Fish & Shellfish Immunology 45 (2015) 800–807

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Effects of dietary *Bacillus cereus* G19, *B. cereus* BC-01, and *Paracoccus marcusii* DB11 supplementation on the growth, immune response, and expression of immune-related genes in coelomocytes and intestine of the sea cucumber (*Apostichopus japonicus* Selenka)

Gang Yang [a], Xiangli Tian [a, *], Shuanglin Dong [a], Mo Peng [b], Dongdong Wang [a]

[a] The Key Laboratory of Mariculture, Ministry of Education, Fisheries College, Ocean University of China, Qingdao 266003, PR China
[b] School of Animal Science and Technology, Jiangxi Agricultural University, Nanchang 330045, PR China

ARTICLE INFO

Article history:
Received 23 January 2015
Received in revised form
18 May 2015
Accepted 28 May 2015
Available online 4 June 2015

Keywords:
Apostichopus japonicus
Bacillus cereus
Paracoccus marcusii
Immune response
Immune-related gene

ABSTRACT

Probiotics have positive effects on the nutrient digestibility and absorption, immune responses, and growth of aquatic animals, including the sea cucumber (*Apostichopus japonicus* Selenka). A 60-day feeding trial was conducted to evaluate the effects of *Bacillus cereus* G19, *B. cereus* BC-01 and *Paracoccus marcusii* DB11 supplementation on the growth, immune response, and expression level of four immune-related genes (*Aj*-p105, *Aj*-p50, *Aj*-rel, and *Aj*-lys) in coelomocytes and the intestine of juvenile sea cucumbers. One group was fed the basal diet (control group), while three other groups were fed the basal diet supplemented with *B. cereus* G19 (G19 group), *B. cereus* BC-01 (BC group), or *P. marcusii* DB11 (PM group). The growth rate of sea cucumbers fed diets with probiotics supplementation was significantly higher than that of the control group ($P < 0.05$). Sea cucumbers in the G19 and PM groups had a significantly greater phagocytic activity of coelomocytes compared to the control group ($P < 0.05$), while those in the G19 and BC groups had a greater respiratory burst activity ($P < 0.05$). The alkaline phosphatase (AKP) activity of coelomocytes in sea cucumbers fed diets with probiotics supplementation was significantly higher than the control group ($P < 0.05$). Comparatively, superoxide dismutase (SOD) activity of coelomocytes for sea cucumber in the PM group was significantly greater ($P < 0.05$). As for the immune-related genes, *B. cereus* G19 supplementation significantly increased the expression level of the *Aj*-rel gene in coelomocytes ($P < 0.05$), while *B. cereus* BC-01 supplementation significantly increased that of the *Aj*-p50 gene as compared to the control group ($P < 0.05$). In the intestine, the relative expression level of *Aj*-p105, *Aj*-p50, and *Aj*-lys genes in the PM group was significantly higher than that in the control group ($P < 0.05$). These results suggested that *B. cereus* G19 and *B. cereus* BC-01 supplementation could improve the growth performance and the immune response in coelomocytes, while *P. marcusii* DB11 supplementation could have a positive effect on the growth performance and immune response in coelomocytes and the intestine of sea cucumbers.

1. Introductions

The sea cucumber, *Apostichopus japonicus* Selenka, is one of the most economically important holothurian species in China [1]. Sea cucumber farming has rapidly grown over the past decade, causing problems, such as water pollution and diseases outbreaks that severely restricted its sustainable development. In addition, the widespread use of antibiotics in aquaculture to control bacterial diseases has resulted in the development of antibiotic-resistant pathogens, the alteration of environmental microbiota, the reduced immunity, and the rise of food safety issues [2–5].

Marine invertebrates, including the sea cucumber, lack adaptive immunity and rely solely on the innate immune system against invading microbes. Coelomocytes play a key role in the defense against pathogens and other invading organisms through cellular (phagocytosis) and cell-free (humoral) immune responses [6,7]; therefore, a strategy for enhance the immune activity of coelomocytes would be an appropriate approach for improving the

* Corresponding author.
E-mail address: xianglitian@ouc.edu.cn (X. Tian).

http://dx.doi.org/10.1016/j.fsi.2015.05.032
1050-4648/

resistance of sea cucumbers against infectious microbial diseases [8]. The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which is involved in the regulation of non-specific immune responses, which controls the expression of various immune-related genes such as antimicrobial peptides, cytokines, reactive oxygen species-generating enzymes, and reactive nitrogen generating enzymes [9–11]. In mammals, the NF-κB family of transcription factors contains five members: RelA (p65), RelB, c-Rel, NF-κB1 (pl05/p50), and NF-κB2 (pl00/p52). The NF-κB family has also been identified in some aquatic animals such as channel catfish (*Ictalurus punctatus*) [12], scallop (*Chlamys farreri*) [13], and sea cucumber [14]. In addition, lysozyme (lys), a natural antimicrobial substances, is one of the most important humoral factors of the innate immune system in aquatic animals, which can effectively remove invading pathogens from the body [15].

Probiotics are live microorganisms, such as *Bacillus* spp. [16–18], *Lactobacillus* sp. [19], *Bifidobacterium* sp. [20], and *Paracoccus marcusii* DB11 [21] that have positive effects on the nutrient digestibility and absorption, immune responses, and growth of aquatic animals [22,23]. Probiotics can improve non-specific immunity of the host, although the regulatory mechanism of the immune system is still understudied. Previous studies showed that the potential probiotic strains *Bacillus cereus* G19 [24], *B. cereus* BC-01 [25] and *P. marcusii* DB11 [26] isolated from the intestine of the sea cucumber positively influenced growth and immune responses; however, the mechanism by which these strains affected the innate immune system of the sea cucumber has not been studied. The objective of this study was to assess the effect of *B. cereus* G19, *B. cereus* BC-01 and *P. marcusii* DB11 supplementation on the growth, immune responses, and expression of immune-related genes (*Aj*-p105, *Aj*-p50, *Aj*-rel, and *Aj*-lys) in coelomocytes and the intestine of the sea cucumber, and investigate the regulatory mechanism underlying the manner in which these strains affect the innate immune system.

2. Materials and methods

2.1. Experimental animals

The juvenile sea cucumbers were obtained from Lian He Yuan Jian Farm (Qingdao, China). Sea cucumbers were cultured in a 1000 L fiberglass tank for 15 d to acclimate them to the experimental conditions. Then sea cucumbers were fasted for 24 h, and 200 similar-sized individuals (4.68 ± 0.07 g) were randomly distributed among 20 aquaria (53 × 28 × 34 cm, 50 L), for a density of 10 sea cucumbers in per aquarium.

2.2. Experimental diets

Basal diet (control diet) was formulated with marine mud, red fish meal and sargasso (Table 1). All ingredients were first ground to fine powder through a 320 μm-mesh, and then thoroughly mixed. On basis of the basal diet, the other diets were supplemented with 10^9 cfu/kg three potential probiotics, i.e., *B. cereus* G19 (G19 group), *B. cereus* BC-01 (BC group), and *P. marcusii* DB11 (PM group), respectively. The control group was fed the basal diet. 5 replicate aquaria were set in per treatment. In the previous study of our laboratory, three probiotic strains were isolated from the intestine of sea cucumber [24–26]. The daily dosages of *B. cereus* G19, *B. cereus* BC-01, and *P. marcusii* DB11 was designed in accordance with the research results of Wang et al. [24], Tian et al. [25], and Yan et al. [21], respectively.

Three probiotic strains were cultured for 48 h at 16 ± 1 °C in shaken bottles containing a liquid tryptone soya marine medium, and then harvested by centrifugation (4000 g for 10 min) and the fresh cells were washed twice in sterile normal saline, added to the basal diet and thoroughly mixed, yielding a dose equivalent to appointed dose equivalent in the diet. Strain diets were prepared daily with the proper moisture content and fed to sea cucumbers within 1 h of preparation to guarantee the vitality of strains.

Table 1
Formulation and proximate composition of the basal diet.

Ingredient/(dry weight)	Percentage (%)
Marine mud[a]	20.00
Red fish meal[b]	10.00
Sargasso powder[c]	69.96
Y_2O_3[d]	0.04
Proximate composition	
Crude protein	16.10
Crude lipid	0.97

[a] Qingdao Great Seven Biotechnology Co., Ltd., Qingdao, China.
[b] Maluha Co. Ltd., Japan. Fish meal contained 71.56% crude protein and 9.17% crude lipid on a dry weight basis.
[c] Shandong Liuhe Group Co. Ltd., Qingdao, China. Sargasso powder contained 12.79% crude protein and 0.71% crude lipid on a dry weight basis.
[d] Qingdao Master Biotechnology Co. Ltd., Qingdao, China.

2.3. Feeding experiment

Sea cucumbers fed once daily at 6:00 pm for 60 days. Uneaten feed in the tank was removed by pipetting before the next feeding. During the last two weeks of the trial, the feces were collected from each tank by pipetting every day at 08:00–10:00 am and 05:00–6:00 pm. After collection, feces were centrifuged (3000 g at 4 °C for 20 min) and frozen daily at −20 °C. During the feeding trial, the environmental conditions were suitable for sea cucumbers (temperature, 17 ± 1 °C; salinity, 28–30‰; pH, 8.0 ± 0.3; dissolved oxygen, 10 ± 0.25 mg/L).

2.4. Sample collection

At the end of the experiment, after being fasted for 24 h, four sea cucumbers of each replicate were dissected using the tail cutting method and coelomic fluid of each sea cucumber was collected for further challenge test. The coelomic fluid (1 ml) was thoroughly mixed with an equal volume of anticoagulant (0.02 M EGTA, 0.48 M NaCl, 0.019 M KCl, 0.068 M Trise-HCl, pH 7.6). The coelomic fluid from four sea cucumbers from each aquarium was pooled for immunological analyses. After an aliquot of the coelomic fluid samples were taken for total coelomocytes counts (TCC), phagocytosis activity test and respiratory burst analysis, the remaining coelomic fluid samples were centrifuged at 3000 ×g, 4 °C for 10 min to collect coelomocytes. Half of the sample of coelomocytes were resuspended in 600 ml 0.85% cold saline and sonicated at 22 kHz for 25 s at 0 °C. Finally, the suspension was centrifugated (4000 g) at 4 °C for 10 min and the cells lysate supernatant (CLS) was stored at −80 °C for lysozyme (LZM) activity assays. The remaining coelomocytes were mixed with 1 ml TaKaRa RNAiso Plus for qPCR. The mid-intestinal tract for immune genes from each sea cucumber was removed and frozen at −80 °C until further analysis.

2.5. Chemical analyses

2.5.1. Crude protein and crude lipid in the basal diet

Crude protein of the basal diet samples was determined by measuring nitrogen (N × 6.25) using the Kjeldahl method (Kjeltec TM 8400, FOSS, Tecator, Sweden). Crude lipid was measured by ether extraction using Soxhlet method [27].