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Effect of potential probiotic *Rhodotorula benthica* D30 on the growth performance, digestive enzyme activity and immunity in juvenile sea cucumber *Apostichopus japonicus*

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ABSTRACT

The effects of dietary addition of yeast *Rhodotorula benthica* (*R. benthica*) D30 which isolated from local sea mud at levels of 0 (control), 10^5, 10^6 and 10^7 CFU/g feed on the growth performance, digestive enzyme activity, immunity and disease resistance of juvenile sea cucumber *Apostichopus japonicus* were investigated. It was shown that dietary addition of *R. benthica* D30 significantly increased the growth rates of sea cucumbers ($p < 0.05$). The amylase activity, cellulase activity and alginase activity were increased for the animals from three probiotics treated groups. And with the supplemented concentration increased, the values of those digestive enzyme activities increased as well. Dietary addition of *R. benthica* D30 at the level of 10^7 CFU significantly increased the lysozyme, phagocytic and total nitric oxide synthase activity of *A. japonicus* ($p < 0.05$). While, the highest values of the phenoloxidase and alkaline phosphatase activity were found in sea cucumbers fed with *R. benthica* D30 at the level of 10^6 CFU. Whereas adding *R. benthica* D30 to diet had no significant effects on the total coelomocyte counts and acid phosphatase activity of *A. japonicus* ($p > 0.05$). It was observed that adding *R. benthica* D30 could significantly decrease the cumulative mortality of sea cucumbers. The present study demonstrated that dietary addition of *R. benthica* D30 could increase growth performance and some digestive enzyme activities, improve immunity and disease resistance of *A. japonicus*. And the medium (10^6 CFU) and high (10^7 CFU) additional levels showed better effects. It suggests that yeast *R. benthica* D30 could be a good probiotic for aquaculture.

1. Introduction

The sea cucumber *Apostichopus japonicus*, belonging to the phylum Echinodermata and with a distribution mainly along the coast of northwest Pacific, is one of the most important farmed species in the northern China. Along with their tonic value knowing gradually by consumers in China, the demand for *A. japonicus* is surging in the domestic market, and the sea cucumber farming has developed rapidly [1]. However, the vigorous development resulted in high density of animals in hatchery tanks and ponds facilitates which caused serious problems especially the outbreaks of epidemic diseases. It is reported that the skin ulceration syndrome had caused large economic losses for *A. japonicus* aquaculture industry [2]. To solve these problems, antibiotics and chemicals are generally used in aquaculture which leads to antimicrobial resistance in animals, meanwhile the antibiotic and bioaccumulation of chemical residues in final product. These problems strongly limit and challenge the development of sea cucumber industry [3].

Recently, probiotics as the environment friendly agents attracted the interest of scientists, they are thought to be potential substitute of antibiotics for sea cucumber farming. Probiotics are live microorganisms providing health benefits to the host by improving its microbial balance and digestive processes, promoting growth, enhancing the immune response and antiviral effects when given in adequate amounts [3]. Among the probiotics used in sea cucumber farming, *Bacillus subtilis* was the most documented [4–6], and limited researches focused on yeast [7,8]. *Rhodotorula benthica* is a pigmented yeast commonly isolated from sea mud and animal digestive tract [9]. Since it is rich in nutrition ingredients, such as

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proteins, vitamins, fatty acids, carotenoid especially powerful antioxidant astaxanthin, it could be a good feed additive for aquaculture industry [9]. It is reported that *R. benthica* has been used in shrimp *litopenaeus vannamei* [10,11], abalone [12] and fish *Scophthalmus maximus L.* [9,13]. farming. While, very little information was available about the effects of *R. benthica* on sea cucumber farming. Therefore, the aims of this study were to evaluate the effects of potential probiotic *R. benthica* D30 which was isolated from sea mud on growth, digestive enzyme activity and immunity in sea cucumber *A. japonicus*.

2. Materials and methods

2.1. Probiotic preparation

The probiotic used in this study was isolated from mud of sea cucumber water bodies, Wafangdian district, Dalian, China. It was identified as *R. benthica* by 16S rRNA sequencing and we named it as D30. The safety of *R. benthica* D30 was tested by immersing sea cucumbers with *R. benthica* D30 at concentration of 10^8 CFU mL^{-1} for 7 days. The safety test showed that *R. benthica* D30 did not induce disease symptoms and mortality of sea cucumbers.

2.2. Experimental diets

The basal diet was a commercial diet provided by Dalian Boss-all Bio-Tech. Ltd., China. The formulation of basal diet was shown in Table 1. The probiotic strain was cultured for 18 h at 28 °C in shaken bottles with a liquid yeast extract peptone dextrose medium. And the fresh cells were adjusted to 2×10^8 CFU mL^{-1} in sterile saline. The experimental diets were prepared by supplementing graded doses of *R. benthica* D30 at 0 (control), 10^5, 10^6, 10^7 CFU/g feed, respectively. All experimental diets were prepared daily in order to guarantee the vitality of *R. benthica* D30.

2.3. Feeding experiment

Juvenile sea cucumbers were obtained from a commercial farm located in Dalian, China. All sea cucumbers were fed with the basal diet for 7 days to make the animals acclimate to the experimental diet and the rearing environment. Then the sea cucumbers were starved for 24 h and 360 individuals (0.467 ± 0.012 g, means ± SD) were selected and randomly distributed into 12 tanks containing 20 L fresh seawater, with 30 sea cucumbers for each tank. The experiment was designed with 4 treatments which dietary addition of yeast D30 at levels of 0 (control), 10^5, 10^6 and 10^7 CFU/g feed respectively, and 3 replicate tanks per treatment. During the 30-day feeding trial, all sea cucumbers were fed diets twice (08:00 and 20:00) daily at a rate of 10% body weight. And 20% water in each aquarium was replaced with fresh seawater every day. The remain diets and faeces of each tank were cleared daily by siphoning. The water temperature ranged from 20 to 23 °C, salinity 28 to 31, pH 7.8 to 8.3 and dissolved oxygen was no less than 6 mg L^{-1}. The survival rate of each tank was recorded daily.

Table 1
The formulation of the basal diet (% dry matter).

Ingredients	Concentration (%)
Sargassum sp. meal	70
Fermented soybean meal	12
Soybean peptide	1.5
Sea mud	5.0
Stone power	5.0
Scallop skirt power	5.0
Yeast cell wall	0.6
Antimicrobial peptide	0.4
Vitamin C	0.5

2.4. Sample collection

At the end of the feeding trial, sea cucumbers were not fed for 24 h. And 15 sea cucumbers from each tank were randomly sampled. The selected animals were weighed and dissected immediately with sterile scalpel in a sterile plate. The coelomic fluid of samples was collected immediately, and then thoroughly mixed with equal volume anticoagulant (0.02 M EGTA, 0.34 M NaCl, 0.019 M KCl, 0.068 M Tris-HCl, pH 8.0). The coelomic fluid from fifteen sea cucumbers of one replication was pooled together for immunological analysis. After an aliquot of the coelomic fluid sample was taken for total coelomocyte counts (TCC) and phagocytic activity test, the left samples were stored in liquid nitrogen until enzyme activity parameters were measured. After coelomic fluid in each tank was thawed respectively, it was centrifuged at 3000 × *g* for 10 min at 4 °C, the supernatants were collected and immediately used for analyzing lysozyme (LSZ), phenoloxidase (PO), superoxide dismutase (SOD), total nitric oxide synthase (T-NOS), alkaline phosphatase (AKP) and acid phosphatase (ACP) activities.

The whole gut was removed by an incision at the esophagus and cloaca. It was then washed thoroughly in ice-cold deionized water and blotted with filter. The guts from fifteen sea cucumbers of one replication were pooled and stored in liquid nitrogen until analysis. After guts were thawed, weighed and homogenized, they were mixed with 10 volumes of ice-cold buffer (phosphate buffered saline, 0.02 M, pH 7.5). The homogenates were centrifuged at 4 °C, 10000 × *g* for 20 min. Then the supernatant was pipetted into clean centrifuge tubes and stored at 4 °C until analysis. The enzyme activities were analyzed within 12 h.

2.5. Digestive enzyme assays

The soluble protein concentration was determined using the classical Bradford method [14] using bovine serum albumin as a standard. Protease activity was evaluated according to Anson [15] using Folin-phenol reagent, and amylase activity was measured according to Rick and Stegbauer [16] using iodine solution to reveal non-hydrolyzed starch. Lipase activity was determined through measuring the free fatty acids production from enzymatic hydrolysis of triglycerides in stabilized emulsion of olive oil [17]. Cellulases and alginase activity was measured according to the method of 3,5-dinitrosalicylic acid (DNS) using carboxymethyl cellulose (CMC, 0.5%) and sodium alginate (0.5%) respectively.

2.6. Immune assays

2.6.1. TCC

Coelomocytes were counted and calculated as cells per mL sample using a hemocytometer (Qiujing Inc., Shanghai, China) under light microscope at 400 × magnification.

2.6.2. Phagocytic activity

Phagocytic activity of coelomocytes was measured through assessing the uptake of neutral red stained zymosan particles according to the method described by Pulsford et al. [18].

2.6.3. LSZ activity

LSZ activity was measured according to the method described by Hultmark et al. [19] with minor modification. The *Micrococcus lysodeikticus* was cultured on nutrient agar plate at 28 °C. And the