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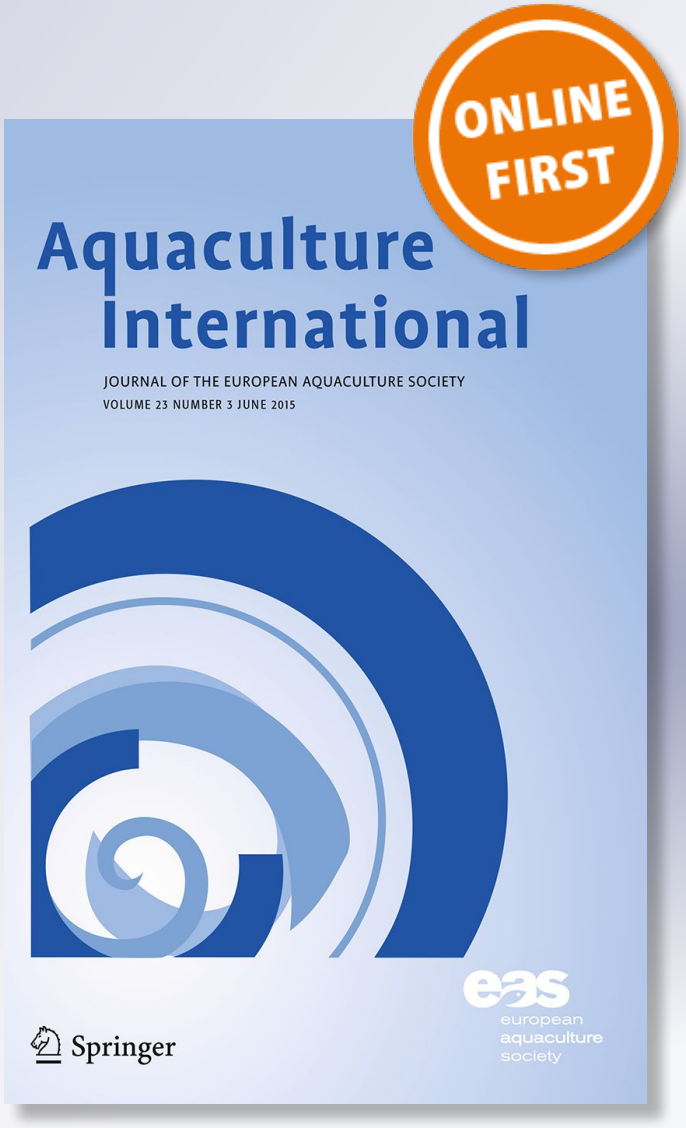
Aquaculture International
Journal of the European Aquaculture Society

ISSN 0967-6120

Aquacult Int
DOI 10.1007/s10499-017-0148-8



Springer

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Alteration of growth, intestinal *Lactobacillus*, selected immune and digestive enzyme activities in juvenile sea cucumber *Apostichopus japonicus*, fed dietary multiple probiotics

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Received: 12 October 2016 / Accepted: 3 April 2017

Abstract To assess the influences on growth performance, intestinal *Lactobacillus*, immune and digestive enzyme activities of juvenile sea cucumber *Apostichopus japonicus* by administrating multiple probiotics. Multiple probiotics (0, 6×10^7 and 9×107 CFU g^{-1}) and normal basal feed were mixed thoroughly at different doses of diet (T0, T1, and T2, respectively) and were administered orally to sea cucumbers for 90 days. After the feeding trial, 20 sea cucumbers were randomly sampled from each pond. The results showed that administration of multiple probiotics significantly affected on the growth performance, non-specific immune enzymes, and microbial ecology of the gut of sea cucumbers ($P < 0.05$). However, the lysozyme activities, the counts of total, and lactic bacteria of sea cucumbers were not significantly altered at dose of 6×10^7 CFU g^{-1} feed compared with control group (T0). Protease activities of sea cucumbers were significantly increased when fed with T1 diet compared with T0 ($P < 0.05$). Under the conditions of mass-scale culture, the present results indicate that the multiple probiotics can benefit growth performance, innate immunity, microbial ecology of the gut, several digestive enzyme activities of sea cucumber. The present study confirmed the potential effects of the multiple probiotics as dietary probiotics in juvenile sea cucumber *Apostichopus japonicus.*

Keywords Aquaculture · Probiotics · Immunology · Intestinal microbiology · Lactic acid bacteria

Introduction

Sea cucumber *Apostichopus japonicus* is an important commercially farmed species in China, Korea, and Japan due to its high nutritional and market value. However, the rapid expansion

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Published online: 18 April 2017

and high intensity of sea cucumber farming has caused many problems. Such as, infectious diseases caused by *Vibrio* sp., showing viscera ejection syndrome (Deng et al. 2009) and skin ulceration syndrome (Becker et al. 2004), which caused decrease of survival rate, considerable economic losses, and limited the sustainable development of this industry (Wang et al. 2006; Wang et al. 2007; Li et al. 2008; Deng et al. 2009). Additionally, disease in sea cucumbers are generally controlled by the antibiotics, however, long-term use of the antibiotics easily leads to many negative impacts such as drug resistance and environmental pollution. Therefore, improving disease control and enhancing their immunity has become urgent need in sea cucumbers.

Several alternative methods have focused on the multiple probiotics (selected bacterial strains) as biological control agents for aquaculture and can used for inhibiting pathogens, improving the growth and immunity (Gatesoupe 1999), promoting vitamin synthesis and digestion, or improving water quality (Chi et al. 2014). Some studies have been conducted for fish, shrimp, crab, and oyster species (Nogami and Maeda 1992; Douillet and Langdon 1994; Wang and Xu 2006; Saeed et al. 2006). As for the sea cucumber, most of the studies on the application of the multiple probiotics are under controlled laboratory conditions (Tang et al. 2007; Wang et al. 2015); however, very limited researches were based on practical farm conditions, and the search for the multiple probiotics that could be used for the sea cucumber is ongoing (Yan et al. 2014).

Currently, there are multiple probiotic formulations commercially available (6×10^7 CFU g^{-1}, powder, a mix of *Lactobacillus, Acetobacter*, and *Sphingomonas*). The amounts and species of bacteria were determined by dilution plate count method and identified by a cluster analysis of the 16S rDNA sequence before the feeding trial. In this study, an attempt was made, accordingly, to evaluate the efficacy of in vivo as a dietary probiotics for growth, intestinal *Lactobacillus*, selected immune, and digestive enzyme activities in sea cucumbers.

Nevertheless, the health of animals might be evaluated by sensitive immunomarkers, such as superoxide dismutase (SOD), catalase (CAT), acid, and alkaline phosphatase (ACP and ALP), as well as lysozyme activity—an important hydrolytic enzyme of the non-specific immune system, can disrupt β-(1, 4) glycosidic bonds between the N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan of bacterial cell walls (Callewaert and Michiels 2010). As for sea cucumber, coelomocytes are the key components of the immune system and can engulf and encapsulate invaded pathogen microorganisms, and then release humoral factors (Zhao et al. 2012). To evaluate the healthy status of experimental sea cucumber, these parameters were analyzed in the current study.

Materials and methods

Probiotics

Multiple probiotics were provided by Dalian B&T biotechnology Co., Ltd. (Dalian, China). It contained *Lactobacillus*, *Sphingomonas*, and *Acetobacter* (identified by gradient gel electrophoresis (PCR-DGGE) methods). Relative abundance is 22.6, 0.7, and 0.5.

Test diets

Test diets were provided by Hongyanhe Aquaculture farm, Dalian, China and were used as the basal diet in the present study. Ingredients in the sea cucumber basal feed diets are shown in

Table 1.The control group (T0) was given a basal diet; the treatment group was prepared by a basal diet mixed with different doses of the multiple probiotics 6×10^7 CFU g^{-1} feed (T1), 9×10^7 CFU g^{-1} feed (T2), and then the diets were dissolved in the sterile seawater and sprayed evenly into the culture ponds (Venkat et al. 2004); all experimental diets were prepared every day to guarantee the vitality of the multiple probiotics (Chi et al. 2014).

Sea cucumber and feeding trial

Sea cucumbers were supplied by Hongyanhe Aquaculture farms, Dalian, China, and this experiment was conducted on this farm. Prior to the initiation of this feeding trial, sea cucumbers were acclimated for 7 days. Then selected sea cucumbers of similar size (0.63 ± 0.13 g, means ± SD) were randomly allocated into nine cement (8 m × 2 m × 1.5 m) culture ponds and stocked at a density of 300 sea cucumbers per cement culture pond. Each diet was assigned to three ponds as a treatment. During the 90 days feeding trial, all sea cucumbers were fed at an amount of 5% of body weight once a day at 13:00, and 30% of the water was replaced every day. Water salinity and pH maintained at 29–31 ppt and 6.9–7.6. Water temperature ranged from 11 to 14 °C. Air supplied to maintain dissolved oxygen near saturation.

At the end of the feeding trial, sea cucumbers were starved for 24 h. Samples were collected from 20 sea cucumbers in each cement pond. The sea cucumbers were immediately dissected on ice to obtain intestine and coelomic fluid. All samples were quickly frozen in liquid nitrogen after collection and stored at −80 °C for further analysis.

Growth performance

The feeding trial was terminated after 90 days. Sea cucumbers were starved for 24 h prior to the sampling. And then the sea cucumbers were selected randomly (20 out of 300 sea cucumbers in each pond) and weighed individually to determine final body weight (FBW), body weight gain (BWG), and specific growth rate (SGR); growth parameters were calculated as follows: BWG (%) = 100 × (final mean weight−initial mean weight)/initial mean weight; SGR (%/day) = 100 × (ln final mean weight−ln initial mean weight)/no. of days (Wang et al. 2015).

Table 1 Ingredients composition and proximate analysis of the experimental diets (%)

Ingredients	Experimental group
Sargassum thunbergiia	44.5
Sea mud	44.5
Soybean meal[a]	2
Brown fish meal[b]	3
Oyster shell powder[c]	4
Yeast[d]	2
Moisture	19.6
Crude protein	14.2
Crude fat	2.4
Crude ash	38.9

[a] Soybean meal: the protein content is 40%

[b] Brown fish meal: the protein content is 65%. Longyuan Fishmeal Inc., Dalian, China

[c] Oyster shell powder: Dalian Fugu Co., Ltd.

[d] Yeast: Hebei Zhongrui Bio-Technology Co., Ltd.

The analysis of intestinal microbiota and digestive enzymes activities

Frozen samples were stored at −80 °C and thawed before analyzing the intestinal microbiota (Ran et al. 2012). The intestinal tissue was removed, weighed, and suspended in sterile saline (0.9%, *w*/*v*), An aliquot of the suspension was taken for the analysis of intestinal microbiota by tenfold serial dilutions method, and 0.1 mL was spread over the surface of triplicate plates of nutrient agar (NA) and modified chalmers agar (MC); all of the plates were incubated at 37 °C for 48 h (Ran et al. 2012) and colony forming units (CFU g^{-1}) were calculated from statistically viable plates (i.e., plates containing 30–300 colonies) (Rawling et al. 2009).

The rest of the suspension was then centrifuged at 4 °C at 3000 *g* for 10 min and collect the supernatant. The supernatants were used for analyzing digestive enzyme activities. The lipase activity was assayed based on measurement of Borlongan (Borlongan 1990); the amylase activity was determined according to Bernfeld (Bernfeld 1955), and the protease activity was determined by the procedure of Lowry (Lowry et al. 1951); all of the digestive enzymes activities were using the detection kit (Nanjing Jiancheng, Bioengineering Institute, China). The lipase was expressed as nanomoles of p-nitrophenol liberated per minute per milligram of protein. The amylase activity was expressed in micromoles of maltose liberated per minute per milligram of protein. The protease activity was expressed as micromoles of tyrosine liberated per hour per milligram of protein.

Non-specific immune enzymes assay

Total superoxide dismutase (T-SOD) activity was determined according to Ōyanagui (Ōyanagui 1984), catalase (CAT) activity was analyzed according to Góth (Góth 1991), lysozyme (LSZ) activity was measured according to Che and Ji (Chen and Ji 1992), and acid and alkaline phosphatase (ACP and ALP) assays were carried out according to Barrett (Barrett 1972); all of the immune enzymes activities were using the assay kit of Nanjing Jiancheng, Bioengineering Institute, China. An SOD unit was defined as the amount of enzyme that will inhibit the superoxide induced oxidation by 50%, and the SOD activity was expressed as the SOD unit mL^{-1}. A CAT unit was defined as the amount that can catalyze 1 μmol H_2O_2 s^{-1}, and the CAT activity was expressed as CAT unit mL^{-1}. An LSZ unit was defined as the absorbance change in M.luteus cell suspension/min, and the LSZ activity was expressed as LSZ unit mL^{-1}. An ACP or ALP unit was defined as the amount of enzyme required to produce 1 μmol phenol, and the ACP/ALP activity was expressed as ACP/ALP King unit mL^{-1}.

Statistical analyses

Data were presented as means and standard deviation (means ± SD). The values were first tested for homogeneity of variances and then compared with a one-way ANOVA followed by Duncan's multiple comparison tests using the computer software SPSS 21.0 (SPSS Inc. Richmond, CA, USA) to determine whether there were significant differences between the treatments. In all cases, a value of $P < 0.05$ was considered significant.

Results

Growth performance

All sea cucumbers receiving supplementary multiple probiotics diets had greater growth performances are illustrated in Table 2, The SGR and BWG of supplementation groups were significantly ($P < 0.05$) higher than the control group.

Intestinal microbiota

Total bacteria counts of sea cucumbers intestinal were significantly increased ($P < 0.05$) in the group T2 than the control group T0; no significant differences were observed between the group T1 and the control group (Table 3).

The number of intestinal *Lactobacillus* of sea cucumbers were significantly increased ($P < 0.05$) in the group T2 than the control group T0; no significant differences were observed in the intestinal *Lactobacillus* of sea cucumbers between the group T1and the control group T0 in Table 3.

Digestive enzymes activities

Lipase activity and Amylase activity of sea cucumbers were not significant among all treatments. Protease activity of sea cucumbers was significantly increased ($P < 0.05$) in the group T1 than the control group T0; no significant differences were observed in the protease activity of sea cucumbers between the group T2 and the control group T0 (Table 4).

Non-specific immune parameter assay

SOD activities, CAT activities, ACP activities, and ALP activities in the coelomic fluid of sea cucumbers presented an increasing trend with increasing doses of the multiple probiotics (Fig. 1). Significantly enhanced activities occurred in all multiple probiotics treatments when compared to the control group ($P < 0.05$).

LSZ activities of sea cucumbers were significantly increased ($P < 0.05$) in the group T2 than the control group T0; no significant differences were observed in the lysozyme activities of sea cucumbers between T1 and control group (Fig. 1).

Table 2 Effects of dietary multiple probiotics on the growth performance of sea cucumber

Treatments	T0	T1	T2
IBW[a]	0.63 ± 0.01	0.62 ± 0.02	0.62 ± 0.02
FBW[a]	4.81 ± 0.64[a]	6.19 ± 0.11[b]	6.38 ± 0.23[c]
BWG[a]	636.44 ± 2.17[a]	892.31 ± 2.37[b]	922.84 ± 8.74[c]
SGR[a]	2.22 ± 0.01[a]	2.55 ± 0.23[b]	2.58 ± 0.27[b]

Values are expressed as mean ± SD ($n = 60$). Different upper-case letters within each row indicate significant differences ($P < 0.05$)

[a] *IBW* initial body weight, *FBW* final body weight, *BWG* body weight gain, *SGR* specific growth rate

Table 3 Effects of dietary multiple probiotics on the intestinal *Lactobacillus* of sea cucumber (CFU mL^{-1})

Treatments	T0	T1	T2
Total bacteria	$(2.68 \pm 0.64) \times 10^{8a}$	$(4.21 \pm 0.88) \times 10^{8ab}$	$(6.36 \pm 1.36) \times 10^{8b}$
Lactobacillus	$(6.45 \pm 0.29) \times 10^{3a}$	$(42.00 \pm 8.91) \times 10^{3a}$	$(104.20 \pm 25.03) \times 10^{3b}$

Values are expressed as mean ± SD (n = 60). Different upper-case letters within each row indicate significant differences ($P < 0.05$)

Discussion

Recently, the multiple probiotics has been environmental friendly solutions to prevent serious diseases from happening in culturing of sea cucumbers (Balcázar et al. 2006). Such studies reported that the multiple probiotics enhanced the growth and disease resistance in shrimp and fish (Rengpipat et al. 1998; Wang and Xu 2006; Wang 2007). However, the study of the multiple probiotics is relatively rare in sea cucumbers. In the present study, the effect of the multiple probiotics (a mix of *Lactobacillus*, *Acetobacter*, and *Sphingomonas*) in sea cucumber was firstly reported.

Sea cucumbers receiving the multiple probiotics diets showed better growth performance compared to the control group, and enhanced SGR, BWG occurred in all treatments fed the multiple probiotics ($P < 0.05$). Wang found that combined use of the multiple probiotics photosynthetic bacteria and *Bacillus sp*. (isolated from carp ponds) had a better effect on growth of *Penaeus vannamei* (Wang 2007) which was similar to the present results. Additionally, Li et al. (2015) observed that the group fed live multiple probiotics at the levels of 10^7, 10^{10} CFU g^{-1} displayed significantly increased weight gain compared to the control fed group. From previous studies, the reason why the growth performance of sea cucumber fed dietary supplemented the multiple probiotics might be attribute to increased health status, stimulation of gastric development, and enzymatic secretion (Hoseinifar et al. 2011). From the present study, it can be assumed that modulation of the intestinal microbial communities, as demonstrated by elevated lactic acid bacteria (LAB) levels, might also be a contributory factor. Another reason why the growth of sea cucumbers fed with the multiple probiotics increased may be attributed to enhance digestive enzyme activities, protease, and amylase that could stimulate and improve host digestibility (Zhang et al. 2010).

The multiple probiotics can modulate the microbial ecology of the gut (Collins and Gibson 1999) and are able to survive in aquatic environment and colonize the intestine by adhering to the intestinal epithelium (Salimaen et al. 1996). Standen et al. (2015) reported that the dietary supplemented a multi-species probiotics had no effect on the total count of intestinal bacteria of tilapia, but the intestinal lactic acid bacteria (LAB) increased in the probiotics group. Ramos et al. (2013) reported that the dietary probiotics supplementation improved the count of total

Table 4 Effects of dietary multiple probiotics on the digestive enzymes activity of sea cucumber (U)

Treatments	T0	T1	T2
Lipase activity	0.21 ± 0.01	0.24 ± 0.07	0.17 ± 0.06
Amylase activity	0.69 ± 0.08	0.77 ± 0.10	0.74 ± 0.12
Protease activity	8.24 ± 0.28^{a}	41.08 ± 10.25^{b}	15.16 ± 5.97^{a}

Values are expressed as mean ± SD (n = 60). Different upper-case letters within each row indicate significant differences ($P < 0.05$)

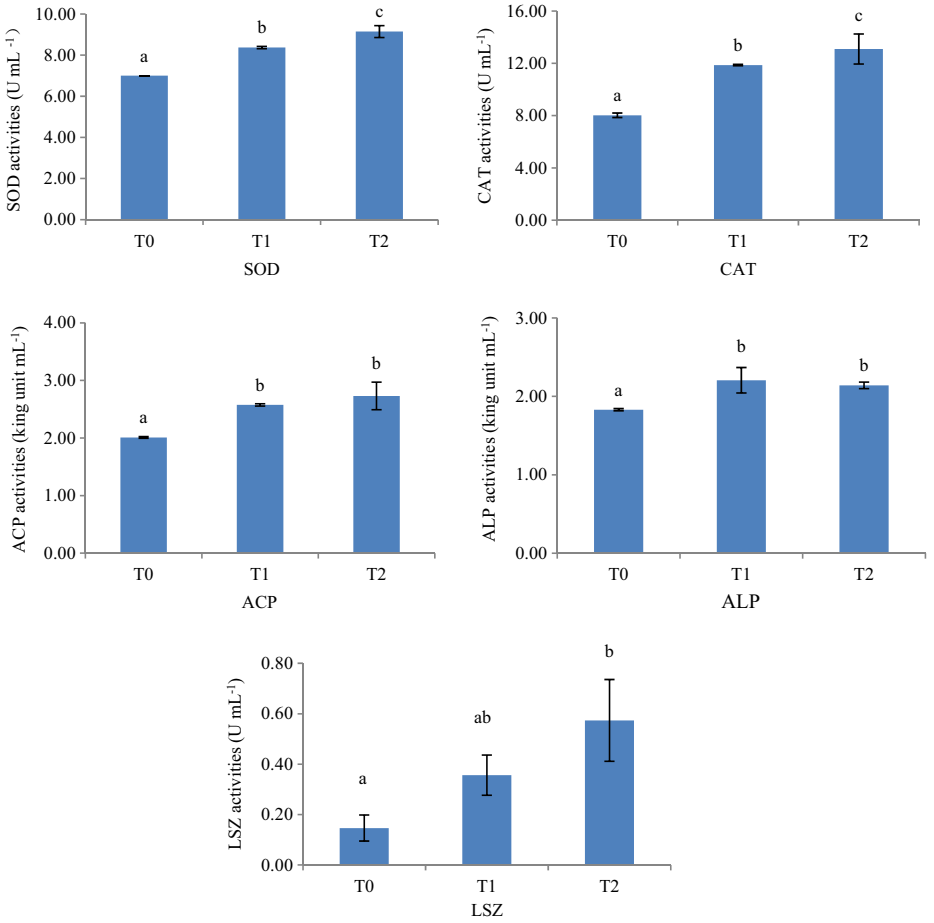

Fig. 1 SOD activities, CAT activities, LSZ activities, ACP activities, ALP activities in the coelomic fluid of sea cucumbers compared with the control. Values are expressed as mean ± SD (n = 60). *Different upper-case letters* within each bar indicate significant differences ($P < 0.05$)

bacteria. In the current study, total bacteria count and intestinal *Lactobacillus* count of sea cucumbers intestinal were significantly increased ($P < 0.05$) in group tested with 9×10^7 CFU g^{-1} feed than the control group; this phenomenon may due to the multiple probiotics have strong adhesion and colonization such as some lactic acid bacteria show good adhesion properties (Salimaen et al. 1996). While no significant differences were observed between the group fed the multiple probiotics (6×10^7 CFU g^{-1} feed) and the control group. Suitable doses of the multiple probiotics in diet may be one of the reasons of this result, but the level of 9×10^7 CFU g^{-1} feed dietary, the multiple probiotics may not be the optimal content for improving the count of total bacteria and intestinal *Lactobacillus* in sea cucumbers. So, further studies needed to find out the optimal content of the multiple probiotics in diet.

Several studies reported that the multiple probiotics were affecting on digestive enzymes of sea cucumbers (Tang et al. 2007; Wang et al. 2015). However, the mechanism of this is not yet clear. In the current study, the lipase activity and amylase activity of sea cucumber were not significantly altered in all experimental groups. Tang et al. (2007) reported that the lipase activity was detected in

digestive tract which was more advanced than the others in early period of larval development of sea cucumber; the lipase activity could not be easily influenced by the multiple probiotics. Wang et al. (2015) reported that the sea cucumber fed the diet supplemented with potential probiotics *Rhodotorula benthica* D30 had increased intestinal amylase activities higher than animals fed the basal diet. However, in this study, no significant differences were observed in sea cucumber between the treatment group and the control group. It could be attributed to the differences in the multiple probiotics, animals (species and size), and experimental conditions (Zhao et al. 2012). Macey and Coyne (2005) reported that the abalone fed the multiple probiotics-supplemented diets had elevated protease activity in the intestinal region of the digestive tract. Ma et al. (2014) reported that sea cucumber fed diet supplemented with *Pseudoalteromonas* sp. BC228 increased intestinal protease activities in comparison with control. Liu et al. (2013) also reported that the sea cucumber fed diet supplemented with *Bacillus* sp. BC26 enhanced protease activities compared with control in sea cucumbers. These results were consist with the current experiment, and protease activity of sea cucumbers fed with 6×10^7 CFU g^{-1} mixed probiotics were significantly altered. As described before, the intestinal tract of sea cucumber constantly interacts with various environmental factors, such as water quality, water temperature, and the environmental microflora, may largely emit their effects on the establishment, proliferation, and function of the multiple probiotics (Das et al. 2008) and could influence digestive enzymes activities. Further studies in regard to histology and physical construction of these enzymes should be conducted to clarify the intestinal digestive enzymes activity.

Phagocytic process is the first line of body's internal defense (Janeway and Medzhitov 2002). Sea cucumber phagocytes could complete degradation of exogenous bacteria by the activity of lysozyme, ACP and ALP (Canicatti 1990), producing reactive oxygen species (ROS), comprising superoxide anions (O_2^-), and hydrogen peroxide (H_2O_2)(Ellis 1999).

SOD and CAT are the paramount component of the antioxidant defense system of the organism (Arabaci 2011; Zhao et al. 2012). SOD catalyzes the dismutation of the extra bactericidal highly reactive O_2^- to O_2 and less reactive H_2O_2; CAT catalyzes the degradation of hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). SOD and CAT activities can reflect the ability of the body's resistance to oxidative stress by reducing the H_2O_2 and O_2^- content in the cell (Alscher et al. 2002) and indirectly reflect the immunity levels of sea cucumber (Zang et al. 2012). This trial showed that SOD activities and CAT activities in sea cucumbers coelomic fluid progressively increased in all multiple probiotics treatments. It was similar with the effects of probiotics mixture (*Bacillus subtilis* YB-1 and *Bacillus cereus* YB-2) (Li et al. 2015) in sea cucumber. Besides, the similar effects of infecting with extra-cellular products of *P. elyakovii* YAAJ6 and *V. ordalii* YASM12 were found to stimulate SOD activities in sea cucumbers (Zheng et al. 2012). The reason for this phenomenon may be an adaptive immune response to oxidative stress and improvement in the antioxidation abilities of the treated sea cucumbers (Yan et al. 2014), or with the continuous feeding of the multiple probiotics; secondary metabolites of the multiple probiotics may contain antioxidant, which had the feature of antioxidant enzymes (Morohoshi et al. 2005).

The stimulation of LSZ activities have been recognized in the coelomic fluid of sea cucumbers fed diets supplemented with the probiotics *Metschnikowia spp.*C14 (Liu et al. 2012). Similarly, in the present study, enhanced LSZ activities were observed in the coelomic fluid, and intestine of sea cucumbers fed the multiple probiotics at 9×10^7 CFU g^{-1} feed when compared to the control. As described before, lysozyme exhibited strong catalytic ability to break the bacteria cell walls, resulting in cell lysis in the hypoosmotic environment. In addition, the expression level of lysozyme gene is considered as important index for monitoring fish immunity. The reason for this phenomenon may be the multiple probiotics stimulate the expression level of lysozyme gene.

The phosphatase enzymes are capable of hydrolyzing organic phosphate esters. ACP is a typical lysosomal enzyme concerned with killing and digesting microorganisms and foreign substances during the immune response (Cheng 1989), while ALP is thought to act as an antibacterial agent and an important enzyme associated with the innate immune system in fishes (Esteban 2012). It has been reported that both ACP and ALP take part in immune defense mechanisms and are correlated with the immune competence of aquatic animals (Blasco et al. 1993; Mazorra et al. 2002). In the present study, the data revealed a significant elevation in ACP and ALP activity in the coelomic fluid when sea cucumbers were fed the multiple probiotics at 6×10^7 CFU g^{-1} feed, 9×10^7 CFU g^{-1} feed. This result was similar to that reported by Ma et al. (2013), where the live dietary yeast *Hanseniaspora opuntiae* C21 significantly increased ACP and ALP activities in the coelomic fluid of sea cucumbers. The increase ACP activity and ALP activity may be attributed to metabolites of the multiple probiotics that stimulate the ACP activity and ALP activity. The expression level of these immune parameters, influenced by the multiple probiotics, may be a factor.

In conclusion, the present results indicate the multiple probiotics are beneficial for sea cucumbers in the growth performance, innate immunity, microbial ecology of the gut, and several digestive enzyme activities. Therefore, applications of this multiple probiotics may be of great interest to those involved in the sea cucumber farming industry.

Acknowledgements This work was funded by the Public Science and Technology Research Funds Project of Ocean of the State Oceanic Administration of the People's Republic of China (Grant No. 201405003) and the Science and Technology Research Funds Project of the Dalian City Oceanic and Fishery Administration, Liaoning Province, China (Grant No. 20140101).

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