Animal Feed Science and Technology 197 (2014) 142–147

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci

Beneficial effects of *Metschnikowia* sp. C14 on growth and intestinal digestive enzymes of juvenile sea cucumber *Apostichopus japonicus*

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ARTICLE INFO

Article history:
Received 21 April 2014
Received in revised form 28 July 2014
Accepted 29 July 2014

Keywords:
Apostichopus japonicus
Metschnikowia sp. C14
Colonization
Growth
Intestinal digestive enzyme

ABSTRACT

We investigated the effects of dietary administration *Metschnikowia* sp. C14 on growth and intestinal digestive enzymes of juvenile sea cucumber *Apostichopus japonicus*. Sea cucumbers were fed with diets containing C14 at 0 (control), 10^4, 10^5 and 10^6 CFU/g feed, respectively. After feeding for 45 days, the specific growth rate was higher in sea cucumbers fed C14-supplemented diets at 10^4 and 10^5 CFU/g feed than in the controls ($P<0.05$). The intestinal trypsin and lipase activities in sea cucumbers were significantly enhanced by C14 administration at 10^4, 10^5 and 10^6 CFU/g feed compared to those of the controls ($P<0.05$). After feeding for 23–42 days, the presence of C14 was evidenced in the intestinal community of sea cucumbers by denaturing gradient gel electrophoresis (DGGE). The intestinal fungal communities of sea cucumbers fed C14-supplemented diets were different from that of the control on day 42. Additionally, sea cucumbers were switched to the control diet from day 16 to day 46. As confirmed by DGGE, C11 colonization of the intestine could be detected until day 46. The present study demonstrates that C14 is able to successfully colonize the intestine through dietary supplementation, and that this colonization improves the growth and intestinal digestive enzymes of juvenile *A. japonicus*.

1. Introduction

The rapidly expanding sea cucumber *Apostichopus japonicus* aquaculture industry has experienced relatively severe setbacks as a result of the frequent outbreaks of epidemic diseases (Ma et al., 2006; Deng et al., 2009; Li et al., 2010). Currently, the use of probiotics as one of the environmental friendly methods controlling diseases has become an important subject of investigation in sea cucumber aquaculture research (Zhang et al., 2010; Zhao et al., 2012; Liu et al., 2012; Ma et al., 2013). Probiotics are preparations of microbial cells or cell components, containing live and/or dead bacteria that confer a health benefit on the host (Salminen et al., 1999). Appropriate probiotics applications have been shown to improve growth and/or digestive enzyme activity of the host organisms, such as fish (Tovar-Ramírez et al., 2002, 2004), shrimp (Ziaei-Nejad et al., 2006; Liu et al., 2009), abalone (Macey and Coyne, 2005; ten Doeschate and Coyne, 2008) and sea cucumber (Zhang et al., 2009, 2010; Zhao et al., 2012). The intake of probiotics has been demonstrated to modify the composition of the microbiota

Abbreviations: DGGE, denaturing gradient gel electrophoresis; MEA, malt extract agar; YPD, yeast-peptone-dextrose; SGR, specific growth rate; BSA, bovine serum albumin; ANOVA, analysis of variance; PCR, polymerase chain reaction; UV, ultraviolet.

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http://dx.doi.org/10.1016/j.anifeedsci.2014.07.013
0377-8401/

and assist in returning a disturbed microbiota to its normal healthy composition (Gómez and Balcázar, 2008). DGGE are used to determine the bacterial diversity (Kim and Austin, 2006; Amaro et al., 2009; Sáenz de Rodrigáñez et al., 2009; Tapia-Paniagua et al., 2010) and the intestinal colonization of probiotics following their administration to rainbow trout (Kim and Austin, 2006). Previously, the marine yeast *Metschnikowia* sp. C14 was found to stimulate the immune response of juvenile *A. japonicus* and enhance their resistance against *Vibrio splendidus* infection (Liu et al., 2012). This study was intended to assess the effects of *Metschnikowia* sp. C14 on growth, intestinal digestive enzymes and microbial community of *A. japonicus*.

2. Materials and methods

Healthy sea cucumbers, *A. japonicus*, were collected from the waters around Bailanzi, Dalian, China. The intestines were removed and homogenized in a sterile glass homogenizer with sterile filtered seawater. Appropriate dilutions were prepared and 0.01 ml volumes were spread over the surface of plates of malt extract agar (MEA) with incubation at 25 °C for 3–5 days. Colonies were picked according to different colonial-morphology, pure cultures were obtained by repeated subculture on the above plate and stored at −80 °C as suspensions in 30% (v/v) glycerol. Using overlay technique, C14 was found to be inhibitory against *Shewanella marisflavi* AP629 *V. splendidus* NB13 which caused the skin ulceration syndrome disease and acute peristome edema disease in sea cucumber. By 26S rDNA sequencing, C14 was identified as *Metschnikowia bicuspidate* (sequence homology = 99%) (Liu et al., 2012). *Metschnikowia* sp. C14 was cultured in yeast–peptone–dextrose broth (YPD medium) at 25 °C with constant aeration until the early stationary phase. The cellular suspension was centrifuged at 1000 × *g* for 10 min at 4 °C and the resultant pellet was washed twice with sterile saline and re-suspended in saline for incorporation into the diet.

A basal diet comprising soybean meal 100 g/kg, fishmeal 80 g/kg, *Sargassum* sp. meal 250 g/kg, 30% degumming kelp powder 300 g/kg, maifanitum 100 g/kg, stone power 165 g/kg and 5 g/kg multidimensional premix was used as the control in this study. The chemical composition of basal diet was crude protein 163 g/kg, crude lipid 55 g/kg, crude fiber 161 g/kg and ash 308 g/kg. The experimental diets were prepared daily by supplementing graded doses of C14 at 0 (control), 10^4, 10^5 and 10^6 CFU/g feed, to ensure the vitality of C14. Juvenile sea cucumbers were obtained from a commercial farm in Dalian, China. Prior to the feeding trial commencing, sea cucumbers were acclimated to the rearing conditions for 2 weeks. Selected sea cucumber (1.37 ± 0.56 g) was randomly distributed into 12 plastic tanks (100-l) at a density of 50 individuals per tank. Three tanks were randomly assigned to each of the dose diets. During the 45–46 day feeding trial, all sea cucumbers were fed once daily (16:00 h) at a rate of 5% of body weight per day. Half of the water in each tank was replaced with fresh seawater every other day. Feces and any remaining food were removed daily by siphoning. Low pressure electrical air pumps provided aeration via air stones. Water parameters remained within the following ranges throughout the experiment: temperature 7–12 °C, salinity 33–34, and pH 7.8–8.2 (Liu et al., 2012). For the colonization study, 20 sea cucumbers from each dietary treatment were removed to separate tanks and fed the control diet from day 16 to day 46.

Growth performance was determined and calculated using the following equation: specific growth rate (SGR) (%/day) = 100 [ln final weight (g) − ln initial weight (g)]/45 days.

At the termination of the feeding trial, sea cucumbers were transferred to another tank containing sea water for 16 h to empty the intestine. Five individuals from each tank were sampled randomly for determining trypsin and lipase activities. The animals were dissected immediately and the whole intestines were removed by incision at the esophagus and cloaca. The samples were blotted dry with filter paper, weighed and homogenized in nine volumes of ice-cold 0.85% NaCl solution using a manual glass homogenizer. Homogenates were then centrifuged and supernatants were pipetted into clean centrifuge vials and analyzed. Total protein of the supernatants was determined according to Bradford (1976) using bovine serum albumin (BSA) as the standard. Trypsin activity was assayed according to Holm et al. (1988) using commercial kits (Nanjing Jiancheng Bioengineering Institute, China). The reaction was based on trypsin's ability to hydrolyze L-arginine ethyl ester, which resulted in an increase in optical density when measured at 253 nm. One unit of trypsin activity was defined as the amount of enzyme causing an increase in absorbance of 0.003 per min per mg protein in intestine materials at 37 °C and pH 8.0. Lipase activity was measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, China) according to the method described by Shihabi and Bishop (1971). The reaction was based on lipase's ability to hydrolyze triglyceride in stabilized emulsion of olive oil, which resulted in a decrease in optical density when measured at 420 nm. One unit of lipase activity was defined as 1 μmol substrate consumed per min per g protein in intestine materials at 37 °C.

Sea cucumbers were transferred to another tank containing sea water for 16 h to empty the intestine before sampling. After the beginning of the feeding trial, the whole intestine of five sea cucumbers (one or two from each tank) from each treatment, and from the additional three tanks used for the colonization study, were aseptically removed on days 23, 27, 37, and also on days 46 or 42 (depending on whether the diet was changed at day 16 or not). Intestines were pooled and stored separately at −80 °C until further analysis. Extraction of DNA from pooled samples followed the method of Martin-Laurent et al. (2001). For analysis of fungal community, the D1 domain of the 26S rDNA was amplified by a nested polymerase chain reaction (PCR) using the method of Prakitchaiwattana et al. (2004). PCR products were analyzed by DGGE using Dcode™ Universal Mutation Detection System (Bio-Rad). PCR samples were directly loaded onto 8% (w/v) polyacrylamide gels in a running buffer (1% TAE). The gels were prepared with a denaturing gradient from 20% to 50% of urea and formamide and a polyacrylamide and bis-acrylamide ratio of 37.5:1. Electrophoresis was performed at a constant voltage of 75 V and a constant temperature of 60 °C for 12 h. Afterwards, the gels were stained with GenFinder according to the manufacturers' instructions and photographed under ultraviolet transillumination.