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Dietary effect of *Rubus coreanus* ethanolic extract on immune gene expression in white leg shrimp, *Penaeus vannamei*

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ABSTRACT

The objective of this study was to evaluate the effect of dietary supplementation of a *Rubus coreanus* ethanolic extract on immunostimulatory response in white leg shrimp *Penaeus vannamei*. Shrimps with an average initial weight of 0.5 ± 0.04 g were collected and acclimatized for 10 days. Four experimental diets including a control diet, a probiotic diet and 0.25 and 0.5% of *R. coreanus* ethanolic extract (*Rc*EE) diets were used to feed the shrimps. After 8 weeks of culture, shrimp fed with probiotic and 0.25% *Rc*EE diet had showed significant enhancement in the growth while shrimp fed with 0.5% *Rc*EE diet showed significantly increased expression of immune genes and antioxidant enzymes activities. One week of challenge experiments for all the four diets fed shrimps showed decreased cumulative mortality in the 0.5% *Rc*EE diets fed shrimps, when compared with the probiotic and 0.25% *Rc*EE diet fed shrimp groups. The results indicates that *R. coreanus* ethanolic extract could be used as a herbal immunostimulant for shrimps to increase its immunity and disease resistance against the bacterial pathogen, *Vibrio alginolyticus*.

1. Introduction

At present, the success of aquaculture depends largely on minimizing the impact of diseases in farmed fish. In recent years, there has been a growing interest in the use of natural immunostimulants, pro- and pre-biotics to prevent and/or control pathogenic microorganisms has an alternative to traditional disease-control treatments, such as chemotherapeutic agents or vaccines [1]. These included additives as dietary supplements minimize the risks associated with chemical drugs; constitute one of the most promising methods of controlling diseases in aquaculture [2]. Studies have demonstrated that they are able to increase resistance to infectious diseases by enhancing nonspecific defense mechanisms, increasing growth rates, and the digestion and absorption of nutrients [3,4].

In Korea, shrimp farming began with two indigenous shrimp species namely *Fenneropenaeus chinensis* and *Marsupenaeus japanicus* in the 1960s and rapid increase in the production since 1990s. Aquaculture production reached 3256 mt from 2600 ha of 437 shrimp farms in 2001. However, due to frequent diseases outbreaks production decreased to 2323 mt (1.02 mt/ha) in 2004 [5]. Hence, in 2003, specific pathogen free (SPF) broodstocks of the Pacific white shrimp, *Penaeus vannamei* were introduced to the Crustacean Research Center, NFRDI for the first time in Korea from Hawaii. The growth rate was 30%–50% higher and the frequency of viral diseases outbreaks was much lower than that of the indigenous shrimp species. However, mass mortality was reported from several shrimp farms with the possible route of infection through imported live feeding materials like *Artemia* or bloodworms [6].

During the last decade, shrimp production has been affected severely by infectious diseases caused by bacteria and viruses. A wide range of antimicrobial drugs, pesticides, disinfectants and chemicals are routinely used to control disease [7]. The excessive and inappropriate use of such antimicrobials in disease prevention and growth promotion caused deterioration in the pond environment and affecting the health status of shrimp [8,9]. A number of preventive approaches such as the use of vaccines [10], probiotics [11] and immunostimulants [12] have been explored in order to reduce the losses due to diseases and mortality of culture stock. Previous studies have tested the dietary effect of immunostimulants to increase their immune activity and resistance against diseases. Oral administration is the most commonly used method for

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shrimp immunostimulants. For example, *Sargassum fusiforme* polysaccharide [13], the hot-water extract of *Gracilaria tenuistipitata* [14], b-1, 3-glucan [15], saponin [16], sodium alginate [17], etc supplemented in diets have been reported to enhance the immune activity and resistance against diseases in shrimp.

Rubus coreanus (bokbunja), Korean rasberry, an astringent herb, is a deciduous broad-leaf *rosaceaen* family herb distributed in Southeast Asian countries, particularly in the southern parts of Korea, China and Japan [18]. The dried fruits of *R. coreanus* have been used for several centuries as human herbal medicine in the management of impotence, spermatorrhea, enuresis, asthma and allergic diseases, and it also has been used as a stomachic and tonic in Korea [19]. The therapeutic effects may be related to various phytochemicals, such as phenolic acids, organic acids, triterpenoids, flavonoids, gallotannin and ellagitannin [20]. Previous studies on phenolics, especially anthocyanins from berries showed several health benefits in higher animals [21]. Recent report on ethanolic extracts of *R. coreanus* (*Rc*EE) had shown to contain *in vitro* antioxidant and antimicrobial activity against aquatic microbes [22].

Present study investigate the immune parameters, and the expression of antioxidant enzyme genes in the white-leg shrimp, *Penaeus vannamei*, after being fed with diets containing anthocyanins rich *R. coreanus* ethanolic extract. Several immune parameters such as total superoxide dismutase (T-SOD) activity, catalase (CAT) activity, glutathione peroxidase (GPx) activity, acid phosphatase (ACP), alkaline phosphatase (AKP) activity, and content of malondialdehyde (MDA), as well as the gene expressions of cytosolic SOD (cyt-SOD), CAT and GPx were determined. This study suggests that *Rc*EE possesses immunostimulant activity, can increase immune enzyme activity and modify expression of immune genes in shrimp.

2. Materials and methods

2.1. Diets preparation

Experimental diets were prepared in the laboratory with the ingredients listed in Table 1. The feed ingredients were mixed with oil and water (30%) until the consistency was suitable for pelleting. The mixture was then extruded in the spaghetti maker through a 3 mm die. Then the feed spreads were dried in a oven until the humidity less than 10% was obtained and then the pellets were broken into small pieces. The animals were fed with either basal diet only (control group), basal diet supplemented with 0.1% of lyophilized *Lactobacillus plantarum* with 10^7 cfu kg^{-1} (probiotic group), 0.25% and 0.5% of lyophilized *R. coreanus* ethanolic extract (*Rc*EE group). Probiotic was selected based on previous report [23].

Table 1
Composition of basal diet (g kg^{-1}) for shrimp.

Ingredients	Control	Probiotic	*Rc*EE 0.25%	*Rc*EE 0.5%
Fish meal	387	387	387	387
Soybean meal	125.04	125.04	125.04	125.04
Yeast meal	17.2	17.2	17.2	17.2
Shirmp shell meal	54.37	54.37	54.37	54.37
Wheat flour	339.39	339.39	339.39	339.39
Gluten	25	25	25	25
Fish oil	10	10	10	10
Soybean oil	12	12	12	12
Mineral mixture	18	18	18	18
Vitamin mixture	6	6	6	6
Cellulose	5	5	2.5	0
Skim milk	1	0.9	1	1
Probiotic	0	0.1	0	0
*Rc*EE	0	0	2.5	5

The dry fruits of *R. coreanus*, were purchased from Korean traditional medicinal market and the pulverized powered was extracted with 70% ethanol in Soxhlet apparatus and concentrated with rotary evaporator. Analysis of the basal diet was approximately 42% crude protein, 8.4% crude lipid, 11.8% ash, and 8% moisture. The finished pellets were stored at 4 °C until used.

2.2. Experimental design

Six hundred healthy shrimp *post-larvae* of average weight 0.53 ± 0.04 g obtained from a commercial shrimp farm (Jeil Shrimp farm, Haenam, Jeolla Province, South Korea) were fed with a basal diet and acclimatized for 10 days before conducting the experiment, and then randomly assigned to four groups with three replicates. Each replicate included 50 shrimps, which were maintained in an aquarium of 80 L capacity, with a daily one-third water exchange and fed a diet at 5% of body weight twice daily. During 8 weeks feeding experimental period, different physico-chemical parameters such as temperature, dissolved oxygen (DO) and pH were routinely monitored. The temperature, salinity and pH of the rearing aquaria were 26 ± 3 °C, 21 ± 5 and 7.5 ± 1.2 respectively. The DO level was maintained above 6 mg L^{-1} by setting the air pump.

2.3. Sampling for analysis

At the end of the experiment, the survival rate and weight gain rate (WGR) of the different groups were calculated according to the following equations:

$$\text{Survival rate}(\%) = 100 \times N_e = N_s$$

$$\text{WGR rate}(\%) = 100 \times (W_e - W_s)/W_s$$

where N_e and W_e are the number and the average weight of shrimp at the end of the experiment, N_s and W_s are the number and average weight of shrimp at the start of the experiment respectively.

2.4. Assessments of immune parameters in shrimp

During the feeding period, eight shrimps from each replicates of group were sampled randomly on 1st, 2nd, 4th, 6th and 8th week for immune enzyme activity and immune genes expression assays. For immune enzyme assay, four shrimp PL were homogenized individually in 10 × (w/v) phosphate buffer solution (0.1 mol L^{-1}, pH 7.2) on ice. The homogenate were centrifuged (6000 rpm, 10 min) at 4 °C and the supernatants were used to determine the activities of acid phosphatase (ACP) and alkaline phosphatase (AKP) and antioxidant enzymes like SOD, CAT, GPx and MDA.

For immune enzyme assays, AKP and ACP activities of the tissues were determined spectrophotometrically at 520 nm (Libra S22 UV/Visible, Biochrom, England), with alkaline phosphatase (AKP) detection kit and acid phosphatase (ACP) detection kit (RANDOX, UK), respectively. One unit of AKP activity was defined as the amount of enzyme that reacted with the matrix and produced 1 mg phenol in 30 min at 37 °C. One unit of ACP activity was defined as the amount of enzyme that reacted with the matrix and produced 1 mg phenol in 15 min at 37 °C.

For antioxidant enzyme assays, SOD, CAT and GPx activities of tissue were determined spectrophotometrically at 550, 405 and 412 nm (Libra S22 UV/Visible, Biochrom, England), respectively. Anti-oxidative enzyme detection kits (SOD, CAT and GPx) were purchased from Randox Laboratories Limited (Country Antrim, UK). One unit of SOD activity was defined as the amount of tissue extracts that inhibited the rate of xanthine reduction at 25 °C by 50%,