Mar Biotechnol (2014) 16:181–192
DOI 10.1007/s10126-013-9532-1

ORIGINAL ARTICLE

Isolation of Lactic Acid Bacteria from Kuruma Shrimp (*Marsupenaeus japonicus*) Intestine and Assessment of Immunomodulatory Role of a Selected Strain as Probiotic

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Received: 6 September 2012 / Accepted: 3 September 2013 / Published online: 18 September 2013

Abstract Fifty-one lactic acid bacteria (LAB) strains were isolated and identified based on 16S ribosomal DNA sequence from the intestinal tracts of 142 kuruma shrimps (*Marsupenaeus japonicus*) collected from Kanmon Strait, Fukuoka and Tachibana Bay, Nagasaki, Japan. Cellular immunomodulatory function of 51 isolated LAB strains was assessed by measuring the level of interferon (IFN)-γ induction in mouse spleen cell culture. The strain *Lactococcus lactis* D1813 exhibited the highest amount of IFN-γ production and also bactericidal activity and was selected for testing its immunomodulatory role as a probiotic in kuruma shrimp. We also assessed the effect of dietary incorporation of this probiotic on resistance to *Vibrio penaeicida* infection in the kuruma shrimp. Our results demonstrate that probiotic *L. lactis* D1813-containing diet-fed (10^5 cfu g^{-1}) shrimps displayed a significant up-regulation of lysozyme gene expression in the intestine and hepatopancreas. However, insignificantly higher expression of anti-lipopolysaccharide factor, super oxide dismutase, prophenoloxidase, and toll-like receptor 1 was recorded in the intestine of shrimps fed the probiotic diet. Moreover, significantly increased ($P<0.01$) resistance to the bacterial pathogen in term of better post-infection survival (61.7 %) was observed in the shrimps fed with the probiotic-incorporated diet compared with the control diet-fed group (28.3 %). The present study indicates the immunomodulatory role of the LAB *L. lactis* D1813 on the kuruma shrimp immune system and supports its potential use as an effective probiotic in shrimp aquaculture.

Keywords Lactic acid bacteria · Immunomodulation · Shrimp probiotic · Innate immune genes · Disease resistance

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Introduction

Probiotic is defined as a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment (Verschuere et al. 2000). Since the last few years, use of probiotics as bio-control agents has become a popular technique to improve and maintain a healthy environment as well as farmed animal health in aquaculture. However, the use of probiotics in aquaculture is a newer concept compared to their usage in human, pig, cattle, and poultry nutrition.

Kuruma shrimp, *Marsupenaeus japonicus* is the highest-priced shrimp species and widely cultured in Japan, China, Australia, and Southeast Asian countries (Rosenberry 2001). However, according to a World Bank report, disease occurrences have led to considerable global losses to the tune of 3 billion US dollars in shrimp farming industry (Farzanfar 2006). Treatment of these diseases using chemotherapeutics and antibiotics at farm level is either infeasible or prohibited due to the potential negative impacts, such as development of resistant bacteria and thereby reduced efficiency of antibiotics for human and animal diseases. Therefore, enhancement of immune status

in farmed animals and control of disease agents using biological means have become a useful, eco-friendly, and hazard-free option in aquaculture.

Shrimps like other exothermic and endothermic animals contain numerous beneficial gut microbes (Moss et al. 2000; Oxley et al. 2002) that are generally less diverse than those of mammals and birds (Liu et al. 2010). Among these, lactic acid bacteria (LAB), the Gram-positive, non-sporulating, and catalase-negative rods or cocci (Vijayabaskar and Somasundaram 2008) constitute a major group. They play a beneficial role in the host gut environment by producing antibacterial substances such as lactic acid, acetic acid, hydrogen peroxide, and bacteriocin that suppress growth of competing bacteria (Ouwehand and Vesterlund 2004). Therefore, they may confer health benefits on the host when administered in an adequate amount from external sources. In the last two decades, application of LAB and their metabolic products as potential probiotics has been tested to improve growth and survival of shrimps (Ajitha et al. 2004; Castex et al. 2008; Chiu et al. 2007; Kongnum and Hongpattarakere 2012; Peraza-Gómez et al. 2009; Vieira et al. 2007). LAB are also well known for their role in improvement of immune status and disease resistance in higher animals (Kimoto et al. 2004; Kimura et al. 2006; Shida et al. 2002; Takeda et al. 2011), fish (Lara-Flores et al. 2003; Panigrahi et al. 2007, 2011; Salinas et al. 2008), and shrimp (Castex et al. 2009; Leyva-Madrigal et al. 2011; Peraza-Gómez et al. 2009; Uma et al. 1999). Most of the LAB used in different probiotic feeding experiments are either from higher animals or allochthonous sources, except a strain isolated from digestive tracts of cultivated and wild shrimps (Kongnum and Hongpattarakere 2012). As a result, these non-shrimp probiotic strains are unable to survive or remain viable at high cell density by overcoming the antagonistic effects in the gut environment during the active growth phase of shrimp. Hence, isolation of putative probiotics from shrimp and use in the same animal would be an appropriate approach because these strains have already adhered to the gut wall and, thus, are well-adapted to compete with pathogens for nutrients (Ghosh et al. 2007). However, immunostimulatory or protective effects conferred by LAB administration in terms of molecular immune functions in shrimp have been barely investigated. One exception is a report by Chiu et al. (2007), which demonstrated higher expressions of prophenoloxidase (proPO) and peroxinectin genes in *Litopenaeus vannamei* fed diets containing *Lactobacillus plantarum* isolated from non-shrimp sources. There is no information on the isolation of LAB from kuruma shrimp intestine and testing their efficacy as probiotics for immunomodulation and expression of innate immune-related genes. Therefore, in this study, we isolated 51 LAB strains from wild kuruma shrimp intestines, selected a strain based on its higher immunomodulatory function in mouse spleen cells, and then investigated its efficacy as a probiotic supplement in induction of immune-related genes, such as crustin, anti-lipopolysaccharide factor (ALF), lysozyme, superoxide dismutase (SOD), proPO and toll-like receptor (TLR)1, and TLR2 in the intestine and hepatopancreas. The intestine and hepatopancreas are important organs to have immune functions in shrimp (Fall et al. 2010; Pan et al. 2005). Additionally, we assessed resistance to *Vibrio penaeicida* infection in the LAB-fed kuruma shrimps to confirm functionality of the elevated innate immune system.

Materials and Methods

Collection of Shrimps

Kuruma shrimps were collected from Kanmon Strait, Fukuoka and Tachibana Bay, Nagasaki, Japan and transported to the laboratory in live condition. A total of 142 wild shrimps were sampled, and they were healthy with an average body weight of 41.5±11.8 g. Shrimps were firstly acclimatized in an aerated seawater tank at 28 °C under a natural photoperiod and then sacrificed and dissected in the same day. The health status of shrimps was checked by culturing hemolymph and hepatopancreas smears on Marine Agar 2216E (Difco, Detroit, MI, USA) plates for presence of any bacterial pathogens. The results showed existence of no pathogenic bacteria in shrimps.

Isolation, Culture, and Storage of Bacteria

Shrimp body surfaces were washed and disinfected with 70 % ethanol, and then the entire intestinal tract was dissected out. Each whole gut sample was then excised separately with sterile forceps and scissors and homogenized in sterilized seawater. This homogenate was used as inoculum for bacterial culture. LAB were isolated using Man, Rogosa, and Sharpe (MRS) agar (Becton Dickinson and Company, NJ, USA) and glucose yeast extract peptone (GYP) agar (1.0 % glucose, 1.0 % yeast extract, 0.5 % peptone, 0.2 % Na acetate, 0.2 % fish extract, 0.05 % Tween 80). All media contained 0.5 % calcium carbonate. Homogenates were serially diluted (10^{-1} to 10^{-8}) with sterilized seawater, and 100 μL of diluted homogenate was spread on agar plates. All of the above steps were performed in a laminar flow hood. Inoculated plates were incubated under aerobic and anaerobic conditions at 27 °C for 5 days. After incubation, colonies were purified by streak-plating on new plates of the same agar. Purified strains were suspended into 10 % glycerol solution in sterilized tubes and stored at −80 °C.

Identification of LAB

For preliminary identification, all isolates obtained from MRS and GYP were Gram-stained and tested for catalase activity. The isolates which exhibited as Gram-positive with catalase-negative were selected and identified based on API test

(bioMérieux, l'Etoile, France) and the 500 base pair (bp) sequences from the 5′-end of 16S ribosomal DNA (16S rDNA). Total DNA was extracted from a colony using a Instagene Matrix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions, and 16S rDNA was amplified by polymerase chain reaction (PCR) in a thermal cycler (Takara Bio Inc., Shiga, Japan). Amplified and purified 16S rDNA was sequenced using a CEQ8800 Automated Sequencer (Beckman Coulter, Inc., CA, USA), and the homology of sequences generated was compared using the BLAST and FASTA programs.

Bacterial Strains and Culture of Mouse Spleen Cells for Interferon-γ Production

A total of 51 LAB strains were isolated and identified and were cultured at 27 °C for 6 days in MRS broth. They were harvested by centrifugation at 13,000×*g* for 10 min, washed with sterilized distilled water, and heated at 70 °C for 15 min. Then the heat-killed LAB were lyophilized. The lyophilized powder was suspended in sterilized saline for treatment of mouse spleen cells. Spleen cells were isolated as a single cell suspension from the spleen of BALB/c female mice. After depletion of erythrocytes, the cells were adjusted to 5×10^6 cell well^{-1} in a 96-well culture plate (Asahi Glass Co., Ltd, Tokyo, Japan) containing 190 μL RPMI-1640 medium (Life Technologies, CA, USA) supplemented with 5 % heat-inactivated fetal bovine serum (FBS; CAMBREX, NJ, USA) and a 1 % solution of 10,000 μg mL^{-1} streptomycin plus 10,000 U mL^{-1} penicillin (Life Technologies). The cells were treated with the heat-killed bacterial strains at a concentration of 10 μg mL^{-1}. Physiological saline and 4 μg mL^{-1} ConcanavalinA (ConA; Sigma, USA) were added to separate wells designated as negative and positive control, respectively. All treated and control wells were replicated thrice. The culture plates were incubated at 37 °C in 5 % CO_2 incubator. Supernatants were collected at day 3 for measuring the level of interferon (IFN)-γ production. As there is no established method for detection of cellular immune function using shrimp cell culture (cell lines) system, we used mouse spleen cell culture to screen 51 LAB strains following an easy and rapid standard method. Our objective was mainly to evaluate the immunomodulatory function of these strains and select the strain causing higher immunomodulation.

Quantification of IFN-γ Using ELISA Method

At day 3, the splenic cell culture was centrifuged and supernatants were isolated. The level of IFN-γ in the supernatants was determined using Mouse IFN-γ ELISA Set (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol.

Selection of LAB Strain that Induced Higher IFN-γ Production and Testing Its Antibacterial Activity

From the cell culture treated with different heat-killed LAB, *Lactococcus lactis* strain D1813 which induced higher level of IFN-γ production was selected for further experimentation. Strain D1813 cell-free culture supernatants were isolated from the culture in brain heart infusion (BHI; Becton Dickinson and Company) agar broth containing 1 % NaCl by filtration (0.22 μm). *V. penaeicida* (NBRC15640) was used as a tested bacterium to assess the bactericidal property of LAB D1813. *V. penaeicida* was cultured in BHI agar broth containing 1 % NaCl at 27 °C for 18 h, and finally, bacterial cell turbidity was adjusted to optical density (OD) 0.1 at 600 nm (0.1 OD_{600}). Antibacterial activity of the D1813 cell-free culture supernatants was determined using a 96-well micro-titer plate according to Khouiti and Simon (1997) with slight modifications. Each well was filled with 100 μL of D1813 cell-free culture supernatants and 100 μL of 0.1 OD_{600} *V. penaeicida*. Wells filled with 100 μL of 0.1 OD_{600} *V. penaeicida* and 100 μL of culture medium served as control. The plates were then incubated at 27 °C for 24 h, and the turbidity of cell suspension was measured spectrophotometrically (600 nm) at 1-h intervals.

Expression Analysis of Immune-Related Genes in Shrimps Fed with LAB Strain

Healthy kuruma shrimps (mean body weight, 4.7±0.3 g) were obtained from Asahi Suisan, Yamaguchi, Japan. Shrimps were firstly acclimatized in an aerated seawater tank at room temperature and fed with a commercial diet (Nihon Formula Feed Manufacturing, Kanagawa, Japan) once a day for an week under a dark enclosure prior to their use in the experiment. *Lactococcus lactis* D1813 strain cultivated in MRS broth was harvested by centrifugation and washed twice with distilled water, and the freeze-dried bacterial cell was mixed to the commercial diet at two concentrations, viz. 10^5 and 10^7 cfu g^{-1} feed by spraying. This bacterium-incorporated feed was then stored at 4 °C for use within 3 days. The same commercial feed served as control diet. For the feeding experiment, shrimps were divided into three groups ($n=80$), viz. two treatments and a control group. Shrimps of the treatment groups were fed with the two diets supplemented with the selected LAB at 10^5 and 10^7 cfu g^{-1} feed, respectively, whereas control group shrimps were fed with the unmixed diet once a day at 3–5 % body weight for 7 days. Shrimps were maintained in a seawater flow-through system at 28±1 °C under the dark enclosure.

At the end of 7-day feeding trial, the intestine and hepatopancreas were dissected out from the kuruma shrimps ($n=5$) fed with LAB-supplemented and non-supplemented diets at 3 and 6 days post-feeding. Total RNA was extracted from the

intestine and hepatopancreas using RNeasy Mini Kit (QIAGEN, Venlo, Netherlands) as per the manufacturer's instructions. 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 (QIAGEN). cDNA synthesis was performed using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, CA, USA), and this cDNA served as a template for quantitative real-time PCR (qPCR). qPCR on cDNA specimens was performed using the Fast SYBR® Green Master Mix (Applied Biosystems). The immune-related genes, internal control, and their respective primers are presented in Table 1. Elongation factor (EF)-1α gene was used as an internal control. All real-time PCR reactions were performed in a reaction mixture containing 10 μL of Fast SYBR® Green Master Mix, 0.8 μL of 10 pM primer set (Crustin/ALF/lysozyme/SOD/proPO/TLR1/TLR2/EF-1α), 2 μL of template DNA (10 ng), and 7.2 μL of nuclease-free water. Amplification was carried out as follows: 20 s at 95 °C, 40 cycles of 3 s at 95 °C, and 30 s at 60 °C. Thermal cycling and fluorescence detection were conducted using the StepOne Real-Time PCR System (Applied Biosystems) with detection run in triplicate. The threshold cycle (C_T) represents the PCR cycle at which an increase in reporter fluorescence above a baseline signal was first detected. The comparative C_T method ($2^{-\Delta\Delta C_T}$ method) (Livak and Schmittgen 2001) was used to analyze the expression level of the shrimp genes.

Table 1 Gene-specific primers of kuruma shrimp EF-1α and immune-related genes used for real-time qPCR analysis in the study

Gene	Primer sequence (5′→3′)	Accession number
EF-1α-F[a]	TTCGCTGAACTGCTGACCAA	AB458256
EF-1α-R[b]	GCTTGCTGGGAACCATCTTG	
Crustin-F	GGTCGAGCGACTGCAGGTA	AB121740
Crustin-R	GGCAGTCCAGTGGCTTGGTA	
ALF-F	CCAACGCCCAACCTTCTACA	AB453738
ALF-R	GGCTGCGGGTCATAGATCTG	
Lysozyme-F	GCGTGGACTACGGCATCTTC	AB080238
Lysozyme-R	GAGATCGGAGCATGGGATTC	
SOD-F	CCAAGCATCACCAGGGTTACA	AB079877
SOD-R	GGCATTCATAGCGCTGACATC	
proPO-F	TCCAAGTGCCAGAACGAAATG	AB065371
proPO-R	CGATGAGACGCGAGGAAGA	
TLR1-F	TCGCCAGTACAGTGATCAGCAT	AB333779
TLR1-R	CATTCACCACAGCCCACAAG	
TLR2-F	CCTTCTCCTGTTTGCTGTCCTT	AB385869
TLR2-R	GCATCTAATTCGTCCTCTGTTATGG	

[a] F=Forward

[b] R=Reverse

Bacterial Challenge to Shrimps Fed with LAB Strain

V. penaeicida (strain E65) was used for artificial infection in this study. The bacterium was grown as described above, and bacterial cells were harvested from stationary phase cultures and re-suspended in sterile seawater. LAB-supplemented (10^5 cfu g^{-1} diet) and control diet-fed kuruma shrimps were artificially challenged at day 1 after the 7-day feeding trial by immersion in seawater contaminated with 10^8 cfu mL^{-1} of *V. penaeicida* for 6 h. Shrimps from each group were distributed in three replicate tanks (n=20) provided with seawater flow-through system, and the challenged shrimps were fed with non-supplemented commercial diet as mentioned. The survival rate from each group was recorded daily for 14 days.

Statistical Analysis

Differences between different levels of IFN-γ produced in mouse spleen cells induced with different LAB strains and the quantified relative expressions of a particular gene in the intestine and hepatopancreas of two LAB-supplemented diets and control diet-fed shrimps at 3 and 6 days post-feeding were evaluated with one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) for their comparison. Final survival rates of bacteria-challenged shrimps were evaluated with an independent sample t test for equality of means. Statistical analysis was performed using the SPSS for Windows v. 17.0 program (SPSS Inc., Chicago, IL, USA). All data are expressed as mean ± standard deviation (S.D.).

Results

Isolation and Identification of LAB

Purification of LAB from 142 shrimp intestine samples yielded 51 isolates, all of which were Gram-positive and catalase-negative. Some of the bacterial strains did not grow in API test strips. However, final identification of the isolated 51 strains was confirmed based on 16S rDNA sequence analysis, and the strains were classified into 14 species (Table 2). The most predominant species was *Lactobacillus plantarum* (31.38 % abundance) followed by *Lactococcus lactis* (23.54 %), *Vagococcus fluvialis* (11.76 %), and *Lactococcus garvieae* (7.84 %).

Selection of LAB with Higher Immunomodulatory Function

We examined the cellular immunomodulatory function of 51 strains of LAB isolated from kuruma shrimp intestine using the mouse spleen cell culture method and found some strains that induced higher production of IFN-γ (Fig. 1). Mostly, the strains

Table 2 Identified LAB strains based on API test and 16S rDNA sequence analysis

Strain number	Identification		
	API test	BLAST result	Sequence identity (%)
671	–	*Lactococcus* (*Lc*) *lactis*	99
673	–	*Enterococcus* (*En*) *pseudovium*	99
708	–	*En. pallens*	98
732	–	*Lc. lactis*	98
938	–	*En. faecalis*	99
1233	–	*En. raffinosus*	97
1660	–	*Lc. garvieae*	99
1813	*Lc. lactis*	*Lc. lactis*	99
1836	*Lc. lactis*	*Lc. lactis*	98
1837	*Lc. lactis*	*Lc. lactis*	98
1838	*Lc. lactis*	*Lc. lactis*	98
2135	–	*Lc. lactis*	98
2310	–	*Lc. garvieae*	98
2325	–	*Lc. garvieae*	98
2326	–	*En. faecium*	98
2339	–	*Lactobacillus* (*Lb*) sp.	98
2350	–	*En. faecium*	99
2440	*Lb. plantarum*	*Lb. plantarum*	98
2447	*Lb. plantarum*	*Lb. plantarum*	98
2449	*Lb. plantarum*	*Lb. plantarum*	98
2451	*Lb. plantarum*	*Lb. plantarum*	98
2452	*Lb. plantarum*	*Lc. lactis*	98
2455	*Lb. plantarum*	*Lc. lactis*	98
2458	*Lb. plantarum*	*Lb. plantarum*	98
2461	*Lb. plantarum*	*Lb. plantarum*	98
2465	*Lb. plantarum*	*Lb. plantarum*	97
2467	*Lb. plantarum*	*Lc. garvieae*	98
2468	*Lb. plantarum*	*Lb. plantarum*	98
2479	*Lc. lactis* sp. *lactis*	*Vagococcus* (*Vc*) *camiphilus*	98
2480	*Lb. plantarum*	*Lb. plantarum*	99
2491	*Lc. lactis* sp. *lactis*	*Lc. lactis*	98
2492	*Lb. plantarum*	*Lb. plantarum*	97
2597	*Lc. lactis* sp. *lactis*	*Lc. lactis*	97
2607	*Lc. lactis* sp. *lactis*	*Lc. lactis*	97
2697	*Lb. plantarum*	*Lb. plantarum*	98
2700	*Lb. pentosus*	*Lb. plantarum*	98
2712	*Lb. brebis*	*Pediococcus* (*Pc*) *pentosaceus*	97
2714	*Lb. brebis*	*Lb. amylophilus*	97
2818	–	*Vc. fluvialis*	98
2819	–	*Vc. fluvialis*	98
2852	–	*Vc. fluvialis*	99
2862	–	*Vc. camiphilus*	97
2902	*Lb. plantarum*	*Lb. plantarum*	97
2903	*Lb. plantarum*	*Lb. plantarum*	97
2905	*Lb. plantarum*	*Lb. plantarum*	97
2906	–	*Lb. nagelii*	97
2908	*Lb. plantarum*	*Lb. plantarum*	98
2910	–	*Vc. fluvialis*	98

– strain did not grow in API test strip

Table 2 (continued)

Strain number	Identification		
	API test	BLAST result	Sequence identity (%)
2917	–	*Vc. fluvialis*	97
2918	–	*Vc. fluvialis*	98
3268	–	*Pc. pentosaceus*	97

of *Lactococcus lactis* induced higher IFN-γ production; among these, *L. lactis* strain D1813 exhibited the highest level of IFN-γ induction in vitro. We selected the strain D1813 for testing its probiotic property as an immunomodulator in shrimp.

Antibacterial Property of *Lactococcus lactis* Strain D1813

The selected *L. lactis* strain D1813 was subjected to assay for its ability to inhibit growth of *V. penaeicida* by a 96-well micro-titer plate method. This LAB strain exhibited antibacterial activity against the tested microorganism as the wells containing D1813 cell-free culture supernatants and *V. penaeicida* had less turbidity of bacterial cells evident from lower optical density value than that of the control wells containing *V. penaeicida* alone (Fig. 2).

Expression of Immune-Related Genes

Innate immune gene expression in the intestine and hepatopancreas of shrimps fed with the 0 (control), 10^5, and 10^7 cfu g^{-1} *Lactococcus lactis* strain D1813-containing diets was measured using real-time RT-PCR at 3 and 6 days post-feeding.

Immune Gene Expression in Intestine

At 3 days post-feeding, although higher expression of ALF, lysozyme, and proPO was observed in the intestine of probiotic-fed shrimps, the expression levels were not different ($P>0.05$) from those of the control diet-fed shrimps (Fig. 3a). Crustin and lysozyme transcripts were significantly increased by probiotic supplementation at 10^5 cfu g^{-1} diet compared to 10^7 cfu g^{-1} and control diet-fed shrimps ($P<0.05$) at 6 days post-feeding (Fig. 3b). However, insignificantly higher expression of ALF, SOD, proPO, and TLR1 was recorded in the intestine of shrimps fed with the 10^5 cfu g^{-1} diet at 6 days post-feeding.

Immune Gene Expression in Hepatopancreas

In the hepatopancreas, the transcript level of lysozyme in 10^5 cfu g^{-1} probiotic diet-fed shrimps was significantly higher

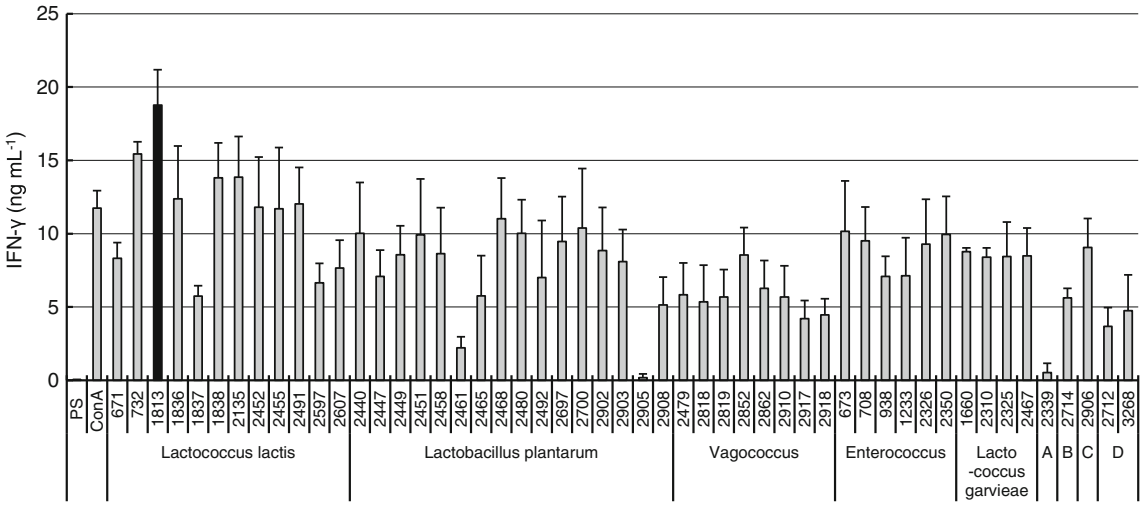

Fig. 1 Levels of IFN-γ induction by 51 LAB strains isolated from wild kuruma shrimp intestines. Heat-killed 51 strains were added at 10 μg mL^{-1} to cultured mouse spleen cells. At day 3, IFN-γ levels in the culture supernatants were measured by ELISA. Physiological saline (PS) and ConcanavalinA (ConA) were added to separate wells as negative and positive control, respectively. *Numbers* on the *X* axis represent the strain number of bacterial species mentioned. Letters *A*, *B*, *C*, and *D* represent *Lactobacillus* sp., *Lactobacillus amylophilus*, *Lactobacillus nagelii*, and *Pediococcus pentosaceus*, respectively. The *black bar* indicates the highest level of IFN-γ induction by strain *Lactococcus lactis* D1813. Data are presented as mean ± SD of three replicates

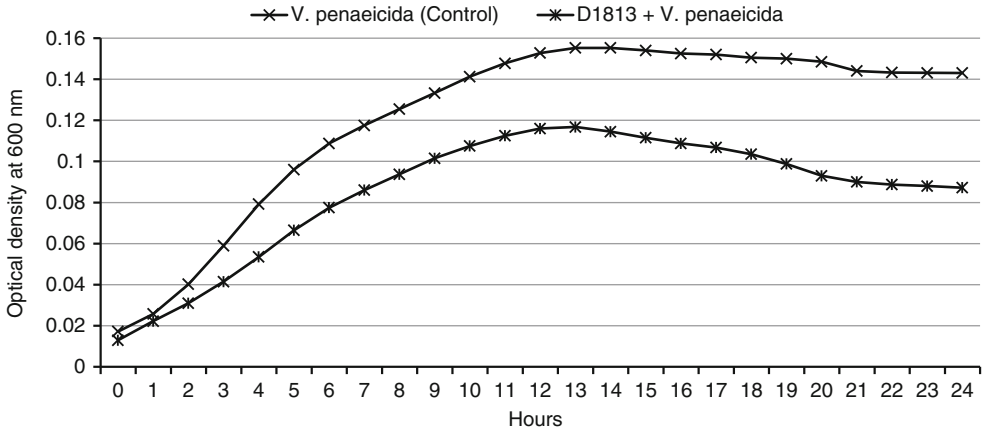

Fig. 2 Antibacterial activity of the LAB *Lactococcus lactis* D1813 to the tested bacterium *V. penaeicida* (strain NBRC15640). Wells of a 96-well micro-titer plate were filled with 100 μL of D1813 cell-free culture supernatants and 100 μL of 0.1 OD (optical density) $_{600}$ *V. penaeicida* (D1813 + *V. penaeicida*). Control wells were filled with 100 μL of 0.1 OD_{600} *V. penaeicida* and 100 μL of culture medium. The plate was incubated at 27 °C for 24 h, and OD of cell suspension was measured at 600 nm at 1-h intervals

($P<0.01$) than in 10^7 cfu g^{-1} and control diet-fed shrimps ($P<0.05$) at 3 days post-feeding (Fig. 4a). There was an increased expression of ALF, lysozyme, SOD, and TLR1 genes in 10^5 cfu g^{-1} diet probiotic-fed shrimps, but the expression levels were not significantly higher than those of 10^7 cfu g^{-1} and control diet-fed shrimps at 6 days post-feeding (Fig. 4b). However, proPO expression could not be detected in the hepatopancreas of probiotic-treated and control shrimps.

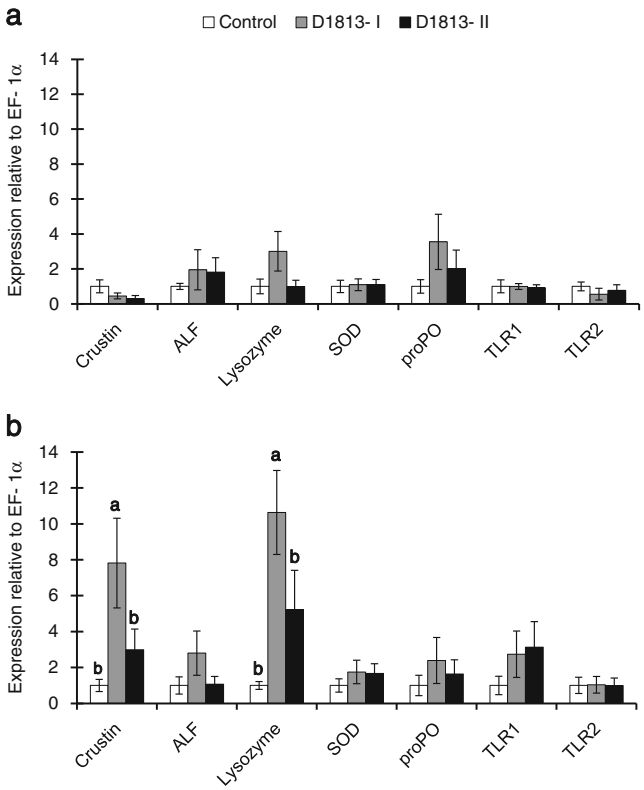

Fig. 3 Quantitative real-time PCR analysis of immune-related gene expression relative to the elongation factor (EF)-1α gene transcript in the intestine of control and *Lactococcus lactis* D1813 probiotic diet-fed kuruma shrimps. Control diet contained no probiotic, while two diets contained the same D1813 probiotic at 10^5 cfu g^{-1} (D1813- I) and 10^7 cfu g^{-1} (D1813- II), respectively. Gene expression at **a** 3 days and **b** 6 days post-feeding. Different *letters* for each gene indicate a significant difference ($P<0.05$) in expression levels among control and probiotic diet-fed shrimps. Data are presented as mean ± SD of five shrimp samples

Resistance of Probiotic-Fed Shrimps to *V. penaeicida*

At the end of 14 days post-challenge trial, shrimps fed with probiotic-incorporated (10^5 cfu g^{-1}) diet exhibited significantly better survival ($P<0.01$) compared with the control diet-fed group (Fig. 5). The LAB probiotic-fed shrimps had more than double survival rate (61.7 %) than the control shrimps (28.3 %). Dead shrimps and those that survived were tested for bacterial infection by growing the bacterial inoculum from muscle in TCBS agar (Nissui Seiyaku, Tokyo, Japan) plates, and it was confirmed that all dead shrimps were infected with *V. penaeicida*, while the survived ones were devoid of bacteria.

Discussion

In this study, we isolated 51 LAB strains from wild kuruma shrimp intestine and investigated the immunomodulatory property of a selected strain when supplemented to shrimp diet as a probiotic. Upon challenge of the experimental animals to a virulent bacterial strain, *V. penaeicida*, higher survival could be obtained in kuruma shrimps fed with the selected LAB-incorporated diet compared to that of the control diet. However, to the best of our knowledge, no studies have been conducted on the isolation of LAB from kuruma shrimp intestine and testing of their probiotic property for immunomodulation and expression of innate immune-related genes.

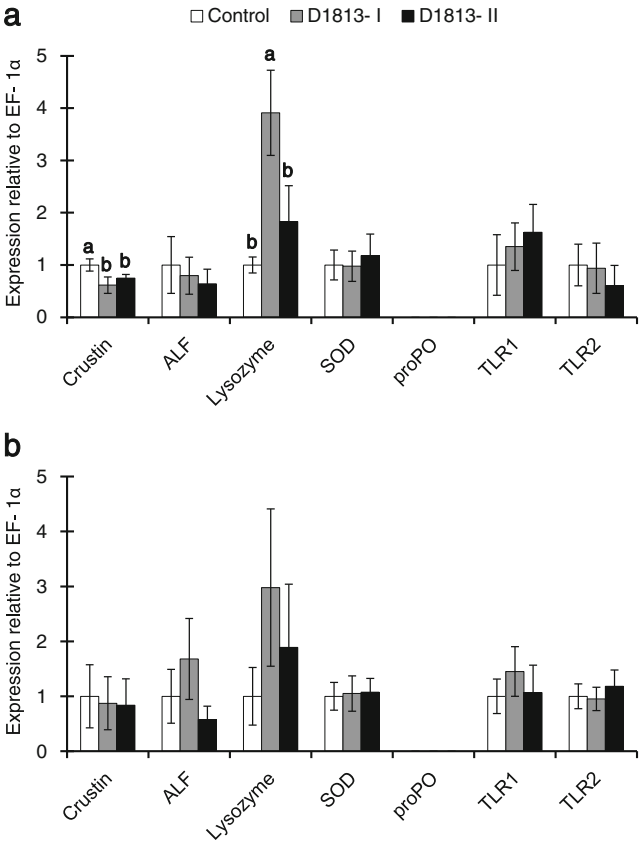

Fig. 4 Quantitative real-time PCR analysis of immune-related gene expression relative to the elongation factor (EF)-1α gene transcript in the hepatopancreas of control and *Lactococcus lactis* D1813 probiotic diet-fed kuruma shrimps. Control diet contained no probiotic, while two diets contained the same D1813 probiotic at 10^5 cfu g^{-1} (D1813- I) and 10^7 cfu g^{-1} (D1813- II), respectively. Gene expression at **a** 3 days and **b** 6 days post-feeding. Different *letters* for each gene indicate a significant difference ($P<0.05$) in expression levels among control and probiotic diet-fed shrimps. Data are presented as mean ± SD of five shrimp samples

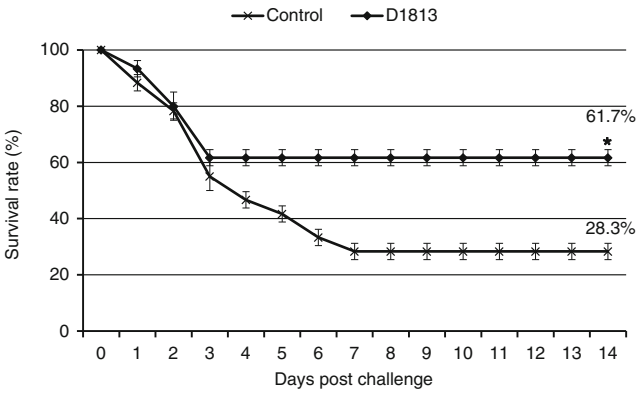

Fig. 5 Survival rates in control and *Lactococcus lactis* D1813 probiotic (10^5 cfu g^{-1}) diet-fed kuruma shrimps challenged by immersion for 6 h in seawater contaminated with 10^8 cfu mL^{-1} of *V. penaeicida* (strain E65). Shrimps from each group were distributed in three replicate tanks ($n=20$), and the survival was recorded for 14 days. *Asterisk* indicates significant difference ($P<0.01$) in the final survival rates. Data represent mean ± SD of three replicated tanks

Out of 51 LAB isolates found in this study, coccoid-shaped strains accounted for 67.75 %, whereas rod-shaped ones were only 32.25 %. Among cocci strains, the most abundant one was *Lactococcus lactis*, and *Lactobacillus plantarum* was the predominant one among rods. Similarly, LAB isolated from wild and cultivated white shrimp guts consisted of 78.23–79.49 % cocci (Kongnum and Hongpattarakere 2012). *Streptococcus*, *Lactococcus*, and *Enterococcus* are very common and abundant coccoid LAB in the digestive tract of aquatic animals (Ringø and Gatesoupe 1998). In line with our observation, Michel et al. (2007) investigated the LAB diversity from aquatic animals and environment, employing amplified rRNA gene restriction analysis and isolated coccoid LAB as the most abundant ones, particularly *Streptococcus* (22 strains), *Lactococcus* (16 strains), and *Enterococcus* (7 strains). However, in an examination on intestinal microbiota diversity from farm-raised kuruma shrimp using molecular analysis of the 16S rDNA, Liu et al. (2010) identified most of the organisms as *Vibrio*, indicating this species as the dominant one in the intestine of kuruma shrimp. Therefore, LAB population under normal circumstances may not be numerically dominant in the digestive tract of aquatic animal (Ringø and Gatesoupe 1998), and this fact resembles the observation of Ringø (1993), who reported that LAB from the stomach of Arctic charr, *Salvelinus alpinus* (L), were approximately 10 %. On the contrary, a high population of LAB was isolated from the warm-water fish gastrointestinal tracts, attributing variation among fish species and geographical location (Buntin et al. 2008).

We measured IFN-γ production as the marker of cellular immunomodulatory function in the murine spleen cells and screened one strain showing higher immunomodulatory property. Increased IFN-γ production due to stimulation with heat-killed LAB indicated activation of natural killer cells (Takeda et al. 2011). Thus, *Lactococcus lactis* (strain D1813) inducing a large quantity of IFN-γ production appeared to have an immunomodulatory function. Although shrimp does not possess the IFN-γ pathway, this strain would possibly have immunostimulatory function in the shrimp defense system also. Previously, heat-killed LAB isolated from Mongolian dairy products also induced IFN-γ production in mice (Kimura et al. 2006; Takeda et al. 2011). Therefore, our result also suggests the capability of this LAB strain in eliciting IFN-γ-mediated immune response in mammals for removal of intracellular pathogens and production of pro-inflammatory cytokines (Schoenborn and Wilson 2007).

In the co-culture, *Lactococcus lactis* D1813 showed antagonistic activity against *V. penaeicida*. This is an important selective criterion for a potential probiotic to be used as an immunomodulator and disease-preventing agent. The antibacterial effect could be due to the production of organic acids, hydrogen peroxide, adhesion inhibitors, or competition

for nutrients (Apún-Molina et al. 2009; Farzanfar 2006; Mota et al. 2006; Ouwehand and Vesterlund 2004). Similar bacterial inhibitory activity to *Vibrio harveyi* was exhibited by *Lactobacillus plantarum* MRO3.12 (Kongnum and Hongpattarakere 2012) and LAB (strain B6 and C2) (Vieira et al. 2007) isolated from white shrimp guts. However, no LAB strains isolated from *L. vannamei* and *Fenneropenaeus californiensis* guts showed antagonistic activity against *Vibrio sinaloensis* (Leyva-Madrigal et al. 2011).

Anti-microbial peptides/proteins (AMPs), the cationic and amphipathic proteins of low molecular weight (<10 kDa), play a major role in innate immunity in shrimp lacking adaptive immunity, and studying their functions enriches basic knowledge on immunity and provides possible avenues in formulating disease management strategies in aquaculture (Bachère et al. 2004). AMPs engage mainly to offer an early and localized first line of defense against pathogens (Selsted and Ouellette 2005; Zasloff 2002). Several AMP families such as penaeidins, crustins, ALFs, histones, and fragments of hemocyanin have so far been described in penaeid shrimps. Crustin, a cysteine-rich peptide containing a 4-disulphide core or a whey-acidic protein (WAP) domain at the carboxy terminus, had been proven to be active against marine Gram-positive bacteria and appeared to require high salinity to express this activity (Rattanachai et al. 2004). Several studies demonstrated the protective responses of crustin induced by various bacterial infections (Amparyup et al. 2008; Shockey et al. 2009; Soonthornchai et al. 2010; Zhang et al. 2007), with a few reports on immunostimulatory effects of probiotics to its expression in shrimp. The elevated expression level of crustin was recorded in *Penaeus monodon* fed with probiotics, viz. *Bacillus*, *Micrococcus*, and a combination of the both (Antony et al. 2011a, b). Similarly, in the current study, the in vivo expression of crustin was enhanced in the LAB probiotic-fed kuruma shrimp intestine at 6 days post-feeding. It is therefore possible that transcriptional activation of this gene might be involved in the protective immunity induced with the oral administration of the LAB probiotic. However, crustin expression in kuruma shrimp may be tissue specific as higher expression was in the intestine. In agreement with our observation, Antony et al. (2011b) reported the highest expression of crustin in the gill followed by the intestine, muscle, and heart and the lowest in the hepatopancreas of *P. monodon*. On the contrary, in *M. japonicus*, crustin mRNA was only detected in hemocyte, not in any other tissue (Rattanachai et al. 2004). Another AMP, ALF, initially isolated and characterized from hemocytes of the horseshoe crab *Limulus polyphemus* (Muta et al. 1987), has the endotoxin- or lipopolysaccharide (LPS)-mediated coagulation system. In the present study, ALF was found to be up-regulated in the intestine and hepatopancreas of shrimps fed with LAB probiotic-incorporated diet, which indicated the immunostimulatory role of this probiotic to elicit ALF in the host defense mechanism. Similarly, probiotic feeding in combination of *Bacillus* and *Micrococcus* supported maximum up-regulation of the ALF gene followed by single probiotic-treated groups in *P. monodon* challenged with white spot syndrome virus (WSSV) (Antony et al. 2011a). This finding along with our results indicated that probiotic administration imitates a microbial challenge in shrimps and thus stimulates the ALF-mediated defense.

Lysozyme is one of the earliest known antibacterial proteins, omnipresent among eukaryotes and prokaryotes (Tyagi et al. 2007). It is an important component of the non-specific innate immune system to provide protection to microbial infections by degrading the β-1,4 glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in the peptidoglycan of bacterial cell walls (Ringø et al. 2012). 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; Tyagi et al. 2007; Soonthornchai et al. 2010), 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 lysozyme transcription in the intestine and hepatopancreas of LAB probiotic-fed shrimps at 6 and 3 days post-feeding, respectively. Deng et al. (2012), in a 60-day experiment, exhibited higher lysozyme concentration in the hemolymph of *L. vannamei* fed with a yeast culture feed supplement (YK-6). This result coupling with our findings, therefore, indicates that lysozyme is elicited by different immunostimulating substances and acts as an integral component of the shrimp antibacterial defense mechanism.

The antioxidant enzyme, SOD, is involved in the removal of superoxide anions (O_2^-) by converting them into hydrogen peroxide and water, and the antioxidant enzymes, catalase, and glutathione peroxidase convert hydrogen peroxide into oxygen and water (Campa-Córdova et al. 2002; Holmblad and Söderhäll 1999). Therefore, these antioxidant enzymes finally provide a post-phagocytosis self-protection to the hemocytes of oxygen-respiring organisms (Lin et al. 2010). In our study, slightly higher SOD expression was noticed in the intestine of LAB probiotic-fed kuruma shrimps compared to control diet-fed shrimps at 6 days post-feeding. *L. vannamei* fed diets containing β-1,3-glucan also had significantly up-regulated cytosolic manganese SOD expression in hemocytes at 6 and 12 h and in the hepatopancreas at 12 h (Wang et al. 2008). These results suggest that glucan and LAB probiotic administered orally may enhance the release of superoxide anion, which would increase the immune capability and protect the host in conjugation with SOD activation. However, the response of SOD gene expression in our study was not acute and strong, which is contrary to the observation of dietary glucan supplementation resulting in the up-regulation of SOD activity at 24 h (Campa-Córdova et al. 2002). In agreement with our findings, healthy shrimps (*Litopenaeus stylirostris*) fed

with a probiotic (*Pediococcus acidilactici*) diet did not exhibit any obvious differences in SOD compared to control animals, emphasizing no effects of this probiotic under standard conditions in the absence of any stress (Castex et al. 2009). Similarly, no significant differences in SOD activities were established in hemocytes of shrimps (*L. vannamei*) fed with experimental diets incorporated with *Bacillus subtilis* (Tseng et al. 2009).

The proPO system is activated through recognition molecules in the hemolymph of invertebrates, and it recognizes and responds to invading agents via LPS or peptidoglycan (PG) from bacteria and β-1,3-glucans from fungi (Söderhäll and Cerenius 1998). Chiu et al. (2007) reported that up-regulation of proPO resulted in increased PO activity in shrimps fed with *Lactobacillus plantarum*-supplemented diet, which enhanced resistance against the pathogen, *Vibrio alginolyticus*. Likewise, white shrimps fed with a higher concentration (10^8 cfu kg^{-1}) of the probiotic (*B. subtilis*) exhibited significant increase in proPO activity (Tseng et al. 2009). The up-regulation of proPO was also recorded in white shrimps fed with *B. subtilis*-incorporated diets after challenge with *V. harveyi* (Zokaeifar et al. 2012). In our study, similar but weak up-regulation of proPO was recorded only in the intestine of kuruma shrimps fed with LAB-supplemented diet compared to the control group. This fact suggests that *Lactococcus lactis* D1813 may enhance the shrimp humoral immune response through elevation of the proPO system.

TLRs are single, membrane-spanning, non-catalytic pattern recognition receptors that recognize structurally conserved molecules derived from microbes (Li and Xiang 2012). Microbes are recognized by TLRs once they have ruptured the host's physical barriers, such as the cuticle or intestinal mucosa, and consequently immune cell responses are activated through signaling pathways (Kumagai et al. 2008). From our result of TLR expressions, TLR1 was slightly up-regulated in the intestine and hepatopancreas of LAB probiotic-fed kuruma shrimps at 6 days post-feeding. Kuruma shrimp TLR gene was expressed in various tissues including the gill, gut, lymphoid organ, heart, hematopoietic organ, hemocytes, ventral abdominal nerve cord, eyestalk neural ganglia, and brain in response to PG treatment (Mekata et al. 2008). The expression of TLR1 gene upon probiotic feeding indicates that the intestine, a prime route of pathogen intrusion, is able to respond to the invaders through activation of systemic innate immunity.

In several studies, dietary probiotic supplementation enhanced shrimp resistance to bacterial and viral pathogens (Chiu et al. 2007; Kongnum and Hongpattarakere 2012; Leyva-Madrigal et al. 2011). In kuruma shrimps, feeding of PG derived from *Bifidobacterium thermophilum* enhanced resistance to *V. penaeicida* as evident from higher survival in PG-fed shrimps than the control group (Itami et al. 1998). In our study, significant resistance to the virulent bacteria *V. penaeicida* in term of higher survival has been provided in kuruma shrimps by the oral administration of the LAB probiotic. *V. penaeicida*, which we used for disease resistance study, is a Gram-negative short rod-shaped bacterium with high pathogenicity to kuruma shrimps, displaying typical brown spots in lymphoid organs and gills and cloudiness of the sixth abdominal segment (Takahashi et al. 1998). The increased shrimp resistance to *V. penaeicida* infection, together with the increased expression of immune-related genes, corroborates that the probiotic diminished the population of this pathogenic strain in the shrimp intestine through competitive exclusion along with activation of the innate immune system.

The present findings of isolation and identification of LAB from kuruma shrimp intestine and dietary supplementation of a selected strain showing increased or induced expression of innate immune genes, enhanced resistance to a bacterial pathogen indicate the immunomodulatory property of the LAB probiotic *Lactococcus lactis* D1813 incorporated at 10^5 cfu g^{-1} diet. Therefore, it is an effective probiotic with a promising potential to control and prevent *V. penaeicida* infection due to its inhibitory and immunostimulant effects. An understanding on the effect of this probiotic on viral pathogens, especially WSSV, and the contribution of various cellular immune parameters in immune responses of shrimp may be the subject of further research.

Acknowledgments This work was supported by JSPS Asian CORE Program and Scientific Research (23580257) and grants from the Research and Development Program for New Bio-industry Initiatives, Japan. During this study, GB received a scholarship from the Ministry of Education, Culture, Sports, Science and Technology (Monbukagakusho), Japan.

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