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Effects of tussah immunoreactive substances on growth, immunity, disease resistance against *Vibrio splendidus* and gut microbiota profile of *Apostichopus japonicus*

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ABSTRACT

Tussah immunoreactive substance (TIS) comprises a number of active chemicals with various bioactivities. The current study investigated the effects of these substances on the sea cucumber *Apostichopus japonicus*. The specific growth rate (SGR) of TIS-fed sea cucumbers was significantly enhanced, whereas no significant difference in SGR was observed between those soaked in antibiotics and those fed with basal diet only. TIS also improved the immune response of the animals when given at a dose of 1.0% or 2.0%, as shown by increased phagocytic, lysozyme, superoxide dismutase, alkaline phosphatase, acid phosphatase, and catalase activities following injection with live *Vibrio splendidus*. At a dose of 1.0% or 2.0%, TIS significantly enhanced the immune ability ($P < 0.05$) of the sea cucumbers, but except for lysozyme activity, other immune indices were reduced one day after the animals were injected with *Vibrio splendidus*. However, the values of these immune indexes were still significantly higher compared to those of the control groups ($P < 0.05$). Intestinal micro flora counts and high-throughput sequencing showed that dietary TIS could improve the amount of probiotic bacteria, yielding a 6-fold increase in *Bacillus* and 10-fold increase in *Lactobacillus* for sea cucumbers fed with 2.0% TIS diet compared to the control. Furthermore, TIS-containing diet also greatly reduced the number of harmful bacteria, with the number of *Vibrio* in sea cucumbers fed with 1%TIS diet decreased by 67% compared to the control. The results thus indicated that TIS increased the growth of sea cucumbers and enhanced their resistance to *V. splendidus* infection by improving the immunity of the animals. TIS also improved the gut microbiota profiles of the animals by increasing the probiotics and reducing the harmful bacteria within their guts.
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1. Introduction

Apostichopus japonicus is a sea cucumber that belongs to Echinodermata, Holothuroidea. It has long been exploited by the fishery industries in China, Russia, Japan, and Korea [1]. Sea cucumber is rich in protein, vitamins microelements, carotenoids, fatty acids, free sterols, fucan sulfates, chondroitin sulfate, tensilin and glycosides [2–7]. *Apostichopus japonicus* is highly prized in China, both as a delicacy and traditional medicine [8]. However, the rapid expansion of sea cucumber farming has led to the emergence of various diseases and serious economic losses. Conventionally, the control of diseases in aquaculture has relied on the use of chemical compounds and antibiotics [9–11]. The overuse of antibiotics has resulted in the spread of drug-resistant pathogens, environmental pollution and unexpected residues in the sea cucumbers and aquaculture water [12–14]. These factors also pose a great threat to human health. One of the most promising methods for controlling diseases in sea cucumbers in aquaculture is to strengthen the defense mechanism of the animals through oral administration of eco-friendly and economically viable immunostimulants [15–17].

Antimicrobial peptides have captured the attention of researchers in recent years because of their efficiency in the fight against pathogens [18]. Since the early 1980s, Cecropin, which was first extracted from the wild silkworm induced by artificial immunization [19,20], has gradually become a research hotspot in

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insect immunology and molecular biology [21–26]. The synthesis of a series of biologically active substances, including cecropin, lysozyme, lectins, and other defense agents can be induced in tussah after exposure to pathogenic microorganisms, chemicals, physical factors and other stimuli or trauma [27]. These substances possess a wide variety of antibacterial activities, and participate in the immune response to the invasion by pathogenic microorganisms. Being harmless and environmental friendly, these natural substances have been regarded as a new generation of natural additives [28–30]. To our best knowledge, the effects of antibacterial peptides on animals have been reported in the literatures [31–33], but the use of tussah immunoreactive substance (TIS) in aquaculture, especially in sea cucumber farming, has not been reported. In this study, sea cucumbers were fed with a diet containing different percentages of TIS for 30 days, and the non-specific parameters, gut microbiota profile and of the animals were evaluated following exposure to *Vibrio splendidus*.

2. Materials and methods

2.1. Preparation of test diets

Tussah immunoreactive substance was prepared in our laboratory as previously described [34]. The preparation process included the homogenization of tussah pupa followed by the removal of skin debris and subsequent drying to yield a powder form. The TIS powder was added to a basal diet to a final concentration of 0.5%, 1.0% or 2.0% (w/w). Basal diet without TIS was used as a control. Four diets were prepared in total, and the approximate compositions of the four test diets are shown in Table 1. The nutrients and amino acid composition of TIS are shown in Table 2 and Table 3, respectively.

2.2. Feeding trial

Sea cucumbers were obtained from an aquaculture farm in Dalian, China, and kept in 250-L (120 cm × 70 cm × 30 cm) glass tanks. The animals were cultured in sea water at 17–19 °C and pH 7.8–8.2 for 30 days under natural illumination. The tanks were continuously aerated and the water was exchanged daily at a rate of 30 %–50%. Feeding trials were performed after one week of acclimatization. A total of 165 sea cucumbers were randomly divided into five groups; two groups were fed with basal diet (BD), but one of the groups was used as an antibiotic control group, in which the sea cucumbers were soaked in 20 ppm Norfloxacin for 20 min once a week. The remaining three groups were each fed with basal diet supplemented with 0.5%, 1.0% or 2.0% TIS. The amount of diet added to each tank was adjusted daily, from 2% to 3% (mean value) relative to the biomass of the sea cucumbers.

2.3. Bacterial challenge

After 30 days of feeding, twenty animals were randomly selected from each tank and each injected with 0.2 mL (1.0×10^9 cfu) of live *Vibrio splendidus* suspension as described previously [16]. The infected sea cucumbers were observed for a period of 15 days.

Table 1
Compositions of the different diets used in this study.
The compositions are expressed in % of dry matter.

Ingredients	Control	0.5% TIS	1.0% TIS	2.0% TIS
Crude protein%	20.7	21.0	21.0	21.1
Crude fat%	1.18	1.19	1.26	1.73
Ash%	43.1	44.9	44.7	46.0
Energy kJ/100 g	855	873	876	918

Table 2
Contents of protein nutrients and elements in TIS.

Ingredients	Amount
Protein g/100 g	54.6
Fat g/100 g	21.2
Crude fiber g/100 g	0.6
Ash g/100 g	5.7
Ca mg/100 g	108
P mg/100 g	423
Hg mg/100 g	0.015
As mg/100 g	0.72
Pb mg/100 g	0.054

Table 3
Amino acid composition analysis of TIS.

Amino acid g/100 g	Amount
Aspartic acid	2.34
Threonine*	4.54
Serine	1.51
Glutamate	5.42
Proline	2.39
Glycine	3.09
Alanine	2.25
cystine	0.71
Valine*	2.98
Methionine*	1.51
Isoleucine	2.17
Leucine*	3.40
Tyrosine	2.43
Phenylalanine*	2.85
Lysine*	3.33
Hlstidine	1.52
Tryptophane*	0.48
Arginine	2.18

Note: "*" indicates essential amino acid.

2.4. Sample collection

After feeding, the sea cucumbers were starved for 24 h before the next experiment. Five sea cucumbers were randomly removed from each group and the coelomic fluid was collected from each animal by the tail-cutting method. The collected coelomic fluid was centrifuged at 3000 × g for 10 min at 4 °C, and the supernatant was used as the serum to determine the status of various immune parameters, including phagocytic (PC), lysozyme (LSZ), superoxide dismutase (SOD), alkaline phosphatase (ALP), acid phosphatase (ACP) and catalase (CAT) activities. At the same time, the intestinal content was removed and stored at −20 °C. The activities of the different immune parameters were determined before and one day after *V. splendidus* exposure. The concentration of C3 was measured before *V. splendidus* exposure. The coelomic fluid samples were subjected to these different assays within 12 h of preparation.

2.5. Phagocytic (PC) activity

Phagocytic activity was evaluated by measuring the phagocytic rate and calculating the phagocytic index of the coelomic fluid according to Zhang et al. [35]. Aliquot (100 μL) of the coelomic fluid containing 10^6 cells was first placed in a well of a 96-well, flat-bottomed microstate plate and incubated at 25 °C for 30 min. Next, the upper fraction was carefully removed and 100 μL of 1 mM neutral red was added to the coelomocyte monolayer and incubated for 30 min. After that, the plate was washed three times with 0.85% normal saline, and the dye within the coelomocytes was then dissolved by the addition of 100 μL mixture of ethyl alcohol-acetic acid (1:1 ratio) followed by 20 min of incubation. The absorbance of

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