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Immune stimulant effects of a nucleotide-rich baker's yeast extract in the kuruma shrimp, *Marsupenaeus japonicus*

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ABSTRACT

Immune stimulant effects of a nucleotide-rich baker's yeast extract (Vertex IG20) were investigated in the kuruma shrimp, *Marsupenaeus japonicus* by examining expression of anti-microbial peptides/proteins (AMPs) such as penaeidin (MjPen), crustin (MjCrus) and lysozyme (MjLyz) genes. Furthermore, to confirm that the baker's yeast extract-induced AMPs were functional, we also assessed the effect of its oral administration on resistance to *Vibrio nigripulchritudo* infection in the kuruma shrimp. Our results demonstrate that baker's yeast extract-injected and fed shrimps displayed a significant up-regulation of MjPen, MjCrus and MjLyz gene expression in the lymphoid organ. Moreover, significantly increased ($P<0.01$) resistance to the bacterial pathogen in term of better post infection survival (66.6%) was observed in the shrimp fed with the yeast extract-incorporated diet compared with the control diet fed group (8.3%). The present study indicates the immunostimulatory effects of the nucleotide-rich baker's yeast extract on the kuruma shrimp immune system and supports its potential use in aquaculture.

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1. Introduction

Aquaculture represents the fastest-growing animal based primary food producing sector with 63.6 million tons (MT) production and 8.8% annual growth rate in 2011 (FAO, 2012). The contribution from crustacean aquaculture is 5.7 MT. Kuruma shrimp, *Marsupenaeus japonicus* is the highest priced shrimp species among the farmed crustaceans and widely cultured in Japan, China, Australia and Southeast Asian countries (Rosenberry, 2001). However, disease occurrences have led to considerable economic loss in shrimp farming industry. Treatment of diseases using chemotherapeutics and antibiotics at farm level is either infeasible or prohibited. Therefore, enhancement of immune status using bio-products could render resistance to diseases and thus prevention of these disease occurrences is possible.

Shrimps, like other invertebrates, depend on innate immune system rather than on non-existent acquired immunity for protection against invading pathogens (Lee and Söderhäll, 2002; Loker et al., 2004). Cationic anti-microbial peptides/proteins (AMPs) play a major role in innate immunity in shrimp. AMPs are amphipathic proteins of low molecular weight (<10 kDa) and mainly offer an early and localized first line of defense against pathogens (Selsted and Ouellette, 2005; Zasloff, 2002). To date, several AMP families including penaeidins (Destoumieux et al., 1997), crustins (Bartlett et al., 2002), anti-lipopolysaccharide factors (Somboonwiwat et al., 2005), histones (Patat et al., 2004), and fragments of hemocyanin (Destoumieux-Garzón et al., 2001) have been described in penaeid shrimps. Penaeidins with chitin-binding properties (Destoumieux et al., 2000a) are ubiquitous in penaeid shrimps (Gueguen et al., 2006) and act against Gram-positive bacteria, filamentous fungi (Destoumieux et al., 1997), viruses and protozoans (Bachère, 2003). Crustins, first identified in shore crab *Carcinus maenas* (Relf et al., 1999) and later also described in *M. japonicus* (Rattanachai et al., 2004), have antimicrobial activity against Gram-positive bacteria. Lysozyme acts against Gram-negative bacteria by degrading the cell wall mucopolysaccharides, allowing their recognition by phagocytic cells (Aguirre-Guzmán et al., 2009). Kuruma shrimp lysozyme also displayed glycolytic activities to the pathogenic *Vibrio* species (Hikima et al., 2003). Therefore, studying the functions of AMPs enriches basic knowledge on shrimp immunity and provides possible avenues in formulating disease management strategies in aquaculture (Bachère et al., 2004).

Nucleotides are low molecular weight biological compounds that play important role in essential physiological and biochemical functions (Carver and Walker, 1995; Cosgrove, 1998). Nucleotides are synthesized *de novo* in most of the tissues, but some immune and intestinal cells lack this process and depend on exogenous supply (Quan, 1992). Hence, administration of pure nucleotides guarantees

Abbreviations: RT, reverse transcription; PCR, polymerase chain reaction.

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increased availability to the body at the time of high demand for various physiological activities (Whitehead et al., 2006). The immunomodulatory effects and genetic expression due to dietary nucleotides supplementation have been reported in higher animals (Gil, 2002; Singhal et al., 2008). Since last three decades, in several studies involving aquatic species, dietary nucleotides have elevated cellular and humoral immune responses in Atlantic salmon (Burrells et al., 2001), catla (Jha et al., 2007), common carp (Sakai et al., 2001), grouper (Lin et al., 2009), hybrid striped bass (Li et al., 2004), rainbow trout (Leonardi et al., 2003; Tahmasebi-Kohyani et al., 2011) and red drum (Cheng et al., 2011). However, few nucleotide nutrition researches in Pacific white shrimp have shown beneficial results on growth, survival (Li et al., 2007) and cellular immunity (Murthy et al., 2009), lacking elucidation of molecular immune functions. There is no information regarding the expression of innate immune genes, especially AMPs in kuruma shrimp administered with a baker's yeast extract preparation. Therefore, we tested the efficacy of a nucleotide-rich baker's yeast extract (Vertex IG20) administration in regulation of AMP genes, such as penaeidin (MjPen), crustin (MjCrus) and lysozyme (MjLyz) in the lymphoid organ (LO) of kuruma shrimp. Additionally, we assessed resistance to *Vibrio nigripulchritudo* infection in the Vertex IG20 fed kuruma shrimps to confirm functionality of the elevated innate immune system.

2. Materials and methods

2.1. Experimental shrimp

Healthy kuruma shrimps, *M. japonicus* (mean body weight, 10 ± 1 g) were obtained from Matsumoto Fisheries Farm, Miyazaki, Japan. Shrimps were firstly acclimatized in an aerated seawater tank at 20 °C and fed with a commercial diet (Higashimaru, Japan) once a day for 2 weeks under a natural photoperiod prior to their use in the experiment. The health status of experimental shrimps was checked by culturing hemolymph and hepatopancreas smears from few sampled animals on Marine Agar Broth 2216E (Difco, Detroit, Michigan, USA) for the presence of any bacterial pathogens. The results showed existence of no pathogenic bacteria in shrimps.

2.2. Preparation, injection and feeding of baker's yeast extract to shrimp

In this study, we used a commercial baker's yeast extract, Vertex IG20 (TableMark Co., Ltd., Tokyo, Japan). Vertex IG20 was processed as per the protocol described by Biswas et al. (2012). However, the final product was in the form of fine powder and it contained nucleotides (36.7%) as major components (Table 1). This yeast extract did not contain β-glucan measured using mushroom and yeast β-glucan assay kit (Megazyme, Bray, Ireland). Presence of any microbial contamination in the baker's yeast extract was examined by culturing the yeast extract dissolved in phosphate-buffered saline (PBS) on Marine Agar Broth 2216E (Difco) and growth of no viable bacteria was detected. For injection experiment, shrimps were divided into two groups (n = 20), treatment and control. Shrimps of treatment group were injected with 0.1 mL of Vertex IG20 dissolved in PBS at 5 mg shrimp^{-1}, whereas control group shrimps received an injection of 0.1 mL PBS. Shrimps of both the groups had an intramuscular (i.m.) injection in the second abdominal segment. For feeding experiment, the yeast extract was mixed at 5% (w/w) with the commercial diet mentioned above. Shrimps were divided into two groups (n = 140), *viz.* treatment (Vertex IG20 fed) and control group. Shrimps of the treatment group were fed with the yeast extract mixed diet, whereas control group shrimps were fed with the unmixed commercial diet once a day at 10% body weight for 7 days. Shrimps in both the experiments were maintained in seawater flow-through system at 22 ± 2 °C under a natural photoperiod.

Table 1
Composition of baker's yeast extract (Vertex IG20) used in the study.

Component	%
Nucleotides[a]	36.70
Total amino acids	38.02
Minerals	12.94
Organic acids	2.95
Total vitamins	1.55
Crude fat	0.30
Moisture	3.71
Others	3.83

[a] Nucleotides (36.70%) composed of disodium inosine-5′-monophosphate (IMP)- 10.59%, disodium guanidine-5′-monophosphate (GMP)- 10.02%, disodium cytidine-5′-monophosphate (CMP)- 7.33%, disodium uridine-5′-monophosphate (UMP)- 8.68% and disodium adenosine-5′-monophosphate (AMP) - 0.08%.

2.3. Expression analysis of innate immune-related genes by semi-quantitative RT-PCR

The lymphoid organ (LO) was dissected out from the PBS and Vertex IG20 injected kuruma shrimps (n = 5) at 1, 3 and 5 days post injection, whereas LO from control and Vertex IG20 mixed diet fed shrimps (n = 5) was collected at 0, 1, 3 and 5 days after feeding. Total RNA was extracted from the LO using ISOGEN (Nippon Gene, Osaka, Japan) as per the manufacturer's instructions. Poly (A) RNA was then purified using a quick prep micro mRNA kit (Amersham Pharmacia Biotech, Sweden). To avoid the presence of DNA, RNA samples were treated with recombinant DNase (RNase-free) at 37 °C for 30 min according to the manufacturer's protocol (Takara Bio Inc., Shiga, Japan). Quantity and quality of RNA in all samples were checked using a NanoDrop spectrophotometer, ND-1000 (Thermo Scientific, Wilmington, DE, USA). cDNA synthesis was performed using ReverTra Ace qPCR RT kit (Toboya, Osaka, Japan) and this cDNA served as a template for PCR. All PCR reactions were performed as per the protocol described by Biswas et al. (2012). Amplification of elongation factor (EF)-1α gene was used as an internal control. The immune-related genes, internal control, their respective primers and annealing conditions are presented in Table 2. PCR products were electrophoresed on a 1.5% agarose gel to detect specific bands. To conduct a semi-quantitative approach of gene expression, kuruma shrimp innate-immune related genes and EF-1α gene were amplified using a series of cycle numbers (20–35) under the above mentioned conditions. After determining the optimal cycle number, specific PCR was conducted and the expression ratio of each innate immune

Table 2
Gene specific primers and annealing temperature of kuruma shrimp EF-1α and innate-immune related genes used for PCR analysis in the study.

Gene	Primer sequence (5′→3′)	Annealing temperature (°C)	Accession number
MjEF-1α Fw[a]	GTCTTCCCCTTCAGGACGTA	55	AB458256
MjEF-1α Rv[b]	GAACTTGCAGGCAATGTGAG		
MjPenaeidin Fw	GCTGCACCCACTATAGTCTTT	60	AU175636
MjPenaeidin Rv	CTACCATGGTGATGAAACAAA		
MjCrustin Fw	CACCTTCAGGGACCTTGAA	62	AB121740
MjCrustin Rv	GTAGTCGTTGGAGCAGGTTA		
MjLysozyme Fw	TCCTAATCTAGTCTCGCAGGGA	58	AB080238
MjLysozyme Rv	CTAGAATGGGTAGATGGA		

[a] Fw = Forward;
[b] Rv = Reverse.

related gene (35 cycles)/EF-1α gene (25 cycles) was determined by densitometry, which was performed by measuring photostimulated luminescence values using Science Lab99 Image Gauge software (Fujifilm, Tokyo, Japan) (Biswas et al., 2012; Kono et al., 2010).

2.4. *V. nigripulchritudo* infection test

V. nigripulchritudo (strain E15) was used for artificial infection in this study. The bacterium was grown in Marine Broth 2216E (Difco) at 27 °C with continuous shaking overnight. Bacterial cells were harvested from stationary phase cultures and re-suspended in sterile saline solution. Cell counts were estimated from optical density (O.D.) values at 600 nm and the corresponding colony forming units (cfu) were determined from a serial dilution of bacterial culture grown on marine agar plates. Vertex IG20 and control diet fed kuruma shrimps were artificially challenged on 1 day after the feeding treatment with an i.m. injection of 0.1 mL of bacterial suspension containing 1×10^5 cfu mL^{-1} as determined by Fall et al. (2010). Injection was made in the second abdominal segment. Injected shrimps from each group were distributed in 3 replicate tanks (n = 20) and maintained in seawater flow-through system. The survival rate from each group was recorded daily for 13 days.

2.5. Statistical analysis

Differences between the quantified relative expressions of a particular gene in Vertex IG20 treated and control group shrimps at each time interval and final survival rates of bacteria challenged shrimps were evaluated with an independent sample *t*-test for equality of means and ANOVA using SPSS for Windows v. 17.0 program (SPSS Inc., Chicago, IL). All data are expressed as mean ± standard deviation (S.D.).

3. Results

3.1. Expression of immune-related genes in yeast extract injected shrimp

In the injection study, significant increase ($P<0.01$) of MjPen was recorded in the yeast extract injected shrimp all through the 1–5 d post injection period compared to that of PBS (control) injected shrimp (Fig. 1A). The highest expression level of this gene was observed at 1 d post injection. The transcript level of MjCrus in injected shrimp LO was significantly higher ($P<0.01$) than in control (PBS injected) shrimp at 3 and 5 d post injection (Fig. 2A). There was a significant increase of MjLyz gene expression in injected shrimp at 1–5 d post injection period (Fig. 3A).

3.2. Expression of immune-related genes in yeast extract fed shrimp

In the Vertex IG20 fed shrimps, MjPen transcript was increased compared to control diet fed shrimps ($P<0.01$) on 1, 3 and 5 d post feeding with a whopping 10 times higher at 5 d and almost similar expression levels on 1 and 3 d post feeding (Fig. 1B). Significantly elevated expression level of MjCrus ($P<0.01$) was alike in Vertex IG20 fed shrimps all through the 0–5 d post feeding (Fig. 2B). The LO of yeast extract fed shrimp had significantly higher expression ($P<0.01$) of MjLyz compared with that of the control diet fed shrimp (Fig. 3B). The MjLyz transcripts reached a peak value with 4.5 times as much as that of the control group at 1 d post feeding in the Vertex IG20 fed group.

3.3. Resistance to *V. nigripulchritudo*

At the end of 13 d post challenge trial, shrimps fed with Vertex IG20 mixed diet exhibited significantly better post infection survival ($P<0.01$) compared with control diet fed group (Fig. 4). Baker's

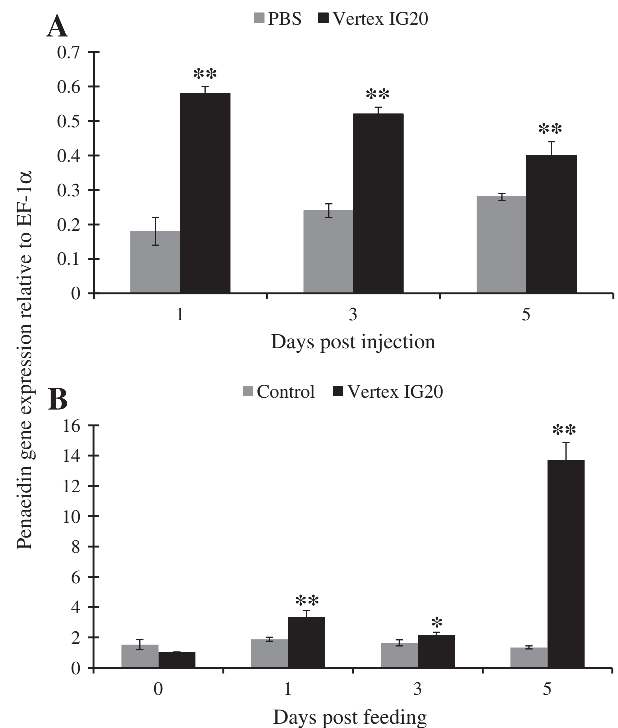

Fig 1. Densitometric quantification of PCR-analysis of penaeidin gene expression relative to the elongation factor (EF)- 1α gene transcript in the lymphoid organ of kuruma shrimp. Comparison of expression level in A) PBS (control) and Vertex IG20 injected, B) control and vertex IG20 diet fed shrimps (n = 5) was made for each time point. **$P<0.01$; *$P<0.05$.

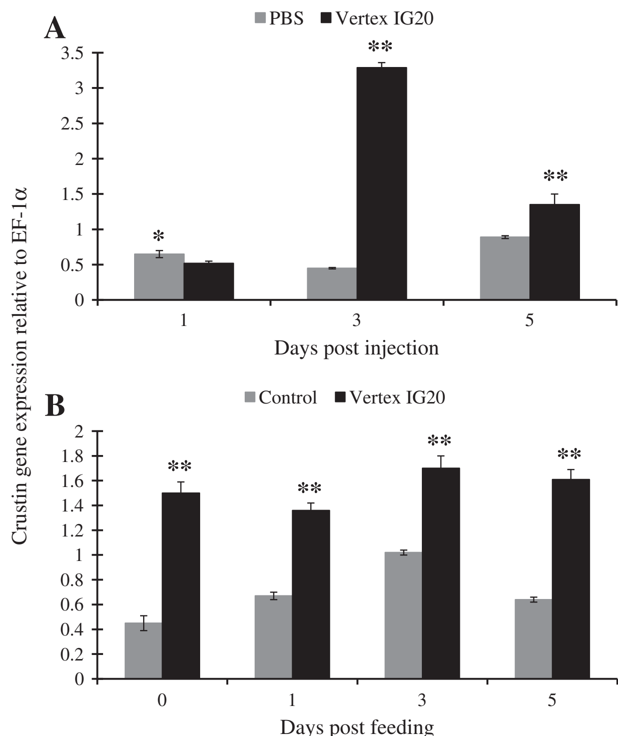

Fig. 2. Densitometric quantification of PCR-analysis of crustin gene expression relative to the elongation factor (EF)- 1α gene transcript in the lymphoid organ of kuruma shrimp. Comparison of expression level in A) PBS (control) and Vertex IG20 injected, B) control and vertex IG20 diet fed shrimps (n = 5) was made for each time point. **$P<0.01$; *$P<0.05$.

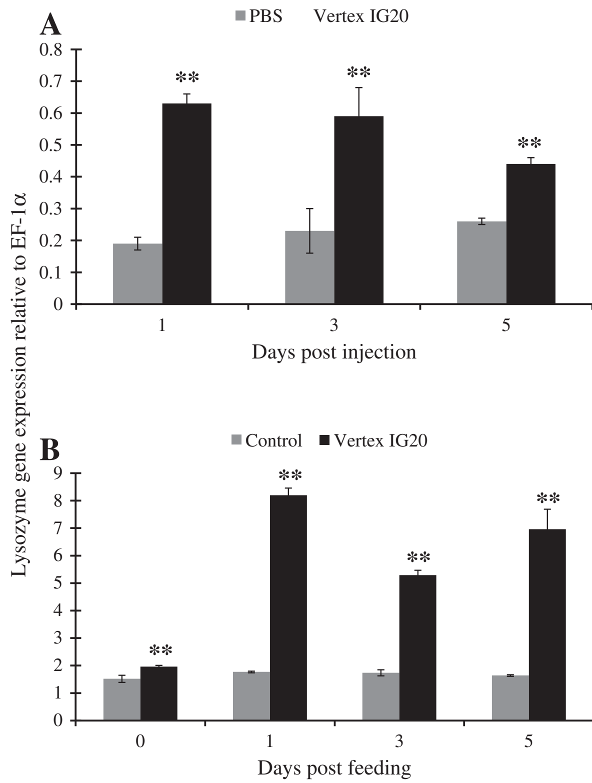

Fig. 3. Densitometric quantification of PCR-analysis of lysozyme gene expression relative to the elongation factor (EF)- 1α gene transcript in the lymphoid organ of kuruma shrimp. Comparison of expression level in A) PBS (control) and Vertex IG20 injected, B) control and vertex IG20 diet fed shrimps (n = 5) was made for each time point. $^{**}P<0.01$.

yeast extract fed shrimps had almost 8 times higher survival rate (66.6%) than the control shrimps (8.3%). Dead and survived shrimps were tested for bacterial infection by growing the bacterial inoculum from LO in marine agar plates and further by PCR, and it was confirmed that all dead shrimps were infected with *V. nigripulchritudo*, while survived ones were devoid of bacteria.

4. Discussion

In this study, the efficacy of a nucleotide-rich baker's yeast extract preparation as immunostimulant was tested by examining expression of innate immune-related genes, *viz.* MjPen (penaeidin), MjCrus (crustin) and MjLyz (lysozyme), and resistance to a virulent bacterial

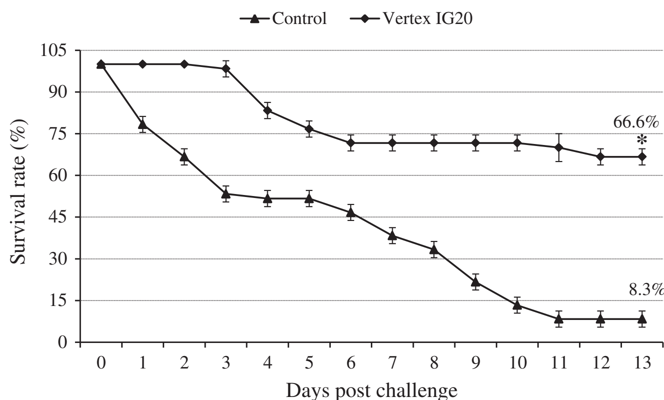

Fig. 4. Survival rates in control and Vertex IG20 diet fed kuruma shrimps challenged with the i.m. injection of *Vibrio nigripulchritudo* at 1×10^5 cfu mL^{-1}. Asterisk indicates significant difference ($P<0.01$) in the final survival rates.

strain, *V. nigripulchritudo* in kuruma shrimp, *M. japonicus* fed the baker's yeast extract incorporated diet.

Penaeidins, major components of immune response, are constitutively synthesized and stored in the shrimp hemocytes, localized in granulocyte cytoplasmic granules, released after stimulation and have antimicrobial and chitin binding properties which may play an important role in coordination between immune function and metamorphosis function with the synthesis of exoskeleton (Destoumieux et al., 2000b). To date, several studies describe 39 penaeidins identified from 8 different shrimp species including *M. japonicus* (Rojtinnakorn et al., 2002; Vasaheeran et al., 2012). Significant up-regulation of MjPen gene expression with expression peak at 5 d post feeding in the yeast extract-treated shrimp compared to the control shrimp indicated the important role of this gene in local immune response in LO and its expression enhanced upon stimulation with suitable substance such as baker's yeast extract. Similar up-regulation of MjPen gene expression was observed in the LO of kuruma shrimp at 3 and 7 d post vaccination with a DNA vaccine encoding viral envelope protein (VP28) (Kono et al., 2010). The role of penaeidins in systemic innate immunity has been well documented in several microbial challenges to different penaeid shrimps such as *Litopenaeus vannamei* (Muñoz et al., 2002), *M. japonicus* (Fall et al., 2010; Kono et al., 2009) and *Penaeus monodon* (Soonthornchai et al., 2010). Therefore, the increased MjPen expression in the present study would indicate the involvement of penaeidins in local defense reaction through their release by hemocytes and binding to shrimp cuticle surfaces (Muñoz et al., 2002).

Crustin, a cysteine-rich peptide containing a 4-disulphide core or a whey-acidic protein (WAP) domain at the C-terminal, acts against marine Gram-positive bacteria and appears to require high salinity to express this activity (Rattanachai et al., 2004). Since its discovery about 50 crustin-like genes have been identified in 20 different crustacean species (Smith et al., 2008). Several studies demonstrated its antimicrobial properties in shrimps when challenged to various bacterial pathogens such as *Staphylococcus aureus* (Zhang et al., 2007a,b), *Vibrio harveyi* (Amparyup et al., 2008; Soonthornchai et al., 2010) and *Vibrio penaeicida* (Shockey et al., 2009). Furthermore, MjCrus gene was up-regulated in the LO of kuruma shrimp after injection of a DNA vaccine at 1–7 d post vaccination period (Kono et al., 2009, 2010). Similarly, in the current study, the *in vivo* expression of MjCrus was enhanced in the baker's yeast extract injected and fed kuruma shrimp LO. It is therefore possible that transcriptional activation of MjCrus gene might be involved in the protective immunity induced with the yeast extract treatment.

Lysozyme is one of the earliest known antibacterial proteins, omnipresent among eukaryotes and prokaryotes (Tyagi et al., 2007). The expression and functions of various lysozymes against bacteria and their expressive responses to pathogenic challenge have been established in different penaeid shrimps (Burge et al., 2007; de-la-Re-Vega et al., 2006; Fall et al., 2010; Soonthornchai et al., 2010; Tyagi et al., 2007) and they mostly perform lytic activity against Gram-negative bacterial cell wall (Yao et al., 2008; Zhao et al., 2007). We observed the up-regulated MjLyz transcription in both yeast extract injected and fed shrimp groups, although higher fold change in expression was recorded in feeding group at 1–5 d post feeding. Consistent to our results, the up-regulated expression of MjLyz gene was noticed in the LO of kuruma shrimp after administration of a DNA vaccine at 3–7 d post vaccination period (Kono et al., 2009, 2010). However, in *L. vannamei* lysozyme transcript levels were enhanced both in hemocytes and hepatopancreas after laminarin, LPS and poly I:C injection, suggesting possible broad spectrum activity of lysozyme against various kinds of microorganisms (Ji et al., 2009). Deng et al. (2012) observed higher lysozyme concentration in the hemolymph of *L. vannamei* fed with a yeast culture feed supplement (YK-6). These results coupled with our findings, therefore, indicate that lysozyme is elicited with different immunostimulating substances

and acts as an integral component of shrimp antibacterial defense mechanism.

Until now, few efforts were undertaken to assess the role of nucleotides on shrimp health management. Particularly, in a few trials involving fish, increased expression of nonspecific immune genes, *viz.* cytokines was observed in turbot (Low et al., 2003) and common carp (Biswas et al., 2012) fed a nucleotide supplemented diet and a diet incorporated with a baker's yeast extract containing both nucleotides and β-glucan, respectively. Li et al. (2007) demonstrated no effects of a purified nucleotide mixture as a dietary supplement on the growth and survival of Pacific white shrimp, *L. vannamei*. On the contrary, in a low saline (5 ppt) rearing of 30 days, the Pacific white shrimp had better growth and immune responses in terms of higher total hemocyte counts and respiratory burst activity, when fed with a nucleotide supplemented diet (Murthy et al., 2009). Similarly, our results of elevated innate immune gene expressions substantiate the necessity of the incorporation of nucleotides to shrimp diet for better animal health management.

It is quite pertinent to assess the elevated resistance level conferred to kuruma shrimp administered with the nucleotide-rich baker's yeast extract. Dietary nucleotide supplementation can enhance resistance to bacterial and viral pathogens in several fish species (Leonardi et al., 2003; Li et al., 2004; Sakai et al., 2001; Tahmasebi-Kohyani et al., 2011). In shrimps, disease resistance studies involving nucleotide administration are non-existent. However, few experiments report about the effects of different yeast species on shrimp resistance to *Vibrios* (Deng et al., 2012) and white spot syndrome virus (WSSV) (Sarlin and Philip, 2011). In our study, significant resistance to the virulent bacteria *V. nigripulchritudo* has been provided in kuruma shrimp by the oral administration of the nucleotide-rich yeast extract. *V. nigripulchritudo* is a Gram-negative motile bacterium known to cause high mortality in shrimps including *M. japonicus* displaying sluggish behavior and spiral swimming pattern without any gross external pathological signs (Sakai et al., 2007). The increased shrimp resistance to *V. nigripulchritudo* infection, together with the increased expression of MjLyz gene corroborates the involvement of lysozyme in the killing of this Gram-negative bacterium in LO that serves as a prime location for bacteria-hemocyte interactions (Burge et al., 2007).

The present findings of increased or induced expression of innate immune genes in the LO, enhanced resistance to a bacterial pathogen that resulted from treatment of the kuruma shrimp with a baker's yeast extract indicate the immunostimulatory effects of this yeast extract that contains nucleotides. Therefore, this extract may be used as a potential shrimp immunostimulant. An understanding on the contribution of various innate immune parameters in immune responses in shrimp treated with the nucleotide-rich baker's yeast extract using different doses and treatment methods may be the subject of further research.

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