photobacteriosis caused by *Photobacterium damselae* subsp. *piscicida* (*Pdp*), especially in Taiwan. Antibiotics and vaccines have been applied to control *Pdp* infection, but the efficacy has been inconsistent. One species of lactic acid bacteria, *Pediococcus pentosaceus* strain 4012 (LAB 4012), was isolated from the intestine of adult cobia, and its culture supernatant can effectively inhibit *Pdp* growth *in vitro*. The acidic pH derived from metabolic acids in LAB culture supernatant was demonstrated to be an important factor for the suppression. After a 2-week feeding of LAB 4012, the growth rate of the fed cobia was 12% higher than that of the non-fed group, and the relative percentage of survival (RPS) of the fed cobia was found to be 74.4 in *Pdp* immersion challenge. In addition, the respiratory burst (RB) of peripheral blood leukocytes (PBL) in the LAB 4012-fed group was significantly higher than that of the non-fed group. Although feeding LAB 4012 did not improve specific antibody response in cobia after immunization with *Pdp* vaccine, it still significantly raised the survival rate by 22% over that of the non-fed group after *Pdp* immersion challenge. Judging by the quick induction of high protection against *Pdp* infection and promotion of growth in larvae, LAB 4012 was considered to be a viable probiotic for cobia aquaculture.

1. Introduction

Cobia (*Rachycentron canadum*) is a tropical and subtropical marine fish, and has only one species under Genus *Rachycentridae*. Due to its good-quality meat and extraordinarily high growth rate, cobia became an important cultured fish in Taiwan during 1990s [1]. Following the successful development of cobia aquaculture technology in Taiwan, cobia now becomes one of the highest priority species for large-scale commercial aquaculture in the Americas and the Caribbean [2,3]. However, the outbreaks of photobacteriosis in sea-cage reared cobia have frequently occurred in Taiwan since 1999, which resulted in vast economic loss [4].

The causative agent of photobacteriosis is *Photobacterium damselae* subsp. *piscicida* (*Pdp*), a rod-shaped gram-negative bacterium, which can induce whitish tubercles in the internal organs of chronically infected fish [5,6]. Photobacteriosis is a worldwide disease, and has provoked mass mortality in many other cultured marine fish, especially at the early grow-out stage, including cobia, sea bass, flounder, breams and yellowtail [7–10]. The major control of photobacteriosis has been usage of antibiotics, but more and more drug-resistant strains of *Pdp* were isolated from medicated or moribund cobia cultured in Taiwan [7]. Several *Pdp* vaccines and vaccination programs were developed [11–15]; however, the efficacy of the vaccines available on market is not consistent in the field. Consequently, probiotic is considered an alternative for the control of photobacteriosis.

Probiotics have been applied in aquaculture, and there are multiple mechanisms of health improvement by probiotics [16–18].

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For example, some probiotics can interact or antagonize with other enteric bacteria through colonization resistance, or direct inhibit and reduce the incidence of opportunistic pathogens. Probiotics may also enhance the health of host via physiological or immune modulation. Some probiotics can assist the process of digestion via producing extracellular enzymes or products necessary for hosts.

Lactic acid bacteria (LAB) have been used as probiotics in many studies, and 1–10% of enteric bacteria were reported to exhibit the probiotic potential [19]. The production of organic acid and bacteriocin of LAB are able to inhibit or directly kill many microbes. The resistance capability of LAB against gastric acid and bile enhances its livability through the digestive tract. In this study, a strain of lactic acid bacteria LAB 4012 was isolated from the intestine of adult cobia, and its classification was identified. In order to evaluate the probiotic potential of LAB 4012 in cobia, the inhibition ability of LAB 4012 metabolites on *Pdp* growth *in vitro*, and the feeding impact of LAB 4012 on cobia growth rate, respiratory burst, the resistance against *Pdp* immersion challenge, and the synergetic protection efficacy with *Pdp* vaccine were examined.

2. Materials and methods

2.1. Bacteria

Pdp strain P40, a gift from Dr. Chen-Chun Ku (National Penghu University, Taiwan), is cultured in brain heart infusion (BHI) broth (Becton, Dickinson and Company) with 20‰ salinity at 28 °C. The identification and characterization of *Pdp* strain P40 has been described in previous paper [7].

The LAB 4012 strain was provided by SynBioTech company (Taipei, Taiwan). The LAB 4012 was isolated from the intestine of a 3-day fasted adult cobia by Dr. Hung-Hsi Hu, and cultured in MRS broth (Fluka) at 37 °C, and was proved to be able to resist the treatments of gastric acid (pH 2.0) and 0.3% bile salt. The species name of LAB 4012 was determined by sequencing LAB 16S ribosomal DNA (rDNA) and comparing with the reference strains derived from the GenBank. PCR was carried out using forward primer 5′-AGAGTTTGATCATGGCTCAG-3′ and reverse primer 5′-AAGGAGGTGATCCAGCC-3′ [20] on PCR System 2700 (Applied Biosystems), and PCR products were sequenced by ABI 3730 (Applied Biosystems). Phylogenetic analysis of LAB 4012 16S rDNA with reference strains was performed using software MEGA 5.0.

2.2. The anti-Pdp activity of LAB 4012 metabolites

To examine the impact of LAB 4012 culture supernatant on the growth of *Pdp*, LAB 4012 was cultured in MRS broth at 37 °C for 18 h. Then, the culture broth was collected by centrifugation at 1600 × *g* for 15 min at 4 °C. The culture supernatant was passed through by 0.45 μm pore-size filter unit (Minisart). After filtrating, 1 mL of tested supernatant was added into 4 mL of Brain Heart Infusion (BHI) (Difco) and then inoculated with 0.1 mL of *Pdp* with the optical density value (OD_{595}) of 1.

The original pH in MRS without LAB 4012 was pH 6.2; however, the pH of the LAB 4012 culture supernatant decreased to 4.1 after 18 h of growth, and the concentration of lactic acid in LAB 4012 supernatant was quantified to be 73 mM by Lactate assay kit (Biovision).

To examine the influence of pH and lactic acid on the *Pdp* growth, additional two tested groups were included. One was an 18 h-culture supernatant of LAB 4012 with adjusted pH value of 6.2 by NaOH, and another tested group contained MRS medium supplemented with lactic acid at a final concentration of 73 mM. Similarly, 1 mL of each test supernatant was added into 4 mL of Brain Heart Infusion (BHI) (Difco) and inoculated with 0.1 mL of *Pdp* ($OD_{595} = 1$). The bacterial mass of *Pdp* after different treatments at each time point was measured at OD_{595} by MRX II ELISA reader.

2.3. The LAB 4012 diet supplementation

The LAB 4012 was cultured in MRS broth at 37 °C until the OD_{595} of culture reached 2.0, and then concentrated by centrifuging at 1500 × *g* for 15 min. After washing with phosphate buffered saline (PBS) three times, LAB 4012 pellet was re-suspended in PBS ($OD_{595} = 2$) and sprayed onto the commercial dry feed at a ratio of 1:3 (w/w). The prepared LAB-containing feed was preserved at 4 °C and used up within five days. The titer of live LAB in the prepared feed was examined by re-dissolving the prepared feed in PBS for CFU counting.

Two groups of cobia were used for LAB 4012 feeding test separately with body weight of 4.6 g and 35 g. All cobia used in this study were raised from Pingtong, Taiwan. Fish were held in FRP (Fiberglass Reinforced Plastics) tanks, supplied with aerated 30‰ salinity sea water at 25 °C, and fed with commercial dry feed (Omega 3rd Marine fish feed, Golden Prawn Enterprise Corp). The fish condition was monitored, and only the healthy fish were used for *in vivo* test. The dose of LAB in the prepared feed was 10^9 CFU g^{-1}, and the fish were fed twice daily.

2.4. Pdp immersion challenge test

All challenge tests in this study were conducted by immersion. The fish were fasted for one day before challenge test. The fish were placed in the sea water at 20 °C, and the immersion time of *Pdp*-containing sea water was 2 min. At the end of challenge test, all dead fish were sampled and examined for white tubercles in the internal organs. Furthermore, *Pdp* was re-isolated from the kidney and spleen, and identified using Bionor Mono-Pp kit. Accumulated mortality was recorded for ten days, and the relative percentage of survival (RPS) was calculated according to the formula: RPS = [1 − (Mortality of test group/Mortality of control group)] × 100.

2.5. The experimental design of feeding trial among the cobia without vaccination

The fish with average body weight of 4.6 g were randomly divided into LAB-fed group (LAB+) and control group (LAB−). To detect the LAB 4012 in the intestine of cobia, microbial analyses were performed at three time points: before the feeding trial, at the end of the 2-week feeding, and one week post the end of feeding trial. Five fish were sacrificed at each time point. The intestine was dissected into pieces and put into 5 mL of MRS (the selective medium for lactic bacteria) to release the bacteria into medium. After appropriate dilution, the supernatant was plated onto the MRS agar plate, and incubated at 37 °C. The isolated bacteria colonies grown on MRS agar were identified by sequencing the 16S rDNA as described in Section 2.1.

The number of fish in LAB+ and LAB− group was 51, and the fish in each group were further divided into 3 subgroups (17 fish per subgroup) so that all tests were performed in triplicate. In LAB+ group, the fish were fed with 5% body weight of LAB 4012-mixed feed (10^9 CFU g^{-1}) for two weeks, while the fish in control group were fed with feed without LAB 4012. The growth rate of the fish, the RB of PBL, and the mortality after *Pdp* immersion challenge test were monitored at the end of the 2-week feeding trial.

The RB of PBLs was determined by the nitroblue tetrazolium (NBT) assay described by Choudhury et al. (2005) [21]. To prepare PBLs, blood samples were collected from the cobia. Six fish taken from respective group were sacrificed after bleeding. The blood