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Effects of dietary supplementation of *Lactobacillus pentosus* PL11 on the growth performance, immune and antioxidant systems of Japanese eel *Anguilla japonica* challenged with *Edwardsiella tarda*

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ABSTRACT

The aim of this study was to determine the efficacy of dietary administration of *Lactobacillus pentosus* PL11 on growth performance and the immune and antioxidant systems in Japanese eel *Anguilla japonica* challenged with *Edwardsiella tarda*. A total of 75 Japanese eels (24.63 ± 0.83 g) were grouped into 5 treatment diets which were a control diet (C) without *E. tarda* and 4 treatment diets with *E. tarda* challenge, including C for *E. tarda* challenge (NC), C plus *L. pentosus* PL11 supplemented diet (10^8 cfu g^{-1}) (T-PL11), C plus *L. pentosus* KCCM 40997 supplemented diet (10^8 cfu g^{-1}) (T-Lp) and C plus *Weissella hellenica* DS-12 supplemented diet (10^8 cfu g^{-1}) (T-Wh) for 5 weeks (4 week before and 1 week after challenge). The results showed enhanced growth performance in fish fed the diet containing *L. pentosus* PL11 compared to others. The growth performance parameters including specific growth rate (SGR) and weight gain (WG), feed intake (FI), feed conversion ratio (FCR) and survival were significantly ($P < 0.05$) higher in fish maintained on *L. pentosus* PL11 supplemented diet compared to C and NC. T-PL11 group also shows a significant increase in the levels of plasma immunoglobulin M, CAT and SOD activities compared to NC. Hematological parameters and mieloperoxidase were significantly better in fish fed the *L. pentosus* PL11 supplemented diet than in the control. *L. pentosus* PL11 supplementation recover the reduced expression of SOD, CAT and heat shock protein 70 genes in liver and intestine in pathogen challenged fishes. In conclusion the result of the current study demonstrated *L. pentosus* PL11 potential as an alternative to antibiotic supplementation to improve the growth and health performance of Japanese eel (*A. japonica*).

1. Introduction

Edwardsiella tarda is the etiological agent of several pathologic symptoms particularly causing septicemia of freshwater and marine fish and affects a wide range of hosts including mammals [1,2]. The ability of the bacteria to infect hosts is partly due to detoxification of reactive oxygen species by enzymes such as catalase, peroxidase and superoxide dismutase [3].

To date, antibiotic therapy is the principal means of controlling disease caused by *E. tarda.* However, intensive use of antibiotics is being discouraged for reasons of the emergence of antibiotic resistance [4]. Therefore, alternative methods of disease prevention are being investigated to minimize such risk [5]. Among these, the use of probiotics in aqua feed has been studied [6,7], especially for protection against infectious diseases [8].

The use of probiotics is an alternative treatment option that contributes to the healthy development of aqua-cultured animals mainly through its role played in the immune and antioxidant systems. Numerous studies focused on adult or juvenile fish anti-oxidant enzyme protection when ROS are generated by xenobiotics or fish exposure to pathogens [9]. Recent studies show that dietary *Debaryomyces hansenii* stimulates both immune and antioxidant responses in Gilthead Sea bream and leopard grouper *Mycteroperca rosacea* [9,10] after exposure to pathogens.

Most of the probiotic studies have used lactobacillus and bacillus isolated from non-marine sources or marine bisources [11].

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Table 1
The percentage composition of the experimental diet used in the experiment.

Ingredients	%	Index	Approximate composition (% wet weight)
Fish meal	71.4	Crude protein	51.66
Potato flour	23	Crude fat	6.35
Mineral mix[a]	0.5	Crude fiber	0.39
Vitamin mix[b]	0.6	Crude ash	11.36
Mono-calcium phosphate[c]	0.5	Calcium	2.23
Choline chloride[d]	0.2	Phosphorus	1.41
Lecithin[e]	0.3	0.0002% Pepsin digestibility	80.08
Casein (China)	1		
20% Trehalose[f]	2.5		
Total	100		

[a] Mineral mix composition : Vitamin A 50,000 IU, vitamin D3 5000 IU, $MnSO_4$ 8000 mg, $ZnSO_4$ 25,000 mg, $FeSO_4$ 50,000 mg, peptide Fe 2000 mg, $CuSO_4$ 2000 mg, $MgSO_4$ 40,000 mg, KCl 192,310 mg, $AlOH_3$ 250 mg, $Ca(IO_3)$ 2625 mg, $CoSO_4$ 100 mg. Woosungvet (Nonsan, Choongnam, Korea).
[b] Vitamin composition: vitamin A 2,700,000 IU, vitamin D 500,000 IU, vitamin E 30,000 mg, vitamin K 15,000 mg, vitamin B1 5500 mg, vitamin B2 10,000 mg, vitamin B6 3800 mg, vitamin B12 30 mg, vitamin C 80,000 mg, niacin 30,000 mg, folic acid 1250 mg, biotin 75 mg, inositol 25,000 mg, cal-pan 18,000 mg. Woosungvet (Nonsan, Choongnam, Korea).
[c] Yara, Helsingborg, Sweden.
[d] Kofavet special, Seoul, Korea.
[e] Eromfeed, Seoul, Korea.
[f] TREHA, hayashibara, Okayama, Japan.

Here, we hypothesized probiotic bacteria of marine origin, such as marine sediment, seaweed, fish farm, and sea-water could represent a new and effective treatment for fish disease.

Therefore, in the current study, we investigate the efficacy of dietary administration of *Lactobacillus pentosus* PL11 isolated from Japanese eel *Anguilla japonica* on growth performance and the immune and antioxidant systems in Japanese eel *A. japonica* challenged with *E. tarda*.

2. Materials and methods

2.1. Microorganisms

The strain *L. pentosus* (PL11) was isolated from intestinal tract of the healthy Japanese eel (*A. japonica*) according to its morphological and biochemical characteristics as described previously [12]. The positive control strain (*L. pentosus* KCCM 40997) was obtained from the Korean Culture Center of Microorganisms (Seoul, Korea) and *Weissella hellenica* DS-12, which is a commercial fish probiotic was obtained from Daesung microbiolgical lab (Fishlac, Daesung microbiolgical Lab, Korea). These LAB strains (*L. pentosus* PL11, *L. pentosus* KCCM 40997 and *W. hellenica* DS-12) were cultured in MRS broth at 30 °C with constant aeration until the stationary phase (21 h). The viability of LAB strains was determined by counting the number of colonies plated on MRS agar (BD, Sparks, MD, USA).

The fish pathogens *E. tarda* (*E. tarda*) FP2018 was obtained from the National Fisheries Research and Development Institute (Busan, Korea) during natural outbreaks.

2.2. Fish, diet and experimental design

Juvenile Japanese eel *A. japonica* (24.63 ± 0.83 g body weight) were provided by "Myung-Il" farm, Jeon-nam province, Korea. Fish were acclimated to laboratory conditions for two weeks prior to experiments at 26 ± 2 °C with 24 h dark period. They were fed 3% body weight twice a day with a fish diet without antibiotics.

In a 5-week study, fish (25.62 ± 2.54 g) were randomly placed in 1 of 5 dietary treatment groups. There were 15 fish per treatment group, which were allocated: 5 fishes per running water experimental tank and 3 tanks per treatment group. The five dietary treatment were as follows: (1) a control diet (Table 1) (C); (2) C for pathogenic *E. tarda*-challenge experiment (NC); (3) C plus 10^8 cfu g^{-1} *L. pentosus* PL11 (T-PL11); (4) C plus 10^8 cfu g^{-1} *L. pentosus* KCCM 40997 (T-Lp); and (5) C plus 10^8 cfu g^{-1} *W. hellenica* DS-12 (T-Wh). The fishes were fed 1 of the 5 test diets for 4 weeks before pathogenic *E. tarda* challenge. After 4 weeks of the experimental period, all groups except C were injected 0.1 mL dose of 3.5×10^8 cfu mL^{-1} pathogenic *E. tarda* intra-peritoneally. After the challenge, all fishes were observed for 1 more week to record clinical signs and daily mortality. At the end of the experiment, the liver and whole intestines were subject to microbiological examination to determine the presence of *E. tarda* and Lactobacillus spp.

An initial body weight was taken at the beginning of the experiment with subsequent fish body weight and feed disappearance measurements obtained at the end of the experiment. Body weights and feed intake were used to determine the weight gain (WG), daily feed intake (FI) and feed conversion ratio (FCR).

2.3. Blood and serum analysis

Fishes were starved for 24 h, and then euthanized with ethyl 3-aminobenzoate methanesulfonate (MS-222, Sigma–Aldrich, USA) and blood samples were collected from the caudal vein. Serum and plasma were separated by centrifugation for 10 min at $2000 \times g$ and then stored at 70 °C until further analysis. Erythrocytes and leukocyte counts were achieved using a hemocytometer at a magnification of 400×, plasma protein content using BCA™ protein assay kit, myeloperoxidase (MPO) activity in serum using MPO activity assay kit (Bio-Vision, Mountain View, CA, USA) according to the manufacturer instructions were performed. Serum IgM levels were measured using commercial ELISA kit (Cusabio, Wuhan, Hubei, China) according to manufacturers' instruction.

2.4. Liver and intestine enzyme analysis

Liver enzyme activities including superoxide dismutase (SOD) and catalase (CAT) were examined from supernatants of 0.1 g of liver

Table 2
Oligonucleotides primer sequences used in the detection of fish cytokines and 18S.

Gene specificity	Forward and reverse primers	Accession number
SOD[a]	F: AGGCATGTTGGAGACCTGG R: ATTGCCTGTCTTTAGACTCTC	
HSP70[a]	F: GACGTGTCCATCCTGACCAT R: TCGATGCCCTCAAACAGAGA	
CAT[a]	F: TGACATGGTGTGGGACTTCTGG R: CTTGTAGTGGAACTTGCAGTAG	
SOD[b]	F: GTTGGAGACCTGGGAGATGT R: CTCCTCATTGCCTCCTTTTC	FJ860004
HSP70[b]	F: CCATCCTGACCATCGAAGAC R: GTTCTCTTGGCCCTCTCACA	AY423555
CAT[b]	F: ATGGTGTGGGACTTCTGGAG R: AGTGGAACTTGCAGTAGAAACG	FJ860003
18[c]	F: CGAGCAATAACAGGTCTGTG R: GGGCAGGGACTTAATCAA	AF2434282

[a] Oligonucleotide primers used for primary PCR.
[b] Oligonucleotide primers used for real time PCR.
[c] 18S rRNA as an internal control for real time PCR.