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Dietary Administration of Yeast β 1,3 1,6 Glucan on Immunity and Survival Rate of White Indian Shrimp, Fennerpenaeus indicus Challenged with White Spot Syndrome Disease

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Abstract

The potency of dietary β 1,3 1,6 glucan (BG), derived from Saccharomyces cerevisiae, in stimulating the non-specific immunity of white Indian shrimp, *Fennerpenaeus indicus* (Milne-Edwards, 1837) and improving its resistance to white spot syndrome disease were investigated. *F. indicus* (11.32±1.20 g) were fed for 20 days on a series of treatment diets containing graded levels of BG (blank control, 0 as control, 2, 10, 20 g kg^{-1} feed) and were then challenged by injection of WSSV virus. Total haemocyte count (THC), total plasma protein (TPP), phagocytic activity (PA) and Bacterial Clearance activity (BC) were measured at days 0, 7, 14, 21 after BG feeding, and shrimp survival rate was also recorded daily after challenge. THC, TPP, PA and BC of the 10 and 20 g kg^{-1} BG treatments were significantly higher (P<0.05) by day 14 than control and 2 g kg^{-1} treatment shrimp. Survival rate of shrimp fed with the diet containing 10 and 20 g kg^{-1} BG after 21 days, were 53.32±5.77 and 48.32±5.77%, respectively. Accordingly, oral administration of BG at an optimal level of 10 g kg^{-1} diet for 20 days efficaciously stimulate the immune defense and improve the survival rate of WSV-infected *F. indicus*.

Keywords: Fenneropenaeus indicus, beta 1, 3 1, 6 glucan, haemocyte, Phagocytic activity, Bacterial Clearance activity

Introduction

Most immune stimulants are chemical compounds which exist as structural constituent of bacteria, mycelial fungi and yeasts. A number of different immune stimulants have been classified in seven categories by Raa (1996). β Glucans, one of the known immunostimulants, has been successfully used to increase resistance of fish and crustacea against bacterial and viral infections (Itami *et al*., 1994, Sung *et al*., 1994, Su *et al*., 1995, Liao *et al*., 1996; Song *et al*., 1997, Chang *et al*., 1999, Chang *et al*., 2000, Chang *et al*., 2003, Sajeevan *et al*., 2009). *Schizophyllum* β glucan, which is derived from the fungus *Schizophyllum commune*, is a water-soluble β1,3 glucan with some β 1,6-glucosidic side chains. Dietary utilization of β 1,3 glucan enhances the resistance of *M. japonicus* against vibriosis (Itami *et al*., 1994). An increased resistance in *P. monodon* against vibriosis and white spot disease were reported by Sung *et al*. (1994) and Song *et al*. (1997) after immersion or injection of a different glucan, BG extracted from yeast cell wall. In M. japonicus, oral administration of peptidoglycan derived from *Bifidobacterium* thermophilum was also found to infections remission with penaeid rod-shaped DNA virus and penaeid acute viremia disease (Hennig *et al*., 1998, Itami *et al*., 1998). In similar studies on *P. monodon* (Su *et al*., 1995, Liao *et al*., 1996, Chang *et al*., 2000) indicates that oral administration of β 1, 3-glucan at 2 g kg-1 diet for 10–20 days significantly increase immunity of postlarvae, juveniles, and adult shrimp against *Vibrio damsela*, *Vibrio harveyi* and white spot infections. Chang *et al*. (1999), however, have been reported the overall survival rates of the test postlarvae and juveniles were relatively less than 20% when the test shrimps were challenged with WSSV (Chang *et al*., 1999). The juveniles fed on β 1, 3-glucan diet for 20 days had a survival rate of 20% 6 days after challenge, while all the juveniles fed on β 1, 3-glucan for 10 days died. The begin-

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ning of disease resistance in response to β 1, 3-glucan appears to be slow, with up to 20 days of continuous feeding being needed to give raise an efficient resistance to WSSV (Chang *et al*., 1999). White spot syndrume disease outbreak in Bushehr province, Iran, from six years ago (Tokhmafshan *et al*., 2004) aroused us to study on shrimp health and enhancement of shrimp immunity as a primary concern. One of the most assuring groups of immune-stimulants is BG, because they have a well-defined chemical structure and mode of action on the immune defense, described in a great number of scientific papers. On the other hand, BG are non-toxic universal "alarm signals" which actuate the immune system by the same basic mechanism in all animals, from uncomplicated invertebrates to man. BG is active not only when injected, but also when administered in oral manner, or on mucosal surfaces (Dalmo and Bøgwald, 2008).

The aim of the present study was to contribute to a better understanding of oral administration of yeast BG to stimulate immune system of *F. indicus* and enhance resistance of shrimps against white spot viral disease in native condition in Bushehr province, Iran.

Materials and methods

Diet preparation, Chemicals and solutions

The BG, derived from *Saccharomyces cerevisiae*, was bought from Southeastern Pharmaceutical Wholesale (Birmingham, Alabama, US). The feed ingredients were thoroughly mixed, cold extruded and cut into 3 mm × 5 mm pellets. Pellets were air-dried and stored at -10°C until feeding.

The glassware and solutions were pyrogen-free to avoid enzymatic interruption and all chemicals were of analytical reagent grade. The anticoagulant that avoids clotting after collection of haemolymph was prepared according to (Vargas-Albores *et al*., 1993): (27 mM Na citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7).

Experimental design and Feeding

350 shrimp from the Helleh research station adjacent to the Bushehr city in Bushehr province, Iran, were transferred to the Iran Shrimp Research Center (ISRC). Shrimp were placed in glass aquariums (155 L), and acclimated to room temperature (25.0±1.0°C) for two weeks. During the acclimation period, shrimp were fed four times daily with a normal formulated shrimp diet without immunostimulants (Havoorash feed Company, Bushehr, Iran). Only shrimp in the intermolt stage were used to the study (Liu *et al*., 2004). The molt stage was determined by examination of uropod (One of the last pair of posterior abdominal appendages of certain crustaceans, such as the lobster and shrimp) in which partial retraction of the epidermis could be distinguished (Liu *et al*. 2004; Robertson *et al*., 1987). The shrimp ranged from 10.33 g to 15.02 g, averaging 11.32±1.20 g (mean ± SD, n=50) with no significant size differences among the treatments. Initially, shrimp were fed for 20 days on a series of treatment diets containing graded levels of BG (0 as control, 2, 10, 20 g kg^{-1} feed, in triplicate and 20 shrimps in each replicate) and were then challenged by injection of WSSV virus. For each replicate, shrimp survival rate was recorded daily since WSSV injection. Moreover, THC, PA, TPP and BC parameters in treatment and blank control (fed BG free diet and not challenged to WSSV) groups were determined, at first day and after 7, 14 and 21 days since virus injection.

Preparation of the WSSV stock solution

The WSSV stock solution was prepared following the method of Chang *et al*. (1999). Shell and epidermis appendage collected from frozen (-80°C) pond-grown shrimp, which had infected to WSSV, were homogenized in saline (NaCl 0.9×g100 ml-1) solution (1:9, v v-1) at 4°C. After centrifugation at 10108×g for 10 min (Sigma 3-16PK, Rotor No. 19776, Germany) the supernatant was filtered through a 0.45 μm milipore and used as the WSSV stock solution. The outcomes of preliminary experiments indicated that the optimum dilution level of the WSSV stock solution to induce 48 h LD50 in *F. indicus*, when injected intramuscularly, was 10 times.

WSSV diagnostic PCR

All shrimps were tested for WSSV infection, using the two-step WSSV PCR diagnostic procedure (Lo *et al*., 1997) before the feeding, and before and after the viral challenge. As Lo *et al*. (1997) have reported, prawns that are detected as 1-step WSSV positive are more seriously infected with WSSV

than the prawns that are 2-step positive but 1-step negative. For each treatment, organs and tissues from 5 surviving shrimps were sampled and individually tested.

Haemolymph preparation

Haemolymph (100 µL) was withdrawn from the ventral sinus located at the base of the first abdominal segment of each shrimp into a 1 mL sterile syringe containing 0.9 ml precold (4°C) anticoagulant solution (prepared as mention before) and transferred into the eppendorf microfuge (Vargas-Albores *et al.*, 1993).

Assay of immunological parameters

THC and TPP

A drop of withdrawn haemolymph after gently mixed with anticoagulant solution was placed on a hemocytometer and the THC was measured using a light microscope (Nikon Photolab Eclipse E200, Japan) with magnification of 40×.

Concentrations of plasma protein for each shrimp sample were ascertained by the Biuret method. All samples were measured in triplicate.

PA and BC

Phagocytic activity was determined by the method of Jiang, Yu and Zhou (2004). 25mL of solution A was placed on a dichromate-cleaned glass slide and incubated for 30 min at room temperature. Subsequently, 25 µL of *Staphylococcus aureus* at a concentration of 1×10^8 cells mL^{-1} was added to each solution A sample and the preparation was incubated for an additional 30 min. Then, each slide was washed with anticoagulant, fixed with 4% glutaraldehyde in the solution of anticoagulant for 1 min, rinsed in distilled water for 1 min, post fixed with 95% ethanol for 1 min, and air-dried. The slides were then stained with toluidine blue for 5 min and decolorized in running tap water. Numbers of ingested *S. aureus* and numbers of haemocytes that have ingested *S. aureus* were counted from any 200 haemocyte observed using a light microscope at a magnification of ×100 (Nikon Photolab Eclipse E200, Japan). Phagocytosis percentage was calculated as below (Weeks-Perkins *et al*., 1995): Percentage phagocytosis= (number of cells ingesting bacteria/number of cells observed) ×100.

To BC assay, after injection challenge with 1×10^5 cfu Vibrio spp. per shrimp and were then kept for 1.0 h in a separate tank containing 40 L of water, 100µL haemolymph samples were taken from ventral sinus of shrimps, the samples were immediately added to 1.9 ml of precold (4°C) sterile Van Harrevald's salt solution (VHS). Haemolymph (100µl) in VHS were spread onto TCBS agar plates for enumeration of numbers of total V. harveyi in haemolymph on TCBS plates were counted after incubation time for I8 hours (Liu *et al*., 2004).

Statistical analysis

ANOVA and Tukey's honest significant difference test were used by SPSS software to compare the significant differences in the shrimp survival rate, THC, TPP, PA, BC, BA and WSSV PCR results between the challenged and the control groups for statistically significant differences ($p < 0.05$).

Results

After feeding the BG containing diets, a glucan content of 10 g kg-1 was evaluated as optimum level for utmost protection against WSSV during experimental infection. The survival rate of the shrimp fed on the BG diets were significantly higher ($P<0.05$) than that of the BG-free group on day 2 after viral challenge.

Survival rate

Survival rate after challenged to WSSV on the 7th day with the 20 g kg^{-1} BG (81.67±2.88%) diet was found to be significantly different from the other treated groups including the BG free (28.32±5.77 %), 2 (55.00±7.6%) and 10 (66.67±7.63%) g kg-1 (Fig. 1). By day 10, however, survival rates of the groups fed on the BG-free or 2 g kg-1 diets were naught and 36.67±7.63%, respectively, but the survival rates of shrimp fed on the diets containing 10 and 20 g kg^{-1} were over 53.33 and 61.67%. In contrast, the day15 survival rate of the 10 g kg^{-1} BG diet decreased to 53.32±5.77%, while survival rate of the 20 g kg^{-1} BG diet had more decline and reached to 48.32±5.77%. In spite of substantial mortalities in the free BG replicates, no shrimp in the blank control group died up to day 21.

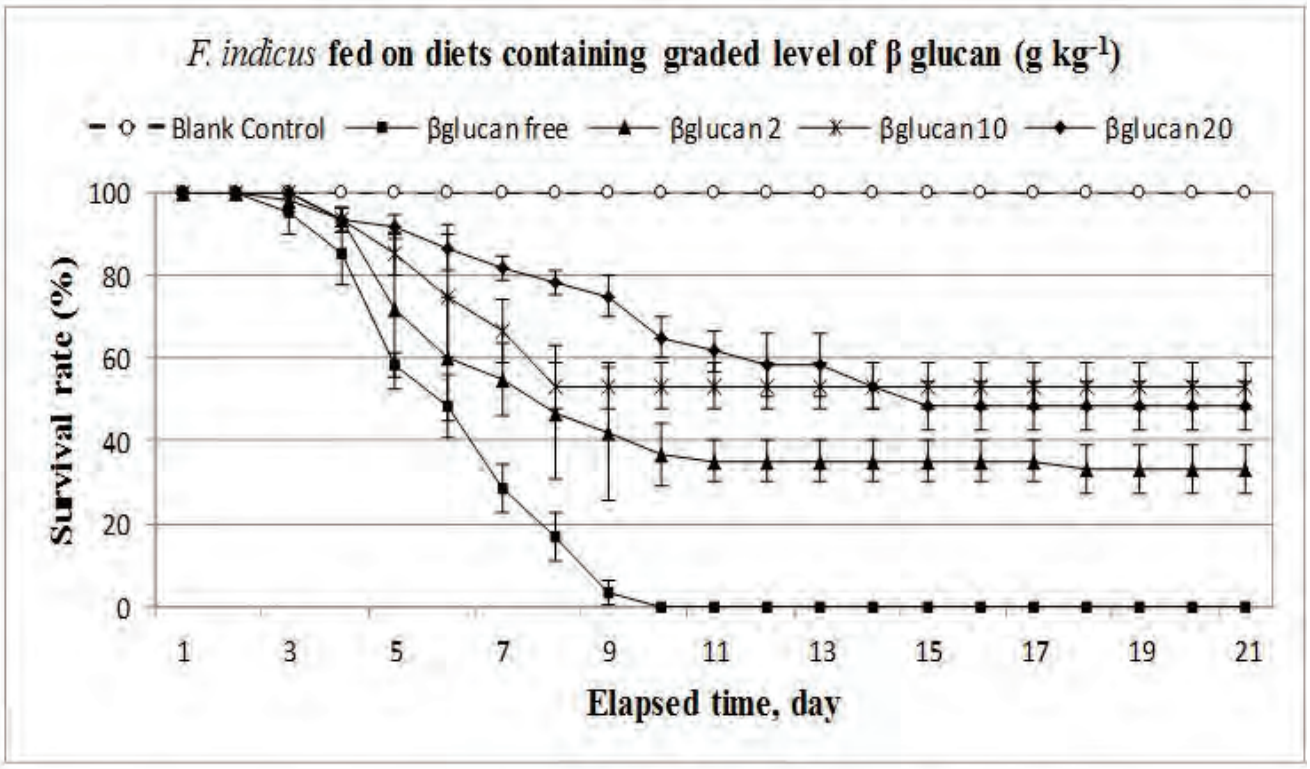

Fig. 1. Survival rates of F. indicus fed on diets containing graded levels of BG for 20 days and then challenged by injection of WSSV (no viral challenge in blank control).

Confirmation of WSSV

All initial shrimps were tested to be 2-step negative in WSSV PCR diagnosis while all of the dead or moribund shrimps, after the WSSV challenge, were 1-step positive. A snap-shot examination of gill, midgut, hepatopancreas and abdominal muscle at day 21 indicated that the 10 and 20 g kg^{-1} diets were 55, 65 and 65% 2-step negative, respectively (Table 1). Tukey's honest significant difference test revealed that the rate of 1-step WSSV positive in the 2 g kg^{-1} group was significantly higher than that of the 10 and 20 g kg^{-1} groups although the rate of 2-step WSSV negative in 10 and 20 g kg^{-1} treatments was significantly higher than the 2 g kg^{-1} treatment.

THC and TPP

Administrated BG diets showed significant immune enhancement after 20 days feeding but both of them were significantly (P<0.005) decreased after WSSV challenge. All infected shrimps had a THC of 28.77–45.32×10^5 cells ml^{-1}, which was less

Table 1. Detection of WSSV on the 21th day in F. indicus fed on diets containing graded levels of BG for 20 days and subsequently challenged by WSSV injection.

Dietary BG g kg^{-1}	Shrimp number	Detection of WSSV infection, number of shrimp (%)			
		1 step PCR positive	2 step PCR positive	2 step PCR negative	Overall positive
2	20	6 (30)	5 (25)	9 (45)	11 (55)
10	20	3 (15)	5 (25)	12 (60)	8 (40)
20	20	3 (15)	6 (30)	11 (55)	9 (45)

than that of the control group (72.89±8.87×10^5 cells ml^{-1}) at day 7 after the challenge. By day 7 the BG free and 2 g kg^{-1} diets showed a THC less than 50% that of the control group and shrimps were totally died from 3th to 7th day subsequently in all replicates of BG free group (Fig. 2). These shrimps were all 1-step WSSV positive. In contrast to the forceful and continuous reduction of THC and TPP in two mentioned groups, the groups fed with diet containing 10 and 20 g kg^{-1}, made partial and total recovery in THC and TPP in 2th and 3th week after challenge, respectively.

PA and BC

PA and BC was significantly higher in the BG diets treatments than the BG free group on day 0, before challenge. Following the challenge, PA of the 10 and 20 g kg^{-1} groups brought back to the level of the control group by day 14. All infected shrimps had a PA and BC of 4, 34-8.77% and 9.55-18.14%, respectively which was less than that of the control group (27.87%) at first week after challenge Figure 3. As showed in figure 3, PA and BC in BG diets groups were higher than BG free and 2 g kg^{-1} treatments. In second week after challenge, PA recovered entirely in 10 and 20 g kg-1 groups.

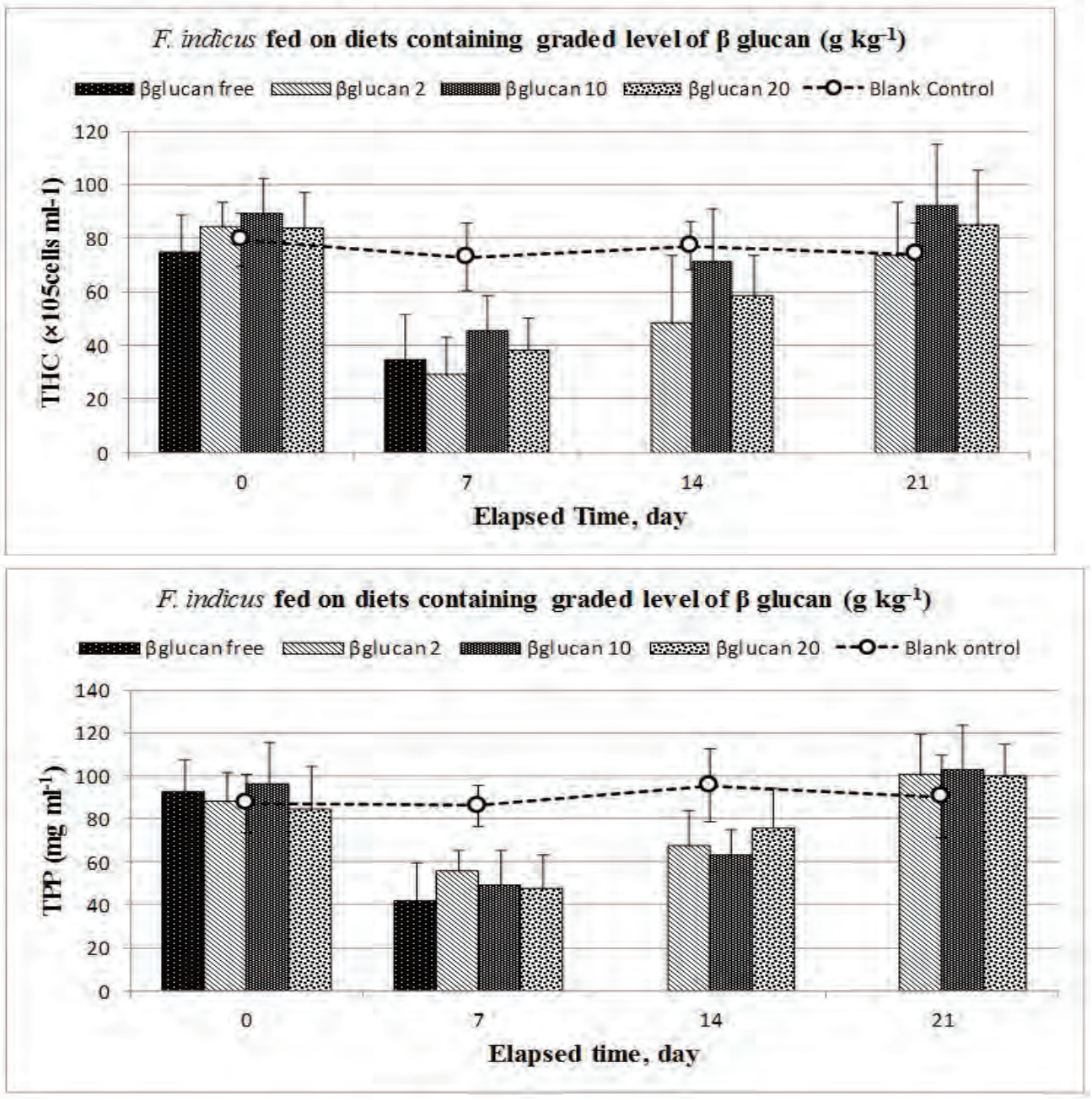

Fig. 2. THC (A) and TPP (B) of F. indicus fed on diets containing graded levels of BG for 20 days and then challenged by injection of WSSV (no viral challenge in blank control).

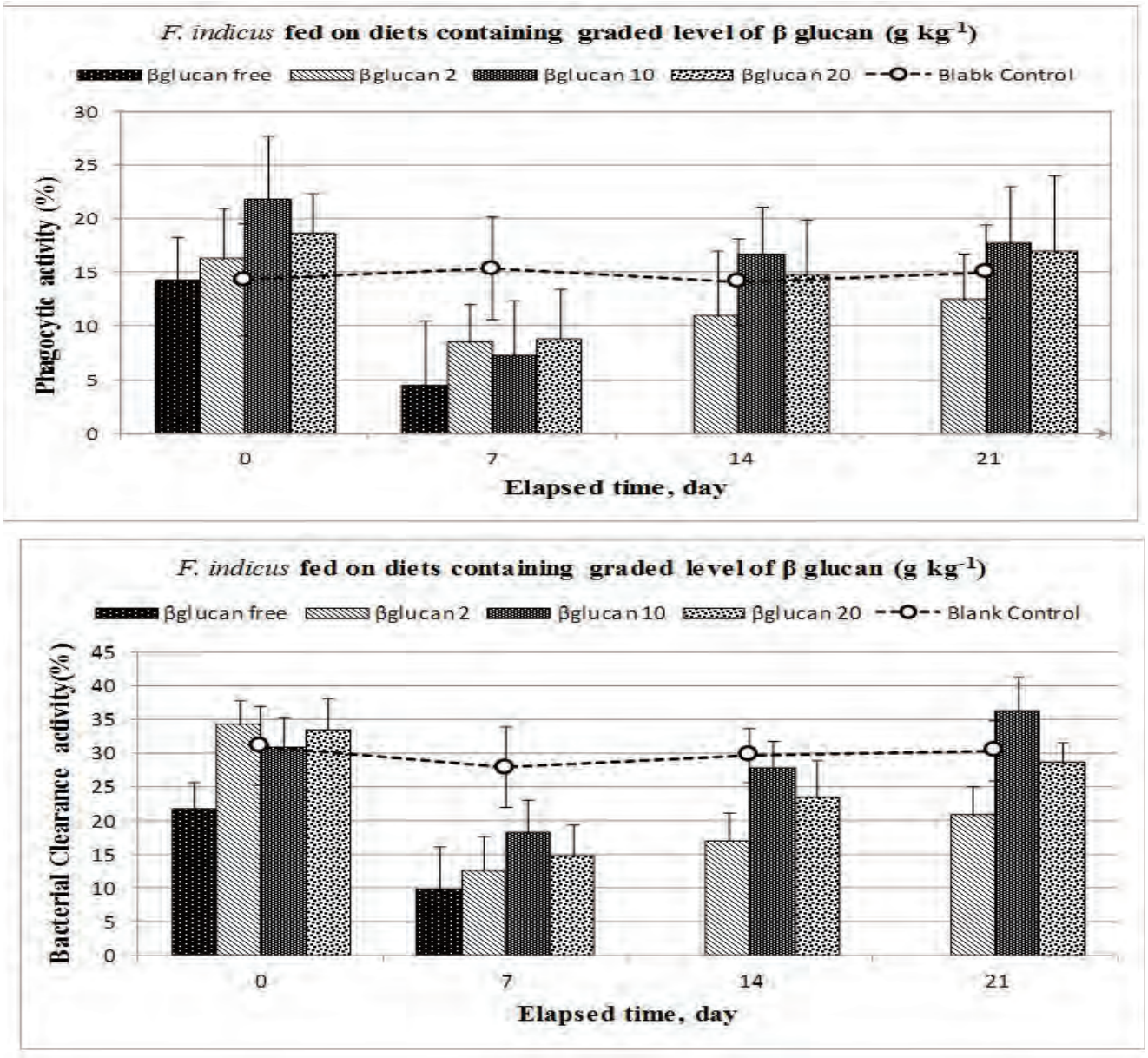

Fig. 3. PA (A) and BC (B) of F. indicus fed on diets containing graded levels of BG for 20 days and then challenged by injection of WSSV (no viral challenge in blank control).

Discussion

The hard cuticle, like a physical barrier, can be considered as the external defense in crustacean. Although hard cuticle is helpful but is not adequate and haemocytes play an important and central role in the internal defense. Crustaceans have three categories of haemocytes consist hyaline cells, semigranular cells and granular cells. Each type of haemocytes has distinctive morphological characteristics and physiological functions (Le Moullac *et al.*, 1997; Johansson *et al*., 2000). The first and essential internal defense role is the recognition of invading microorganisms, which is mediated by the haemocytes and plasma proteins (Vargas-Albores and Yepiz-Plascencia, 2000). Nowadays, Immunostimulants receiving most attending and claims to promoting survival of shrimps against experimental exposure to viral and bacterial pathogens.

Analyzed results were confirmed with Bliznakov and Aldler (1972) thought that unlike many chemotherapeutics, immunostimulants do not show a linear dose–effect relationship. They often show a clear cut maximum at intermediate concentrations and sometime a complete absence of effect or even contrary toxic effect at higher concentrations (Floch *et al*., 1987).

According to the results, BG when dietary administrated at 2 g kg^{-1} of diet was not as effective

in protecting the shrimp from WSSV infection as the dosages of 10 and 20 g kg^{-1} of diet. As for one and two step PCR results, shrimp that survived from the WSSV challenge were less seriously infected with WSSV in the 10 and 20 g kg^{-1} treatments than the 2 g kg^{-1} treatment. Dietary administrated of BG in 10 g kg^{-1} more effective than other dietary even from 20 g kg^{-1}.

Results showed the THC and TPP of the BG fed groups were 30- 50 % lesser than those of the blank control group on 7th day after WSSV challenge but recovered in 14th day. When THC had dropped to below 30×10^5 cells ml-1, all shrimps died if they fed by BG free diet. Mortality occurred at day 3-10 for the BG-free group and at day 4-11 for the 10 and 20 g kg^{-1} groups. Then, according to Chang *et al.* (1999) viewpoint, Haemocytes play an important role in cellular defense and THC is a useful indicator of shrimp health.

PA and BC showed correlation to THC changes after WSSV challenge and appear point of no return in shrimp immune system, when the THC, TPP and PA was lower than 34.18×10^5 cells ml^{-1}, 41.44 mg ml^{-1} and 4.34% respectively and recovery becomes improbable. Defense against bacterial pathogens in shrimp depends on a number of cellular (THC) and humoral (TPP) activities interrelated in a complex way; we reasoned that following the rate or efficiency of bacterial clearance after a severe challenge would be a simple method of measuring the summational potential of these activities.

In conclusion, Survival rate of shrimp fed with the diet containing 10 and 20 g kg^{-1} BG after 21 days, were 53.32±5.77 and 48.32±5.77 %, respectively. Consequently, oral administration of BG at an optimal level of 10 g kg^{-1} diet for 20 days effectively stimulate the immune defense and improve the survival rate of WSV-infected *F. indicus*.

Finally, emphasize on fact that the dose and frequency of application of immunostimulants in shrimp aquaculture should be standardized and validated before commercialization to attain optimum modulation of the immune system and to avoid immune weaken due to overdose. The short time protection, lack of linear dose–effect relationship and lack of memory immunity, lead to more complication in immunostimulants application in shrimp culture industry. Hence, perfunctory use of an immunostimulant without cognizing the optimum dose and frequency could never be an effective administration strategy.

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