Effects of heat-killed *Lactobacillus plantarum* strain L-137 on growth performance and immune responses of white leg shrimp (*Litopenaeus vannamei*) *via* dietary administration

Pham Minh Duc[1]*, Hua Thai Nhan[1], Tran Thi Tuyet Hoa[1], Hong Mong Huyen[1] Chau Tai Tao[1], Cao My An[1] Dang Thuy Mai Thy, Tran Ngoc Hai[1], Hirose Yoshitaka[2] and Onoda Satoru[2]

[1]College of Aquaculture and Fisheries, Can Tho University, Vietnam
[2]House Wellness Foods Corporation, Japan

Abstract- Cultivation of white leg shrimp *Litopenaeus vannamei* has been faced to unprecedented disease and environmental issues. This study was conducted to determine the effect of heat-killed *Lactobacillus plantarum* strain L-137 (HK L-137) on growth performance and immune responses of *L. vannamei* via dietary treatment. Different levels of 20% HK L-137 (LP20) were added into a commercial feed including 0.0g of LP20 kg^{-1} of feed (control), 0.1g of LP20 kg^{-1}, 0.05g LP20 + 5g β-glucan kg^{-1} and 10g β-glucan kg^{-1}. Shrimp (PL12) were fed with these feeds for 90 days in re-circulation 2 m^3 tanks at stocking density of 300 ind./tank. The results showed that survival and growth rates of shrimp fed with feed supplemental LP20 were significantly (P<0.05) higher compared to other feeds. Immune parameters consist of total haemocyte count, differential haemocyte count, phenoloxidase activity, phagocytic activity and clearance efficiency, *Vibrio parahemolyticus* challenges were increased significantly (P<0.05) for those animals fed with HK L-137. This study demonstrated that HK L-137 would enhanced growth performance and improved immune defense system of *L. vannamei*.

Index Terms: Heat-killed bacteria, *Lactobacillus plantarum*, *Litopenaeus vannamei*, growth performance, immune response.

I. INTRODUCTION

Cultivation of white leg shrimp *Litopenaeus vannamei* has been growing rapidly and becoming an important economic activity in Mekong Delta, Vietnam. Unfortunately, this industry has been encounting with the unprecedented disease since the last decade. Obviously, vibriosis is one of the major diseases in shrimp culture responsible for the massive kill of cultivars. Vibriosis is mainlly caused by a number of *Vibrio* species, including *V. harveyi, V. vulnificus, V. parahaemolyticus, V. alginolyticus* and *V. penaeicida* etc. Among them, *V. harveyi* and *V. alginolysticus* are known as endemic opportunistic pathogens in white shrimp culture. In recent years, white shrimp culture in Southeast Asia is suffering the spread of disease namely early mortality syndrome (EMS) or acute hepatopancreatic necrosis syndrome (AHPNS). In Vietnam, shrimp farmers in the Mekong Delta, particularly in coastal provinces of Soc Trang, Bac Lieu, Ben Tre, Tra Vinh and Ca Mau are suffering losses.

Due to devastation of bactierial and viral diseases in shrimp culture; some remedy strategies have been proposed as biosecurity, application of antibiotics, probiotics, and development of disease resistance strains. However, the development of genetically based host resistance is costly and impossible to attain the specific pathogen resistance. The abuse of antibiotic has resulted in antibiotic resistant microbials as well as environment pollution with antibiotic residues. Therefore, development of feed formulation for shrimp growth improving, shrimp immune enhancement, and prevention of pathogens propagation are primary concerns.

Studies have shown that the metabolites of live or dead bacterial cells function as immunostimulants in gastrointestinal tracts of fish such as increased disease resistance and immune responses (Salminen, et al., 1999; Son et al., 2009; Giri et al., 2013; Giri et al., 2014). Dietary supplementation of inactivated bacteria stimulated fish innate immune response of animals such as increased disease resistance (Biswas et al., 2013a; Cerezuela et al., 2012; Biswas et al., 2013b). For example, when Chinese drum (*Miichthys miiuy*) was fed with diet supplemental heat-killed bacterium *Clostridium butyriumretain* induced immunomodulating properties better than those of fish fed with diet supplemental live bacterium (Pan et al., 2008). Similarly, heat-killed bacterium of *Vibrio vulnificus* induced a higher antibody response than that induced by formalinised bacterins in flounder, *P. olivaceus* (Park *et al*., 2001). Heat-killed *L. plantarum* strain L-137 (HK L-137) functions as a stimulant that stimulates the induction of interleukin and antitumor in mice and to enhance gamma interferon and T-cells and activates macrophages cells (Murosaki *et al*., 1998, 2000). In aquaculture, administration of live *L. plantarum* induced immune modulation, enhanced the growth performance, immune ability, and increased disease resistance in fish (Giri *et al*., 2013, 2014; Son *et al*., 2009). Recently, Madmound et al. (2015) found that diet supplemental heat killed *L. plantarum* induced growth, stress resistance and immune response of red sea bream (*Pagrus major*). Tung et al. (2010) found that stress resistance of larval stage and post larval kuruma shrimp, *Marsupenaeus japonicas* increased when animals were fed with a diet containing HK L-137 compared to those diet without HK L-137. Thus, dietary supplementation of inactivated bacteria stimulated fish and shrimp innate immune parameters, increased disease resistance

and growth response as well. With these observations, we hypothesized that HK L-137 might be effective in growth response and biological defense systems of white leg shrimp *L. vannamei*. Thus, this study aims to investigate the effect of the oral administration of HK L-137 on the growth, survival, immune response, and stress resistance of white leg shrimp *L. vannamei.*

II. MATERIALS AND METHODS

A. Experimental diet

LP20 contains 20% Heat-killed *Lactobacillus plantarum* strain L-137 (HK L-137) and 80% dextrin in dried-weight basis. LP20 is a commercial product which was made by House Wellness Foods Corp. (Itami, Japan). The concentration of HK L-137 in the dry product is 2×10^{11} cfu/g. The HK L-137 was prepared based on the previous method described by Murosaki et al. (1998). Previous study showed that addition of 0. 1 or 1.0 g of LP20 kg^{-1} into feed induced a good growth and survival of juvenile Kuruma shrimp (Tung *et al.*, 2009). In this study, three different test feeds and a control one are described in the Table 1, in which feed 1: Commercial pellet (C) + 0.10 g of LP20 kg^{-1}, feed 2: C + 10 g β-glucan kg^{-1}, additional β-glucan was considered as a immunostimulant control for LP20 and feed 3: C + (0.05 g LP20+ 5 g β-glucan kg^{-1}). The commercial pellet feed only was used as a control diet with proximate analysis of protein (41.3%), lipid (6. 1%), carbohydrate (34.0%), ash (15.6%), and moisture (10.7%). Triplicates were done for each dietary treatment.

B. Experimental animals

This study was conducted at the Weblab and Pathology laboratory of College of Aquaculture and Fisheries (CAF), Can Tho University, Viet Nam. Postlarval shrimp *L. vannamei* 12 days (PL12, approximately 100,000 PL/kg) post metamorphosis were obtained from the hatchery of CAF. The average length of each individual was about 10.8 mm. The feeding trials were conducted in 2 m^3 per tanks at stocking density of 300 PL per tank, in a flow through sea water (10‰) system with biofilter system, which was prepared about 2 weeks prior experimental set up. All tanks were continuous aeration supply. The experiment was run indoor under natural light:dark (12h:12h). During the experimental period, water quality parameters (mean ± S.D.) were monitored.

The experiment was assigned randomly with tri-replicates were run for each test diet for 90 days. Animals were fed four times per day at 7:00AM, 11:00 AM, 4:00PM and 8:00PM. Feeding behavior of animals was observed and extra feed with feces were siphoned daily.

C. Measurement of growth performance and immune parameters

The growth of animals in weight (g), length (cm) were measured monthly. The growth was expressed in terms of specific growth rate (SGR), weight gain and length increasing. The weight was determined with an electronic scale (0.01 g error) for every 4 weeks.

Mean weigh gain (g/individual) = Wf-Wi

Specific growth rate (SGR,%) = {(lnWf-lnWi)/T} x 100,

Daily weight gain (DWG) = (Wf-Wi)/T

Survival (%) = (final no. of shrimp/ initial no. of shrimp) x 100

Where, Wf is final weight, Wi is initial weight and T is total day of the experiment.

Feed conversion ratio (FCR) = total feed eaten (g)/wet weight gain (g).

D. Stress test

Stress test was performed at the end of the experiment. Seven shrimps were simply selected from each of experimental test diet for formalin test at the concentration of 200ppm. Three replicates were done for each diet. The mortality of shrimp was observed and monitored every 10 min up to 60 min.

Color evaluation of shrimp in each experimental treatment was also done by boiling 7 shrimps at 100 ^oC for 10 min. The color was evaluated by visual observation and photographed.

E. Total hemocyte count (THC)

Haemolymph (100 µl) was collected from the ventral sinus and mixed gently with 900 µl of sterile anticoagulant solution. Thereafter, the number of hemocytes were counted and calculated as cell ml^{-1} using hemocytometer with light microscope at 400 x magnification (Le Moullac *et al.*, 1998). THC was calculated according to the following fomular

THC (cell/ml) = Total hemocyte counted x 10 x 10 x 100

F. Hemocyte differential

Haemolymph (100 µl) was collected from the ventral sinus and mixed with 900 µl of sterile anticoagulant solution. Mixed haemolymph were centrifuged at 5,000 rpm for 5 min at 4 °C, washed and re-suspended with 200 µl of formalin-AS pH 4.6 (1:10). After centrifuge, the suspension (50 µl) was spread onto a slide glass and fixed by ethanol for 5 min, stained with Giemsa for 30 min, washed in aceton and xylen (Cornick and Stewart, 1978). Thereafter the numbers of hemocyte were counted under compound microscope.

G. Phenoloxidase activity assay

Following the procedures of Hernandez-Lopez *et al.* (1996), phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenyl-alanine (L-DOPA). The details of the measurements were described previously Liu *et al*. (2004). The optical density of the shrimp's phenoloxidase activity was expressed as dopachrome formation per 50 ml haemolymph.

H. Phagocytic activity assay

Experimental shrimp were injected with 20 µl of bacterial suspension ($1x10^8$ cfu ml^{-1}) and kept for 1hr. Total of 100 µl of haemolymph was collected and mixed with 100 µl of sterile anticoagulant. The methods for the measurements of phagocytic activity were described previously Liu *et al*. (2004). Phagocytic activity, defined as percentage phagocytosis (PR) was expressed as: PR (%) = (phagocytic hemocytes / total hemocytes) x 100

I. Clearance efficiency assay

Experimental shrimp were injected with 20 µl of bacterial suspension ($1x10^8$ cfu ml^{-1}) and kept for 1hr. The 200 µl volume of diluted haemolymph was further diluted to 20 ml with saline solution (Adam, 1991). Three 50 µl portions of this diluted haemolymph sample were spread on separate TSA plates and

incubated at 28°C for 24 hrs. The clearance efficiency, defined as percentage inhibition (PI), was calculated upon the formula: PI (%) = 100 – (cfu in the test group/cfu in the control group) x 100

J. Bacteria challenge

After 45 days and 60 days, shrimp in the feeding experiment (4 treatments) were challenged with *Vibrio parahaemolyticus* by immersion method at 10^8 cells ml^{-1} for 15 min (Tran *et al*., 2013). Shrimp in the negative control group were immersed in sterile Nutrient broth (1.5% NaCl). After immersion the shrimp was transferred directly into experimental tanks containing clean seawater. There were a total of five treatments. Each treatment was conducted with 30 shrimp. The experiment lasted 14 days. PCR assay was performed as previously described (Sritunyalucksana *et al.*, 2014) (Table 2). Experimental shrimps were also collected for histopathology methods (Bell and Lightner, 1988).

K. Gill sampling

Gill samples were sampled after 45 days and 75 days culture period. Two shrimps of each tank were randomly selected. Gill of each shrimp was removed from body and placed into a 1.5 mL tube, thereafter about 1.2 to 1.3 mL of RNAlater was added into each tube. These tubes were stored overnight at 4°C, followed by keeping them in a freezer (-20°C). Different immune index of experiment shrimp were measured from extracted RNA of gill samples using RT-qPCR method (Table 3).

M. Statistical analysis.

Collected data were subjected to One-way ANOVA, followed by Tukey and/or Duncan test to compare the weight gain (g), specific growth rate (% body weight/day), and feed conversion ratio (dry feed eaten/weight gain), Total haemocyte count (THC), Differential haemocyte count (DHC) (version 13.5; SPSS Inc., Chicago. IL, USA).

III. Results and Discussion

Water quality parameters

Water quality parameters are showed in Table 4. Temperatures were similar among the treatments and did not fluctuate much between the morning and afternoon ranging from 28.4-29°C. Christopher (2008) reported that white leg shrimp *Litopenaeus vannamei* can grow within temperature of 14.5-35.0 °C, this indicates that the range of monitored temperatures in our study is suitable for normal growth of animals. Average pH levels among the treatments were from 8.15 to 8.21. The optimal pH value for normal growth of shrimp is 7.5-9.0 (Boyd *et al*., 2002; Whetstone *et al*., 2002). Similarly, alkalinity levels were in good conditions for animals. Overall, these physical water parameters are in good conditions for normal growth of shrimp.

Average $N\text{-}NO_2^-$ levels in all treatments ranged from 0.36-0.47 mg/L (Table 5). According to Chen và Chin (1998) safe levels of $N\text{-}NO_2^-$ for shrimp should be lower than 4.5 mg/L. Average total ammonia (TAN, NH_3/NH_4^+) levels were low for all treatments ranging from 0.04 mg/L to 1.2 mg/L. Whestston (2002) reported that TAN should lower than 2 mg/L. Thus, these chemistry parameter values were safe for shrimp in this study.

Growth performance parameters

Survival rates and growth performance of shrimp are shown in the Table 6 and Figure 1. Survival rates ranged from 60.2% to 87.6%, in which the highest level (87.6%) was obtained from feed supplementation of 0.1g LP20 kg^{-1}, and differed significantly ($p<0.05$) compared to other test feeds. Survival rate in our study is lower than a study by Nguyen Thi Ngoc Anh (2014), when shrimp cultured in integrated system with seaweed, in which the survival rate ranged from 88-95%, it could probably due to the quality of post larvae, as various sizes of shrimp occurred in our experiment.

The highest final weight was obtained from feed supplementation of 0.1g LP20 kg^{-1} and was significantly higher than diet containing β-glucan and control diet, but no differ significantly with feed addition of 0.05g LP20+5g β-glucan kg^{-1}. A slow growth was observed for shrimp in the first 30 days. Similar for DWG and SGR, the highest DWG and SGR were obtained from feed supplemental 0.1g LP20 kg^{-1} (Table 4). DWG and SGR in this study was almost the same as previous study by Nguyen Thi Ngoc Anh (2014), she found that DWG and SGR of *L. vannamei* in co-cultivation with seaweed was about 0.10 g/day and 7.39%/day respectively.

The lowest FCR was obtained from diet supplemental 0. 1 g LP20, and was significantly lower than feed containing β-glucan and control feed, but did not differ significantly with feed addition of 0.05 g LP20+5 g β-glucan kg^{-1}. According to survey data on feed used in semi-intensive and intensive of white leg shrimp culture in the Middle and the Sounth of Viet Nam by Le Thanh Hung và Ong Moc Quy (2010) showed that the FCR values ranging from 1-1.2 accounted for more than 80% farmers. This indicates that our results are quiet match with current pratical shrimp farming in Viet Nam.

Stress resistance against formalin showed that no mortality occurred for shrimp in all treatments after 60 minutes at the 200ppm. Figure 2 showed that shrimp fed with feed supplemental 0.1g LP20 kg^{-1} accumulated dark red color after submerging in boiled water after 10 minutes. Whereas shrimps fed with feed containing beta-glucan had a very light red color on skin and shrimps fed feed without any addition of additive (control). Yu and his colleagues (2003) reported that shrimp *L. vannamei* raise in intensive system normally had a light red skin color after boiling due to lacking of capability of pigmentation especially astaxanthin and/or due to feed supply deficiency of carotenoid. Another study showed that when shrimp were fed with feed supplemental seaweed gave dark red color after boiling (Anh, 2014). It was due to high level of carotenoid in seaweed *Enteromorpha* sp.. Skin color of shrimp after cook was considered as an attractive criteria for consumers. In our study, shrimp fed with feed containing 0.1 g LP20 gave a better color than other feeds, the result indicates that LP20 may also stimulate or act as a co-factor for the synthesis of carotenoid.

Total haemocyte count (THC)

Figure 3 showed total haemocyte count of *L. vannamei* at the 45, 60 and 75 day of oral administrations. It is not clear for THC of animals at the 45-day oral administration. The same observation for those animals at the 60-day oral administration, the average THC of shrimp were $74.67\pm9.98x10^5$ cell/ml (LP20), $61\pm10.62x10^5$ cell/ml (β-glucan group), $69.67\pm7.91x10^5$ cell/ml

(LP20+β-glucan), and 63±9.47x10^5 cell/ml (Control). However, at the 75-day oral administration, the THC of the shrimp fed with LP20 (94.83±14.55x10^5 cell/ml) was higher significantly ($p<0.05$) than all the other groups including LP20+glucan group (79.25±11.76x10^5 cell/ml), β-glucan (59.33±12.32x10^5 cell/ml) and control as well.

Differential haemocyte count.

Differential haemocyte count (DHC) of *L. vannamei* was determined and expressed in the Table 7 and Figure 4. Large granular cell (LGC) of shrimp in the experiment was within the range of 55.52±7.03x10^5 - 65.61±8.63x10^5 cell/ml and no significant difference value ($p<0.05$) was found at the 45 day oral administration. In the other 60 and 75 days oral administrations, however, LGC of the LP20 group was higher than all the other groups ($p<0.05$). Hyaline cell (HC) of the LP20 group and the LP20+ β-glucan were higher than all the other groups ($p<0.05$) at the 45, 60, and 75-day oral administrations.

Phenoloxidase activity

Figure 5 showed that phenoloxidase activity (PO) of the shrimp group fed with feed supplemental LP20 was higher than the control group but no significant difference was found for shrimp fed with feeds supplemental β-glucan and LP20+β-glucan at the 45 day (0.118±0.014 - 0.158±0.021), and 60-day (0.11±0.013 - 0.146±0.025) oral administrations. However, in the 75-day oral administration PO activity of shrimp in all feeds increased value. The PO activity of LP20 group (0.231±0.034) was significantly ($p<0.05$) higher than the other groups ($p<0.05$). Besides that, the LP20+ β-glucan group (0.2±0.018) showed significantly difference value ($p<0.05$) compared to the glucan group (0.16±0.023). The pro-phenol oxidase (proPO) system has been considered to play an important role in the defence system of crustaceans (Söderhäll & Cerenius, 1992). Activation of the proPO system (measured in terms of the phenol oxidase activity) has been used to measure immunostimulation in shrimp (Sung et al., 1996). Previously, it was shown that PO activity, RB and SOD activity were significantly higher in shrimp fed the two immunostimulant diets after 18 days than those in shrimp fed immunostimulant-free diets. The white shrimp *Litopenaeus vannamei*, fed immunostimulant-free, 0.2%β-glucan and 0.06% glycyrrhizin diets for 18 days, respectively, were challenged with *Vibrio alginolyticus* at 6.4×10^4 CFU $shrimp^{-1}$ (Chang et al., 2010).

Phagocytic activity and clearance efficiency of L. vannamei to V. parahaemolyticus

Phagocytic activity of shrimp fed with LP20, LP20+ β-glucan were higher than all the other groups in the first two oral administrations 45 and 60 days (Fig. 6). Specifically, in the 75-day oral administration, phagocytic activity of those shrimp fed with supplemental LP20 was significantly ($P<0.05$) higher than other groups in which phagocytic activity was 19.58±2.89%, 11.33±1.83%, 12.92±2.56% and 7.83±1.03 for the shrimp fed LP20, β-glucan, LP20+β-glucan and control, respectively. Figure 7 shows staining hemolymph under compound microscope.

Clearance efficiency assay

Table 8 showed clearance efficiency of shrimp fed with feed supplemental LP20 was higher significantly ($p<0.05$) than those shrimp fed with β-glucan, but did not differ significantly compare to those animals fed with feed containing LP20+β-Glucan in the 45 and 60-day oral administrations. Similar results were obtained by another study. Itami et al. (1994) suggested that oral administration of β-glucan enhanced disease resistance of *P. japonicus*. Their experiment also noted high phagocytic activity in the hemocytes of *P. japonicus* treated with 0.01% glucan for 3 days or 0.005% glucan for 10 days. Results of the study indicate that oral administration of 0.1% β-glucan for one day can induce vibriocidal activity in both hemocytes as well as plasma. The results in our study showed that the present of LP20 in feed was even acting better β-glucan in term of enhancing disease resistance.

Challenge experiments

During the 14-day challenge experiment, the cumulative mortality of shrimp fed with LP20, β-glucan, and LP20+ β-glucan were lower than the control group in the 45-day oral administration (Fig. 8). Specifically, the cumulative mortality of shrimp fed with LP20 was 23.3%. This rate in the shrimp fed with LP20+β-glucan, β-glucan, and control was 26.7%, 40.0%, and 53.3%, respectively. The negative control group showed no mortality. Similarly, the cumulative mortality of shrimp fed with coating pellets was lower than the control group in the 60-day oral administration (Fig. 9). Specifically, the cumulative mortality of shrimp were 16.7%. 43.3%, 26.7% and 56.7% for the shrimp fed with LP20, β-glucan, LP20+β-glucan and control, respectively. The negative control groups showed no mortality.

Confirmation of bacteria infection

Clinical signs of white leg shrimp challenged with *V. parahemolyticus* were often pale to white hepatopancreas (HP), soft shells and empty gut (Fig. 10). The shrimps were also collected and detected *V. parahaemolyticus* by PCR (Fig. 11) and histopathology methods (Fig. 12). Figure 11 showed that challenge shrimp of 45-day oral administration (lanes 1-4) and challenge shrimp of 60-day oral administration (lanes 6-9) were detected and obtained bright positive band for *V. parahemolyticus*. Since negative control shrimp (lanes 5, 10) were not experimentally infected with *V. parahemolyticus*, they did not show bright bands.

In addition, Figure 12 showed that negative control shrimp (Fig. 12A) not experimentally infected with *V. parahemolyticus*, their HP section contain intact tubules and distinct F, B and R cells. The challenge shrimp of 60-day oral administration of LP20+β-glucan (Fig. 12B) and challenge shrimp of 45-day oral administration of glucan (Fig. 12C) were detected with massive sloughing of HP cells. The challenge shrimp of 60-day oral administration of LP20 (Fig. 12D) was detected with hemocytic infiltration with masssive bacterial infection and complete destruction of HP. From this result, it can be concluded that shrimp mortality in the challenge test was caused by *V. parahemolyticus*. The hemocytic infiltration was representative observed phenomenon in the LP20 and LP20+β-glucan treatments.

In the present study, it was shown that *L. vannamei* shrimp fed with LP20 had an increased susceptibility to *V. parahemolyticus* immersion. In similar study on black tiger shrimp (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 diet for 10–20 days significantly increase immunity of postlarvae, juveniles, and adult shrimp against *Vibrio damsela*, *Vibrio harveyi* and white spot infections.

Gill sampling analysis showed that three was no significant difference in PPAE1, LV- β, LVintergrin and LV Toll parameters among the groups after 45 days feed trials (Table 9). This indicates animals fed with LP20 did not show any advantage for gill analysis after 45 days.

Table 10 shows all gill parameters of PPAE1, LV- β, LVintergrin and LV Toll of all groups increased after 75 days feed trials, however, no differ significantly among the groups. This could due to a high range of mean values were collected among the samples leading overlapping among values.

IV. CONCLUSION

The highest growth in weight, survival rate, DWG, SGR were obtained for shrimp fed with feed supplemental LP20 and differ significantly ($p<0,05$) with other feeds and control. FCR were also significantly lower for shrimp fed with diets supplemental LP20 compared with other diets. Shrimp fed with diets supplemental LP20 had darker purple color compared to other. The results also demonstrate that LP20 seems to play an important role in immune modulation by increasing THC, phenoloxidase activity, phagocytic activity and clearance efficiency, survival rates. The immunostimulation of white leg shrimp is possible through oral administration of LP20 (*Lactobacillus plantarum*). Thus, FEED LP20 is high potential used as feed supplemental ingredient that would enhance growth, survival, immune system for white leg shrimp.

REFERENCES

[1] Adams A. Response of penaeid shrimp to exposure to Vibrio species. Fish & Shellfish Immunology 1991; 1:59 - 70.

[2] Bell TA, Lightner DV. A handbook of normal penaeid shrimp histology. World Aquaculture Society, Baton Rouge LA; 1988.

[3] Biswas G, Korenaga H, Nagamine R, Takayama H, Kawahara S, Takeda S, Kikuchi Y, Dashnyam B, Kono T, Sakai M et al. Elevated cytokine responses to Vibrio harveyi infection in the Japanese pufferfish (*Takifugu rubripes*) treated with *Lactobacillus paracasei* spp. Paracasei (06TCa22) isolated from the Mongolian dairy product. Fish and Shellfish Immunology 2013b; 35: 756-765.

[4] Biswas G, Korenaga H, Nagamine R, Takayama H, Kawahara S, Takeda S, Kikuchi Y, Dashnyam B, Kono T, Sakai M et al. Elevated cytokine responses to *Vibrio harveyi* infection in the Japanese pufferfish (*Takifugu rubripes*) treated with Lactobacillus paracasei spp. paracasei(06TCa22) isolated from the Mongolian dairy product. Fish & Shellfish Immunology 2013b; 35: 756-765.

[5] Boyd CE, Thunjai T, Boonyaratpalin M. Dissolved salts in water for inland low-salinity shrimp culture. Global Aquaculture Advocated 2002; 5 (3): 40–45.

[6] Cerezuela R, Cuesta A, Meseguer J, Esteban MA. Effects of dietary inulin and heat-inactivated *Bacillus subtilis* on gilthead seabream (*Sparus aurata* L.) innate immune parameters. Beneficial Microbes 2012; 3(1): 77-81.

[7] Cornick JW, Stewart JE. Lobster (Hommarus americanus) hemocytes: classification, differential counts and associated agglutinin activity. Journal of Invertebrate Pathology 1978; 31:194-203.

[8] Chang CF, Chen HY, Su MS, Liao IC. Immunomodulation by dietary β-1,3-glucan in the brooders of the grass prawn *Penaeus monodon*. Fish & Shellfish Immunology 2000; 10: 505-514.

[9] Chang J, Zhang W, Mai K, Ma H, Liufu Z, Wang X, Ai Q, Xu W. Effects of dietary β-glucan and glycyrrhizin on non-specific immunity and disease resistance of white shrimp, *Litopenaeus vannamei* (Boone) challenged with *Vibrio alginolyticus*. Aquaculture Research 2011; 42: 1101-1109.

[10] Chen JC, Chin TS. Accute oxicty of nitrite to tiger praw *Penaeus monodon* larvae. Aquaculture 1998; 69: 253-262.

[11] Christopher EM. Evaluation Of Ground Water From The Lajas ValleyFor Low salinity Culture Of The Pacific White Shrimp *Litopenaeus vannamei*. University Of Puerto Rico Mayagüez Campus; 2008.

[12] Giri SS, Sukumaran V, Oviya M. Potential probiotic Lactobacillus plantarum VSG3 improves the growth, immunity, and disease resistance of tropical freshwater fish, Labeo rohita. Fish & Shellfish Immunology 2013; 34: 660–666.

[13] Giri SS, Sukumaran V, Sen SS, Jena PK. Effects of dietary supplementation of potential probiotic Bacillus subtilis VSG1 singularly or in combination with Lactobacillus plantarum VSG3 or/and Pseudomonas aeruginosa VSG2 on the growth, immunity and disease resistance of Labeo rohita. Aquaculture Nutrition 2014; 20: 163–171.

[14] Hernandez-Lopez J, Gollas-Galvan TS, Vargas-Albores F. Activation of the prophenoloxidase system of the brown shrimp (*Penaeus californiensis* Holmes). Comparative Biochemistry and Physiology, Part C 1996; 113:61-66.

[15] Itami T, Takahashi Y, Tsuchihira E, Igusa H, Kondo M. Enhancement of disease resistance of kuruma prawn Penaeus japonicas and increase in phagocytic activity of prawn hemocytes after oral administration of β-1,3-glucan (Schizophyllum). In: Chou, L.M. Munro, A.D., Lam, J.J., Chen, T.W., Cheong, L.K.K., Ding, J.K. Asian Fisheries Society 1994; 375–378.

[16] Le Moullac G, Le Groumellec M, Ansquer D, Froissard S, Levy P. Haematological and phenoloxidase activity changes in the shrimp Penaeus stylirostris in relation with the moult cycle: protection against vibriosis. Fish and Shellfish Immunology 1997; 7: 227–234.

[17] Liao IC, Su MS, Chang CF, Her BY, Kojima T. Enhancement of the resistance of grass prawn *Penaeus monodon* against Vibrio damsela infection by beta-1,3-glucan. Journal of Fish Society Taiwan 1996; 23: 109–116.

[18] Lin YC, Chen JC, Chen YY, Liu CH, Cheng W, Hsu CH, Tsui WC. Characterization of white shrimp Litopenaeus vannamei integrin β and its role in immunomodulation by dsRNA-mediated gene silencing. Development Comp Immunol 2013. 40(2): 167–179.

[19] Liu CH, Chen JC. Eff ect of ammonia on the immune response of white shrimp Litopenaeus vannamei and its susceptibility to Vibrio alginolyticus. Fish & Shellfish Immunology 2004; 16:321- 334.

[20] Murosaki S, Yamamoto Y, Ito K, Inokuchi T, Kusaka H, Ikeda H, Yoshikai Y. Heat killed *Lactobacillus plantarum* L-137 suppresses naturally fed antigen-specific IgE production by stimulation of IL-12 production in mice. Journal of Allergy and Clinical Immunology 1998; 102(1): 57–64.

[21] Anh NTN, Nhung DTK, Hai TN. Feed efficiency of white leg shrimp (Litopeneaus vannamei) in co-culture with gut weed (Enterormorpha sp.) and blanket weed (Cladophoraceae). Cantho Universiy Journal of Science 2014; 31: 98-105.

[22] Pan X, Wu T, Song Z, Tang H, Zhao Z. Immune responses and enhanced disease resistance in Chinese drum, Miichthys miiuy (Basilewsky), after oral administration of live or dead cells of *Clostridium butyrium* CB2. Journal of Fish Disease 2008; 31: 679–686.

[23] Park JH, Park WJ, Joeng HD. Immunological efficacy of *V. vulnificus* bacterins given as an oral vaccine in the flounder, *P. olivaceus*. Aquaculture 2001; 201: 187–197.

[24] Salminen S, Ouwehand AC, Benno Y, Lee YK. Probiotics: how should they be defined? Trends in Food Science Technology 1999; 10, 107-10.

[25] Son VM, Chang CC, Wu MC, Guu YK, Chiu CH, Cheng W. Dietary administration of the probiotic, Lactobacillus plantarum, enhanced the growth, innate immune responses, and disease resistance of the grouper Epinephelus coioides. Fish & Shellfish Immunology 2009; 26: 691–698.

[26] Sritunyalucksana K, Dangtip S, Sanguanrut P, Sirikharin R, Taengchaiyaphum S, Thitamadee S, Mavichak R, Proespraiwong P, Flegel TW. A two-tube, nested PCR detection method for AHPND bacteria. http://enaca.org/publications/health/disease-cards/ap4-pcr-detection-method-announcement-final%202014-02-19.pdf. 19 Feb. 2014.

[27] Su MS, Liu KF, Chang CF, Liao IC. Enhancement of grass prawn Penaeus monodon postlarvae viability by beta-1,3-glucan from Schizophyllum commune. Journal of Taiwan Fish Research 1995; 3: 125–132.

[28] Sung HH, Yans YL, Song YLJ. Enhancement of microbicidal activity in the tiger shrimp Penaeus monodon via immunostimulations. Journal Crustacean Biological 1996; 16: 278 -284.

[29] Tung HT, Koshio S, Traifalgar RF, Ishikawa M, Yokoyama S. Effects of dietary Heat-killed *Lactobacillus plantarum* on larval and post larval kuruma shrimp Marsupenaeus japonicas bate. Journal of the world aquaculture society. Vol41, No. S1. Feb; 2010.

[30] Tung HT, Koshio S, Teshima SI, Ishitaka M, Yokoyama S, Ren T, Hirose Y, Phuong NDT. Effects of heat-skilled *Lactobacillus plantarum* supplementatal diets on growth performance, stress resistance and immune response of juvenile Kuruma shrimp *Marsupenaeus japonicas* Bate. Aquaculture Science 2009; 57 (2): 175-184.

[31] Tran LH, Nunan L, Redman RM, Mohney LL, Pantoja CR, Fitzsimmons K, Lightner DV. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Diseases of Aquatic Organism 2013; 105(1): 45-55.

[32] Jang IK, Pang Z, Yu J, Kim SK, Seo HC, Cho YR. Selectively enhanced expression of prophenoloxidase activating enzyme 1 (PPAE1) at a bacteria clearance site in the white shrimp, *Litopenaeus vannamei*. BMC Immunol 2011; 12:70.

[33] Wang KH, Tseng CW, Lin HY, Chen IT, Chen YH, Chen YM, Chen TY, Yang HL. RNAi knock-down of the *Litopenaeus vannamei* Toll gene (LvToll) significantly increases mortality and reduces bacterial clearance after challenge with Vibrio harveyi. Dev Comp Immunol 2010; 34(1): 49–58.

[34] Whetstone JM, Treece GD, Stokes AD. Opportunities and Constrains in Marine Shrimp Farming. Southern Regional Aquaculture Center (SRAC) publication No, 2600 USDA; 2002.

AUTHORS

First Author[1] – Pham Minh Duc, email: pmduc@ctu.edu.vn
Second Author[1] – Hua Thai Nhan, email: htnhan@ctu.edu.vn
Third Author[1] – Tran Thi Tuyet Hoa, email: ttthoa@ctu.edu.vn
Fourth Author[1] – Hong Mong Huyen
email: hmhuyen@ctu.edu.vn
Fifth Author[1] – Chau Tai Tao, email: cttao@ctu.edu.vn
Sixth Author[1] – Cao My An, email: cman@ctu.edu.vn
Seventh Author[1] – Tran Ngoc Hai, email: tnhai@ctu.edu.vn
Eighth Author[1] – Dang Thuy Mai Thy
email: dtmthy@ctu.edu.vn
[1]College of Aquaculture and Fisheries, Can Tho University, Can Tho City, Vietnam
Nighth Author – Onoda Satoru, House Wellness Foods Corporation, Japan, email: onoda_satoru@house-wf.co.jp
Correspondence Author[1]– Pham Minh Duc
Mobile: (+84) 983152109, Fax: (+84 710) 3830323

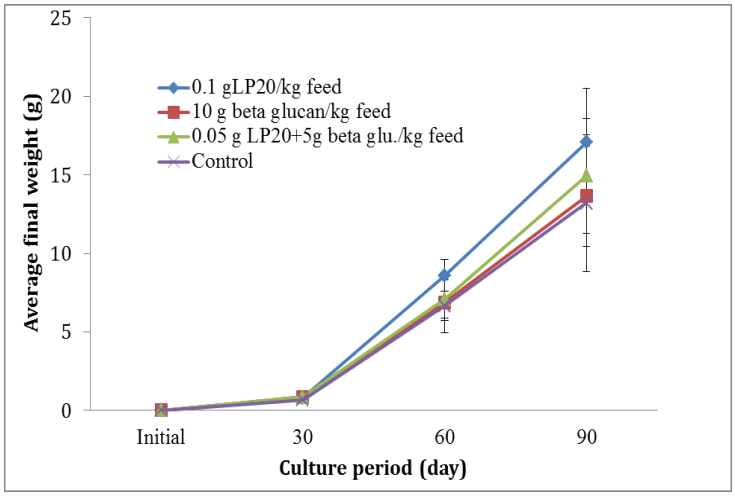

Fig. 1. Growth in weight of animals during the experimental diets.

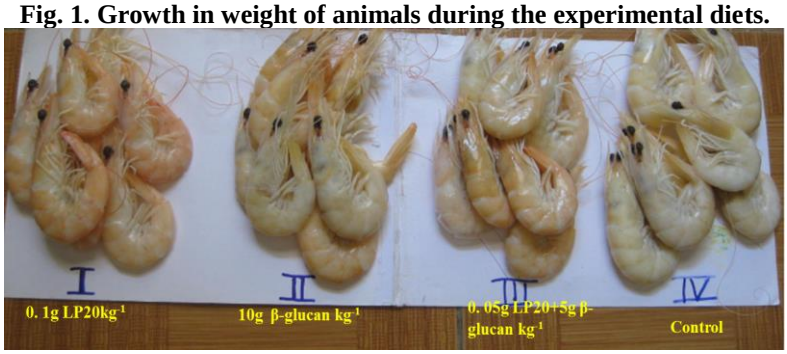

Fig. 2. Skin color of shrimp in all treatments after boiling in hot water

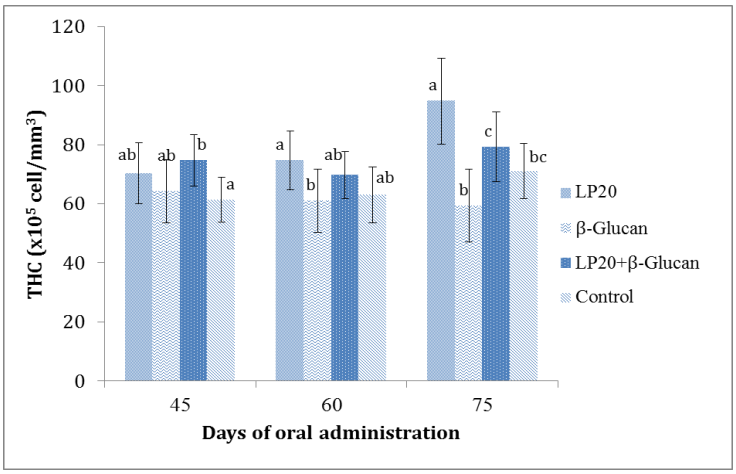

Fig. 3. Total haemocyte count of *L. vannamei* after feeding LP20, β-glucan, LP20+β-glucan and control in the 45, 60, and 75-day oral administrations; the same letters within the same group of the days of oral administration indicates no differ significantly (*P* > 0.05) among the feeds groups

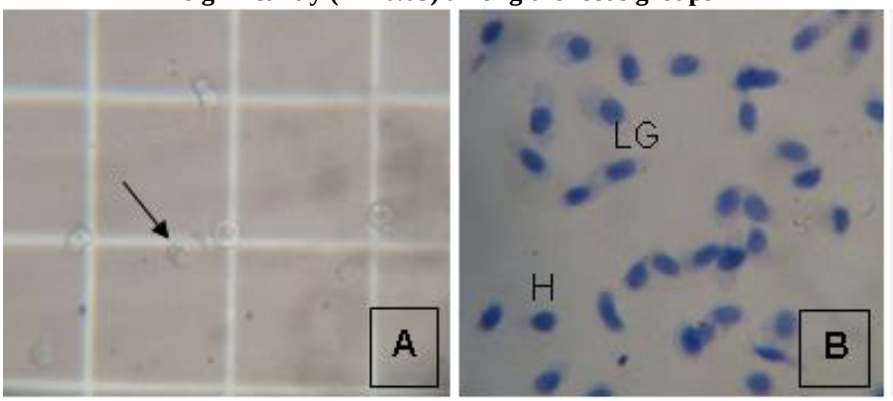

Fig. 4. Analysis of total haemocyte count and differential hemocyte count on a microscope. (A) In a Neubauer (40X); (B) Giemsa stain: HC (Hyaline cell), LGC (Large granular cell) (100X)

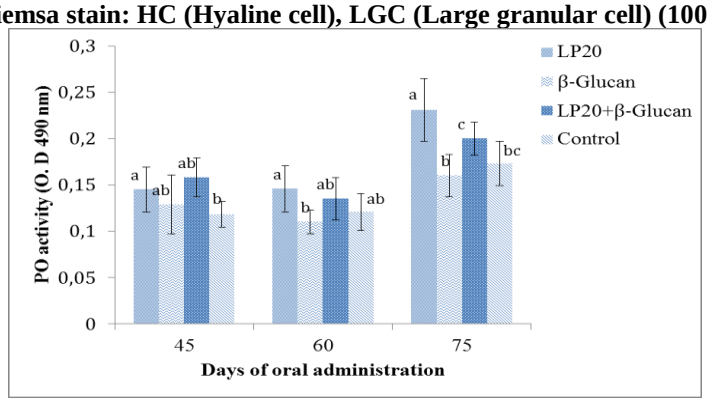

Fig. 5. PO activity of *L. vannamei* after feeding LP20, β-glucan, LP20+β-glucan and control in the 45, 60 and 75-day oral administrations; the same letters within the same group of the days of oral administration indicates no differ significantly (*P* > 0.05) among the feeds groups.

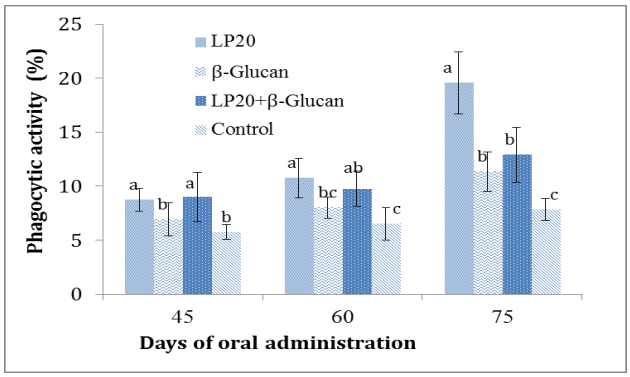

Fig. 6. Phagocytic activity of *L. vannamei* after challenged with *V. Parahemolyticus;* the same letters are not significantly (*P*>0.05) different among feeds of the same days of oral administration.

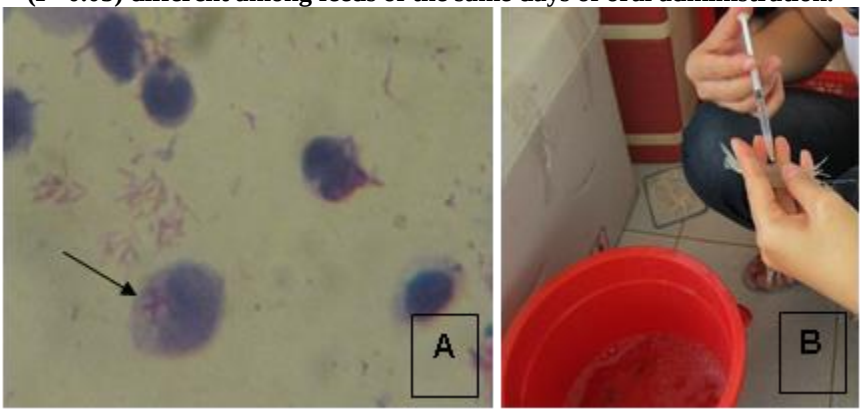

Fig. 7. Analysis of phagocytic activity; (A) staining hemolymph with Giemsa (arrow: phagocytic hemocytes with bacteria); (B) withdrawing hemolymph with a syringe.

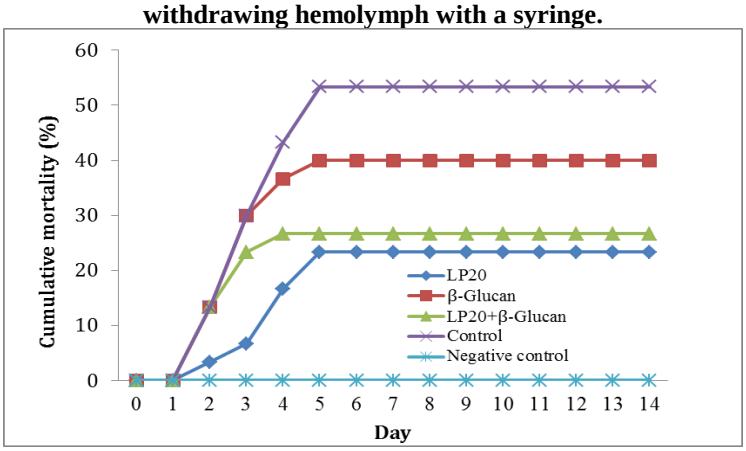

Fig. 8. Cumulative mortality of *L. vannamei* challenged with *Vibrio parahemolyticus* 45 days post challenge

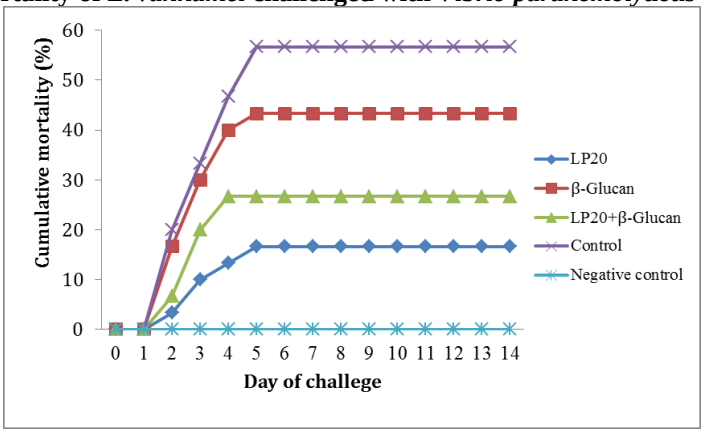

Fig. 9. Cumulative mortality of *L. vannamei* challenged with *Vibrio parahemolyticus*, 60 days post challenge

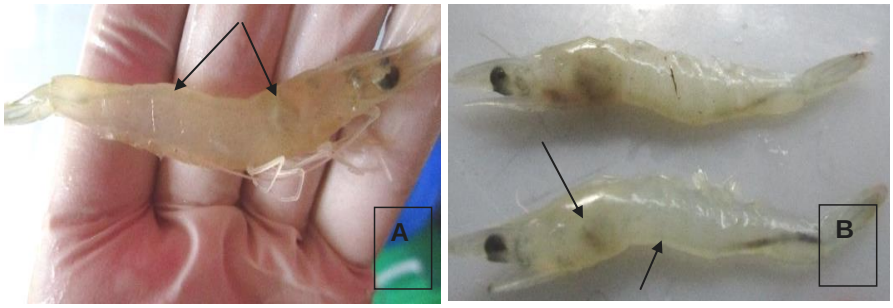

Fig. 10. Clinical signs of experimental shrimp post infection with *Vibrio paraheamolyticus,* (A) & (B) pale hepatopancreas and empty gut (arrows)

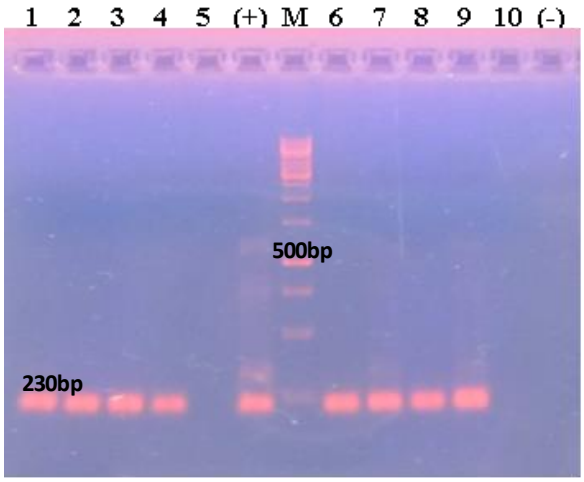

Fig. 11. PCR result of experiment shrimp post infection with *Vibrio paraheamolyticus*. Lane M: 1kb ladder; lane 1, 6: LP20; lane 2, 7: glucan; lane: 3, 8: LP20+glucan; lane 4, 9: control; lane 5, 10: negative control; lane (+): positive control of PCR; lane (-): negative control of PCR

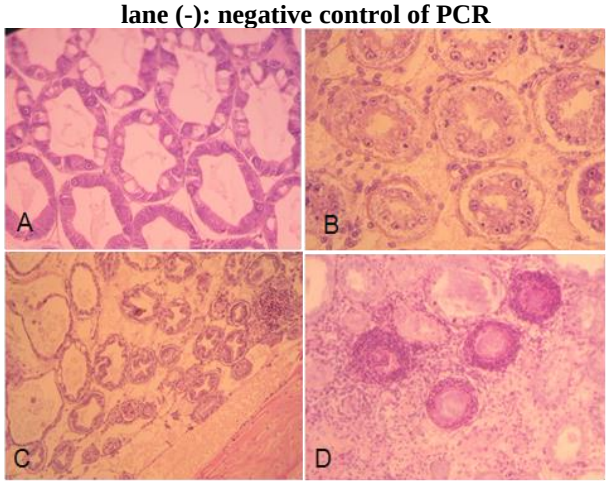

Fig. 12. Histopathological result of experiment shrimp post infection with *Vibrio paraheamolyticus.* (A) HP section of healthy shrimp from negative control group; (B) & (C) massive sloughing of HP cells; (D) hemocytic infiltration with massive bacterial infection and complete destruction of HP.

Table 1: Experimental feed trial

Feed trial	Component
LP20	Commercial pellet + 0.1 g LP20/kg
β-glucan	Commercial pellet + 10 g β-glucan*/kg
LP20+β-glucan	Commercial pellet +(0.05 g LP20+5 g β-glucan/kg)
Control	Commercial pellet only

**Beta glucan 40: 1,3 and 1,6 beta glucan Mannan Oligo Mixture by enzyme extraction, Nanogen Biopharma Ltd., USA.*

Table 2: List of primers for nested PCR detection of *V. parahaemolyticus*

Primers	5'-3'	Expected amplicon
AP4-F1	ATGAGTAACAATATAAAACATGAAAC	1269 bp
AP4-R1	ACGATTTCGACGTTCCCCAA	
AP4-F2	TTGAGAATACGGGACGTGGG	230 bp
AP4-R2	GTTAGTCATGTGAGCACCTTC	

Table 3: Primer lists for detection of different target genes in gill samples

Target gene	Primer name	Sequences	References
PPAE1	qRTPPAE Fw	5'-AGTTCCTACGACACGACCACCTA-3'	Jang *et al*., 2011
	qRTPPAE Rv	5'-TCGACGTTGAAGTTGGTGCTT-3'	
Integrin beta	Liva It beta Fw	5'-TTGGGCATCGTGTTCGGACTC-3'	Lin *et al.*, 2013
	Liva It beta Rv	5'-TGAAGGTGTTGGTCGCAGGTC-3'	
Integrin beta	It beta (P7) Fw	5'-CCAGATTAGGTGGCGGTCAG3'	Zhang et al., 2012
	It beta (P8) Rv	5'-GTGCTGTTTGGCGACTTGATT-3'	
Toll receptor	LvToll Fw	5'-ATGTGCGTGCGGATACATTA-3'	Wang *et al*., 2010
	LvToll Rv	5'-GGGTGTTGGATGTCGAGAGT-3'	
beta-actin	qRT beta Fw	5'- CGAGGTATCCTCACCCTGAAAT-3'	Jang *et al*., 2011
	qRT beta Rv	5'- GTGATGCCAGATCTTCTCCATGT-3'	
18s rRNA	qRT18S Fw	5'- TGCTCAGAGCAGGCTGGTTT-3'	Jang *et al*., 2011
	qRT18S Rv	5'- GAGGTCCTGTTCCAATCATTCCA-3'	

Table 4: Average of temperature, pH, alkalinity after 3 months experiment

Feed treatment	Temperature (^{O}C)		pH		Alkalinity (mg/L)
	AM	PM	AM	PM	
LP20	28.4±0.29	29.0±0.43	8.17±0.33	8.18±0.30	135±19.7
β-glucan	28.4±0.42	29.0±0.40	8.15±0.32	8.21±0.30	137±25.1
LP20+ β-glucan	28.4±0.33	29.0±0.38	8.15±0.31	8.20±0.30	139.9±24.9
Control	28.5±0.32	29.0±0.35	8.15±0.32	8.20±0.30	137±27.5

Table 5: Average $N\text{-}NO_2^-$, NH_3/NH_4^+ levels after 3 months culture period.

Feed treatment	$N\text{-}NO_2^-$ (mg/L)	NH_3/NH_4^+ (mg/L)
LP20	0.36±0.34	0.05±0.08
β-glucan	0.43±0.55	0.07±0.11
LP20+ β-glucan	0.47±0.6	0.05±0.11
Control	0.40±0.58	0.04±0.08

Table 6: Survival rate and growth performance of shrimp after 3 months.

	LP20	β-glucan	LP20+β-glucan	Control
Survival rate (%)	87.6 ± 2.91^{a}	62.9 ± 4.29^{b}	71.2 ± 4.55^{b}	60.2 ± 5.48^{b}
Final Weight (g)	17.1 ± 3.44^{a}	13.6 ± 3.20^{b}	14.9 ± 3.65^{a}	13.2 ± 4.34^{b}
DWG (g/day)	0.19 ± 0.003^{a}	0.15 ± 0.009^{c}	0.17 ± 0.004^{b}	0.15 ± 0.009^{c}
SGR (%/day)	7.02 ± 0.013^{a}	6.82 ± 0.054^{c}	6.90 ± 0.021^{b}	6.78 ± 0.057^{c}
FCR	1.08 ± 0.01^{a}	1.16 ± 0.02^{bc}	1.12 ± 0.04^{ab}	1.18 ± 0.04^{c}

^{a,b}mean with difference letters in same row are not significant difference ($P<0.05$)

Table 7: Large granular cell (LGC) and hyaline cell (HC) of *L. vannamei* in the 45, 60, and 75 day oral administrations.

	Treatments	Days of oral administration		
		45	60	75
LGC	LP20	61.81±8.49[a]	65.9±8.68[a]	83.64±13[a]
	β-Glucan	57.54±9.66[a]	54.44±9.46[b]	53.59±11.33[c]
	LP20+β-Glucan	65.61±8.63[a]	61.68±7.44[ab]	70.84±10.86[ab]
	Control	55.52±7.03[a]	56.92±8.97[ab]	64.67±9.34[bc]
HC	LP20	8.52±2.39[ab]	8.77±1.81[a]	11.19±2.2[a]
	β-Glucan	6.63±1.46[bc]	6.56±1.45[bc]	5.74±1.05[c]
	LP20+ β-Glucan	9.05±1.41[a]	7.99±1.5[ab]	8.41±1.5[b]
	Control	5.81±0.86[c]	6.08±1.21[c]	6.42±0.94[c]

Table 8: Clearance efficiency of *L. vannamei* challenged with *V. parahemolyticus*

Treatments	Days of oral administration	
	45	60
LP20	67.44±12.22[a]	73.42±10.47[a]
β-Glucan	34.88±15.07[b]	43.04±19.66[b]
LP20+β-Glucan	58.14±25.35[ab]	51.49±23.86[ab]

Table 9: Gill samples analysis at the 45 day of oral administration

Feed trial	Days oral administration (45 days)			
	PPAE1	LV-β	Lv Integrin	LV Toll
LP20	0.01650±0.014082[a]	0.01833±0.010132[b]	0.07300±0.048058[b]	0.00900±0.004099[b]
β-Glucan	0.07267±0.096827[a]	0.08350±0.106637[a]	0.38300±0.449632[a]	0.04617±0.054308[a]
LP20+β-Glucan	0.01750±0.010055[a]	0.01333±0.007339[b]	0.06983±0.028548[b]	0.00850±0.002588[b]
Control	0.01467±0.005203[a]	0.01050±0.006221[b]	0.05717±0.009368[b]	0.00667±0.001633[b]

Table 10: Gill samples analysis at the 75 day of oral administration

Feed trial	Days oral administration (75 days)			
	PPAE1	LV-β	Lv Integrin	LV Toll
LP20	0.05800±0.043095[a]	0.07317±0.080576[a]	0.16467±0.086553[a]	0.01517±0.007782[ab]
β-Glucan	0.03283±0.016881[a]	0.07000±0.033752[a]	0.10900±0.049421[a]	0.00667±0.004844[b]
LP20+β-Glucan	0.04483±0.017256[a]	0.05217±0.021563[a]	0.10850±0.055146[a]	0.01450±0.006804[ab]
Control	0.04950±0.029798[a]	0.05750±0.028697[a]	0.10450±0.047154[a]	0.02167±0.012404[a]

Means within a column with different letters are significantly different (p<0.05)