Expression of innate immunity genes in kuruma shrimp *Marsupenaeus japonicus* after *in vivo* stimulation with garlic extract (allicin)

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ABSTRACT: In recent times, attention has focused on immunostimulants and plant products which could have beneficial effects in disease control. At present, the application of immunostimulants has been considered a more effective approach to health management in aquaculture through the enhancement of immune capability and disease resistance in shrimp. Garlic possesses bactericidal property against bacteria and can inhibit the growth of protozoa as well as gregarine infection in cultured shrimp. However, its effect on viral disease infection has not been well studied and requires further investigation. Garlic can also stimulate shrimp haemocytes as determined by cellular immune responses (phagocytic activity, superoxide anion production and phenoloxidase activity) suggesting that the immune-stimulatory properties of garlic will be useful for improvement of shrimp health. Here, we determined the expression of the immune-related genes Penaeidin, Crustin, Lysozyme, Toll-like, and tumour necrosis factor in kuruma shrimp, upon stimulation with allicin extract. The expression of these factors was measured for the first time and was found to be elevated in intestine and lymphoid organ after *in vivo* stimulation for 3, 12, 24 and 48 h. We conclude that garlic can be used in shrimp culture as an alternative to antibiotics or chemotherapeutic agents; however, further research is needed under field conditions.

Keywords: allicin; kuruma; *in vivo*; immune genes

The production of cultivated penaeid shrimp species has increased exponentially since the early 1970s. However, there is a rapidly increasing problem with serious disease outbreaks (Tanticharoen et al. 2008). Shrimp are vulnerable to a wide array of bacterial and viral pathogens. As shrimp lack an adaptive immune system, they rely on innate immune responses against microbial invasion (Lee and Soderhall 2002). A better understanding of the innate immune system of shrimp will undoubtedly help us to develop strategies in disease control and sustainable shrimp farming. The lymphoid organ and intestine of penaeid shrimps are thought to have immune function. The lymphoid organ exerts bacteriostatic effects, and is suggested to be the major phagocytic organ in shrimp (Van de Braak et al. 2002; Burgents et al. 2005). The intestine is a favourable site for invasion of pathogens carried in water, food, and sediment (Jayabalan et al. 1982). It was previously demonstrated that an influx of haemocytes enters the intestine of *P. monodon* following exposure to *V. harveyi*. Moreover, the haemocytes associated with the basal lamina of *S. ingentis* were reported to fight pathogens entering the body via the midgut (Chen et al. 1992).

Throughout history, garlic has been considered as a healing agent in many different cultures. It is still used in complementary and alternative medicine for a wide variety of illnesses. Allicin is the active substance of freshly crushed garlic and it is also responsible for the special strong odour of crushed garlic. Allicin was reported to exert different biological functions including antibacterial, antiviral, anti-parasitic and antifungal activities (Ankri and Mirelman 1999). Interestingly, allicin has radical scavenging properties in activated granulocytes

and may also inhibit inducible nitric oxide synthase expression in activated macrophages (Dirsh et al. 1998). Previous research suggested that the above mentioned activities are mainly attributed to the bioactive components of garlic, including sulphur-containing compounds, such as allin, diallysulphides and allicin (Amagase et al. 2001). Allicin (diallythiosulfinate) is the most abundant compound representing about 70% of all thiosulfinates present, or formed in crushed garlic (Block et al. 1992). It is produced by the interaction of the non-protein amino acid allin (= S-allyl-ʟ-cysteine sulfoxide), with the enzyme alliinase (Cavallito et al. 1944).

Screening for efficacy of garlic

Bacteria and fungi. The broth dilution assay and the disc or agar well diffusion assay are the commonly used *in vitro* methods for initial screening of the potential antibacterial properties of a medicinal plant (Cowan 1999). Subsequently, more detailed studies into their antibiotic effects are conducted by determining the minimum inhibitory concentration (MIC) compared to currently used antibiotics. The MIC value of fresh garlic against seven Vibrio strains along with the standard bacteria *V. cholerae ATCC 14035* and *E. coli ATCC 25922* was determined using the disc diffusion method. It showed good bactericidal potent against all seven strains of tested bacteria with MIC values of 0.156 to 0.312 mg/ml as shown in Table 1 (Kasornchandra et al. 2005). Garlic has revealed its huge potential as an antimicrobial agent against pathogenic fish

bacteria (Wei and Najiah 2009). Garlic has a broad-spectrum effect and contains an active compound, allicin, which acts upon various enzymes that can affect the metabolism of virulent bacteria (Ankri and Mirelman 1999).

Much research has been conducted on the inhibitory effects of garlic on the principal pathogenic bacteria of freshwater fish, including *P. fluorescens*, *M. piscicola*, *E. tarda*, *Aeromonas hydrophila*, *A. punctata f. intestinalis*, *Streptococcus agalactiae*, and *Staphylococcus aureus* (Lee and Yang 2012).

Fungi and virus. Garlic has been reported to possess anti-fungal and anti-viral properties (Yoshida et al. 1987; Werber et al. 1992). In fact, fresh garlic extract was proven to exert *in vitro* virucidal effects against viral infection in human cells. However, the effects of garlic against viral infection in shrimp have not been studied. Since virus infection is the major problem for shrimp culture, the virucidal effect of garlic in shrimp warrants investigation.

Parasites and protozoans. The efficacy of fresh garlic paste for reducing the number of parasites (gregarines) in the midgut of black tiger shrimp was tested by mixing 10 g of fresh garlic paste with 1 kg of commercial feed, coated with 20 ml of chitosan. This was fed to shrimp in three earth ponds for five weeks. The shrimps were sampled before the start of feeding the diet containing garlic and every week thereafter. Twenty shrimps each were examined using histological techniques to determine the number of gregarines in the intestinal tract of cultured shrimp (Chatchawanchaipan et al. 2004). The number of shrimp infected with gregarines was 100% reduced after feeding the garlic-containing diet for four weeks (Table 2).

Table 1. The MIC values of fresh garlic against different strains of bacteria (*Vibrio* spp.)

Bacterial strains	Minimum inhibitory concentration (MIC, mg/ml)
V. harveyi	0.156
V. parahaemolyticus	0.312
Photobacterium damselae subsp. *damselae*	0.156
V. alginolyticus	0.312
V. vulnificus	0.156
V. pelagius II	0.156
V. minicus	0.156
V. cholerae ATCC 14035	0.156
E. coli ATCC 25922	0.312

Table 2. Percentages of shrimp infected with gregarines before and after feeding of garlic paste mixed diet (10 g fresh garlic paste was mixed with 1 kg commercial feed, coated with 20 ml of chitosan, and fed to shrimp in three earth ponds for five weeks)

Duration (weeks)	Shrimp with gregarines (%)		
	pond No.1	pond No.2	pond No.3
Before feeding garlic paste mixed diet	100	90	85
	100	100	40
1	30	65	10
2	10	15	10
3	0	15	15
4	0	0	0
5	0	0	0

Immunostimulatory effects

In vitro phagocytosis is one of the screening methods used for the detection of immune-stimulating compounds (Wagner 1990). The effects of fresh garlic extract on phagocytic activity of *P. monodon* haemocytes was tested *in vitro* at the Coastal Aquatic Animal health Research Institute. Higher phagocytic activity was found in haemocytes treated with garlic extract (78.7%) compared to control cells without pre-incubation with garlic extract (64.1%). Application of garlic in fish farming has become popular for enhancing the activity of defence systems, conferring protection against diseases and because of its growth promoting properties (Nya and Austin 2009).

At present, little is known about the response of fish and shellfish to allicin. This study was undertaken to examine the immune response of Kuruma shrimp when stimulated with pure allicin. Several innate immune-related genes were examined including MjCrus, MjPEN, MjLyz, MjTNF, and MjToll.

MATERIAL AND METHODS

Experimental shrimps. Specific pathogen free (SPF) kuruma shrimp (mean body mass 10 ± 1 g) were obtained from Matsumoto Fisheries, Miyazaki, Japan. Shrimp were maintained in an indoor system with re-circulating artificial seawater at 20 °C and fed with commercial diets once a day. Three groups (*n* = 10 each) of *M. japonicus* were kept in a tank supplied with artificial seawater and continuous aeration at 20 °C. The shrimp were fed with a commercial diet (Higashimaru, Japan) at 1% of body weight per day for a week.

Allicin. Allicin as Allimed® liquid was obtained from Allicin International. The product comprised pure allicin in liquid form and aqua and allicin liquidum with a maximum stated allicin content of around 1000 mg/l. In our previous *in vitro* study, the head kidney of carp was stimulated with 6 μg/μl, 12 μg/μl, 25 μg/μl, 50 μg/μl of pure allicin. The lower concentration of pure allicin 6 μg/μl resulted in a significant increase in cytokine production as compared to the control group (unpublished data). Therefore, we decided to use 6 μg/μl of pure allicin for the shrimp experiments.

In vivo **stimulation**. The *in vivo* effects of allicin on kuruma shrimp were tested in the following way: each shrimp was injected with 6 μg/μl of pure allicin dissolved in 100 μl of PBS while the control group was injected with 100 μl of PBS only. Intestines and lymphoid organs were collected from three shrimps and then immediately pooled. This sampling was performed in triplicate. Intestine and lymphoid organ samples of each group of shrimps were collected at 0 h, 1 h, 4 h, 8 h, and 12 h post-injection.

Expression analysis of innate immune-related genes using semi-quantitative RT-PCR. Total RNA

Table 3. Primers sequences used in this study with the accession numbers of each gene and the optimal conditions for PCR

Primers	Sequence (5'-3')	Cycles	Ann-Temp	Amplicon size	Access. No.
MjLyz-F	TCCTAATCTAGTCTGCAGGGA	35	58	512	AB080238
MjLyz-R	CTAGAATGGGTAGATGGA				
MjPen-F	GCTGCACCCACTATAGTCTTT	30	60	339	AU175636
MjPen-R	CTACCATGGTGATGAAACAAA				
MjCrus-F	CATGGTGGTGGCTTAGGAAA	35	62	300	AB12174
MjCrus-R	GTAGTCGTTGGAGCAGGTTA				
MjToll-F	TCTTTCTGGTGTTTTAGCTACTGTAA	30	60	300	AB333779
MjToll-R	TTTGATGAGAGCACGACAATG				
MjTNF-F	AAGAAAACCCCCAGGAAGAA	30	60	324	MNM_165735
MjTNF-R	AACCAGTGTGCACTCCATGA				
MjEF1-α-F	GTCTTCCCCTTCAGGACGTA	25	55	373	AB458256
MjEF1-α-R	GAACTTGCAGGCAATGTGAG				

was extracted from the lymphoid organ and intestine of kuruma shrimp using ISOGEN (Nippon Gene, Japan) in accordance with the manufacturer's instructions. The amount of nucleic acid in the total RNA was determined by measuring the absorbance at 260 nm using a Nano Drop spectrophotometer, ND-1000 (Thermo Scientific, USA). The purity of the total RNA was confirmed by measuring the ratio of OD 260 nm/OD 280 nm. cDNA was synthesised from 1.0 mg of total RNA using a ReverTra Ace qPCR RT kit (Toyobo, Japan) following the manufacturer's instructions and used as a template for polymerase chain reaction (PCR). The EF-α1 gene was used as an internal control. All PCR reactions were performed according to the following protocol: 1 μl cDNA was mixed with 5 μl buffer, 5 μl dNTPs (10μM each dNTP), 0.5 μl *Taq* polymerase (5 IU/μl), 5 μl each of gene-specific primer (5μM), and 28.5 μl distilled water. The immune-related genes, the designed primers and optimised conditions are shown in Table 3. PCR products were analysed using 1.5% agarose gel electrophoresis, followed by staining with ethidium bromide, and visualisation under a trans-illuminator. The expected amplicon sizes of all products are listed in Table 3. The immune-related gene/EF-1α ratio was determined using densitometry, by measuring the photostimulated luminescence values using Science Lab99 Image Gauge software (Fujifilm, Tokyo, Japan).

Statistical analysis. The data obtained from the RT-PCR analysis were subjected to one-way analysis of variance (ANOVA) using SPSS software 14 followed by Tukey's test. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Transcription of shrimp immune genes after stimulation with allicin

Time-course reverse transcriptase polymerase chain reaction (RT-PCR) assays were used to investigate the transcriptional regulation of six shrimp immune-related genes during stimulation with allicin. For this assay, the expression levels of individual genes were first normalised using EF-α1 as an internal control and then expressed relative to the basal expression in the 0 hpi sample, which was collected in the control group. Results are arranged below according to the respective gene's roles in the six main shrimp defence mechanisms.

The Penaedin gene was significantly up-regulated in lymphoid organ at 12, 24 hpi and at 3, 12 hpi in intestine. The expression level of lysozymes was significantly increased at 12, 48 hpi in lymphoid organ and at 12 hpi in intestine. Crustin was up-regulated significantly at 12 hpi in lymphoid organ and at 3, 48 hpi in intestine. The Toll gene was up-regulated significantly early at 3, 12, 24 hpi in lymphoid organ and non-significantly in intestine at 48 hpi. The expression level of the TNF gene was significantly increased at 12, 48 hpi in lymphoid organ and in intestine at 3, 12, 24 and 48 hpi.

The effects of immunostimulants such as glucan, chitin and other polysaccharides have been widely studied in crustaceans (Song and Huang 1999). Administration of β-1,3 to 1,6-glucan extracted from the yeast *Saccharomyces cerevisiae* by immersion has been reported to increase the phenoloxidase activity of tiger shrimp, *Penaeus monodon* (Sung et al. 1994). Oral administration of schizophyllan, a β-1,3-glucan extracted from the fungus Schizophyllum commune, has been reported to increase immune function in *P. monodon* (Liao et al. 1996). It is known that the immune parameters of shrimp receiving immunostimulants are augmented once the shrimp are infected by a pathogen. The administration of hot-water extracts of the red seaweeds *Gracilaria tenuistipitata* and *Gelidium amansii* via injection was reported to enhance the immune function of white shrimp (Hou and Chen 2005; Fu et al. 2007). Furthermore, the protective effects of allicin incorporated into commercial fish feed have been reported in rainbow trout (Nya et al. 2010). This study describes for the first time the expression of shrimp innate immune response genes upon stimulation with the garlic extract allicin.

Antimicrobial peptides (AMPs) are essential effectors in the innate immune response in most organisms. Penaeidins are constitutively synthesised and stored in shrimp haemocytes, localised in granulocyte cytoplasmic granules, and released in response to appropriate stimuli such as infections (Destoumieux et al. 2000). It has been shown that the expression of the MjPEN gene was significantly increased in the lymphoid organ and intestine of kuruma shrimp after injection of DNA vaccine encoding viral envelope protein VP28 of penaeid rod-shaped DNA virus (PRDV) (Kono et al. 2009).
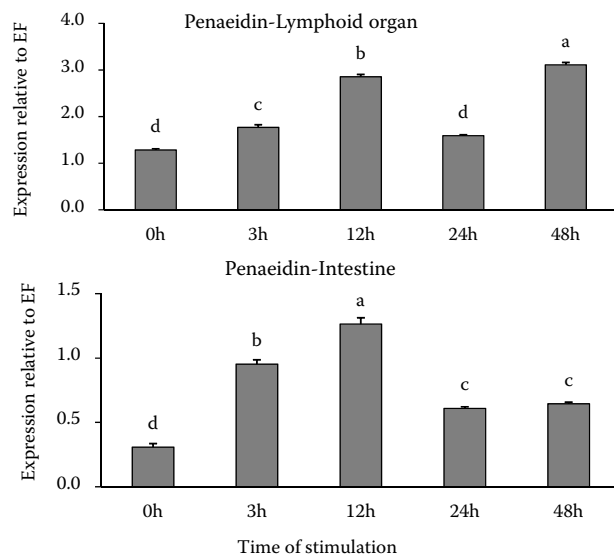
Figure 1. Expression pattern of the shrimp penaeidin gene in the intestine and lymphoid organ at 3, 12, 24 and 48 h post-allicin stimulation. Data are presented as mean ± SD of triplicate samples. Relative expression was normalised to elongation factor-1α (EF-1α) and the basal expression level at 0 hpi. Letters a, b, c, d indicate significant up-regulation of the target gene in the allicin-treated group compared to the control group at the same time point ($P < 0.05$)

Similarly, our results show that the expression of MjPEN in the intestine was significantly increased in both intestine and lymphoid organ as compared to the control group. These results suggest that penaeidins play a major role as effectors of shrimp immune defence as early as 3 hpi (Figure 1).

Crustins are antibacterial proteins found in numerous crustacean species (Rattanachai et al. 2004). They contain a four-disulphide core (4DSC) or a whey acidic protein (WAP) domain. To assess the efficiency of DNA vaccine in kuruma shrimp, the expression of MjCrus in vaccinated shrimp was examined. The results suggested that MjCrus was significantly increased in the lymphoid organ and intestine of kuruma shrimp after DNA vaccination (Kono et al. 2009). Similarly, in the current study, the *in vivo* expression of MjCrus in lymphoid organ was significantly increased at 1 hpi. Further, the expression of MjCrus in the intestine was significantly increased early at 3, 12 hpi in intestine and at 12, 48 hpi in Lo as compared to the control group (Figure 2). Thus, crustin does appear to play an essential role in the kuruma shrimp innate immune system.

Lysozyme is widely distributed among eukaryotes and prokaryotes and is considered to be an integral component of the innate immune system which protects against microbial infections (Ji et al. 2009). Lysozyme catalyses the hydrolysis of bacterial cell walls and acts as a non-specific innate immunity factor, which protects against the invasion of bacterial pathogens (Jolles and Jolles 1984). Lysozyme-like activity was determined in Chinese shrimp haemocytes 48 h after immune stimulation with laminarin. The results showed that haemocyte lysozyme-like activity started to increase at 45 min after injection, peaked at 3 h, and high activity lasted for 48 h (Mekata et al. 2008). Recently, it was found that the expression of Mjlyz was significantly increased in the lymphoid organ and intestine of kuruma shrimp after DNA vaccination (Kono et al. 2009). These results are consistent with our study in which the expression analysis revealed that MjLyz was significantly increased at 3 hpi only in both the lymphoid organ and intestine as compared to the control group (Figure 3). All these results suggest that lysozyme is an important component of the
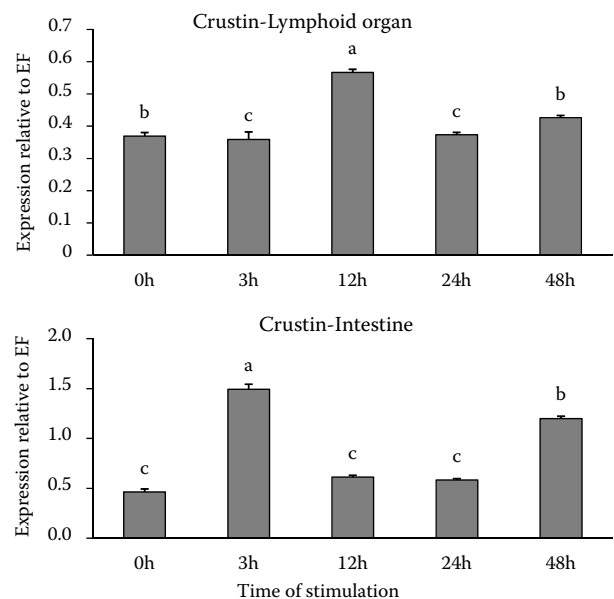

Figure 2. Expression pattern of the shrimp crustin gene in the intestine and lymphoid organ at 3, 12, 24 and 48 h post-allicin stimulation. Data are presented as mean ± SD of triplicate samples. Relative expression was normalised to elongation factor-1α (EF-1α) and the basal expression level at 0 hpi. Letters a, b, c, d indicate significant up-regulation of the target gene in the allicin-treated group compared to the control group at the same time point ($P < 0.05$)
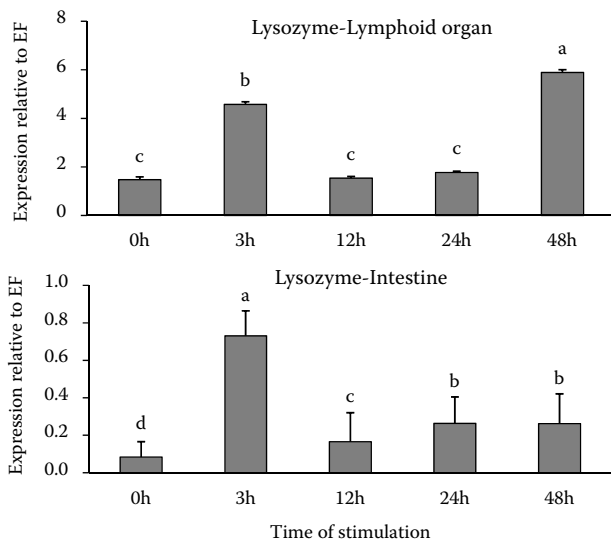
Figure 3. Expression pattern of the shrimp lysozyme gene in the intestine and lymphoid organ at 3, 12, 24 and 48 h post-allicin stimulation. Data are presented as mean ± SD of triplicate samples. Relative expression was normalised to elongation factor-1α (EF-1α) and the basal expression level at 0 hpi. Letters a, b, c, d indicate significant up-regulation of the target gene in the allicin-treated group compared to the control group at the same time point ($P < 0.05$)

shrimp defence system. Further, these data are in agreement with the findings of Woo et al. (2010) who found that the expression of non-specific immune defence factors in olive flounder, *Paralichthys olivaceus*, were significantly elevated after either injection of a 5% garlic extract or immersion in 0.25 g/l garlic juice. In a challenge infection experiment with *Streptococcus iniae* and *E. tarda*, the relative percentage survival values were markedly higher in the 5% garlic extract pre-injected group and 0.25 g/l garlic juice immersed group than in the other groups tested, respectively.

Tolls and Toll-like receptors (TLRs) are recognised as major Pattern Recognition Receptors (PRRs). They are involved in the signalling pathway for the activation of innate immunity and are evolutionarily conserved from insects to mammals (Tanekhy et al. 2010). Upon *in vitro* immunostimulation of shrimp lymphoid organ tissue, a significant increase in MjToll expression after stimulation with PG at 9 and 12 h was detected. However, the expression of the MjToll gene was not modulated by treatment with LPS, CpG DNA, Flagellin, Imiquimod, or Poly I : C (Mekata et al. 2008). Similarly, our results showed that MjToll

expression in the intestine was non-significantly increased from 3 to 48 hpi as compared with the control group, while a significant increase in lymphoid organ could be detected starting at 3 hpi (Figure 4). Taken together, these results suggest that MjToll might be involved in innate host defence of kuruma shrimp.

TNFs are potent inflammatory cytokines implicated in inflammation, apoptosis, cell proliferation, and in stimulation of various aspects of the immune system involved in prophylaxis against pathogens. Recently, it has been demonstrated the MjTNF expression is induced in response to LPS stimulation in the gill tissue of kuruma shrimp. In the LPS-injected group, a high expression level of the MjTNF gene was observed at 2 h, compared to non-treated shrimp, which decreased thereafter (Fall et al. 2010; Mekata et al. 2010). In the present study, the expression level of MjTNF in the intestine was significantly increased in the intestine at 3, 12 and 48 hpi as compared to the control group while it significantly increased at 12 and 48 hpi in the lym-
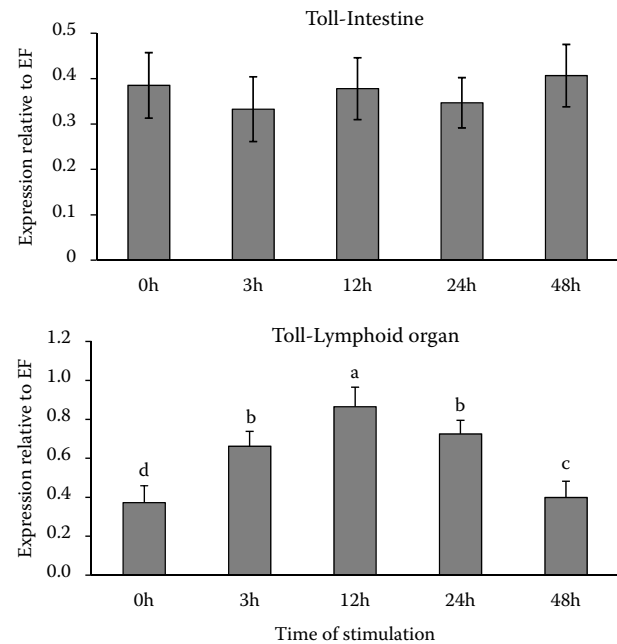

Figure 4. Expression pattern of the shrimp toll-like gene in the intestine and lymphoid organ at 3, 12, 24 and 48 h post-allicin stimulation. Data are presented as mean ± SD of triplicate samples. Relative expression was normalised to elongation factor-1α (EF-1α) and the basal expression level at 0 hpi. Letters a, b, c, d indicate significant up-regulation of the target gene in the allicin-treated group compared to the control group at the same time point ($P < 0.05$)
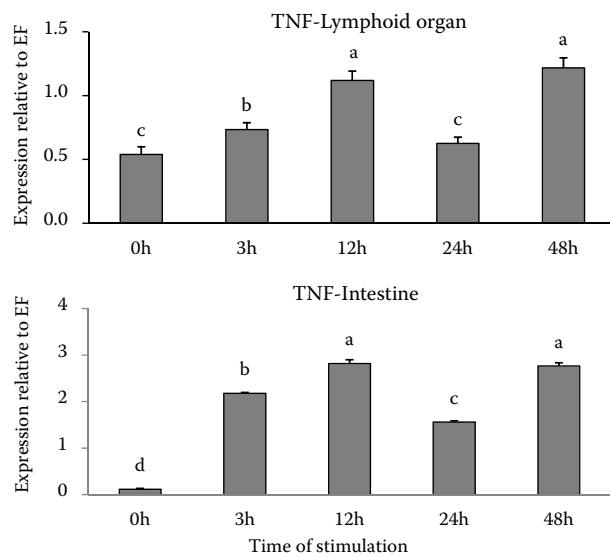
Figure 5. Expression pattern of the shrimp tumour necrosis factor gene in the intestine and lymphoid organ at 3, 12, 24 and 48 h post-allicin stimulation. Data are presented as mean ± SD of triplicate samples. Relative expression was normalised to elongation factor-1α (EF-1α) and the basal expression level at 0 hpi. Letters a, b, c, d indicate significant up-regulation of the target gene in the allicin-treated group compared to the control group at the same time point ($P < 0.05$)

phoid organ (Figure 5). Our results thus confirm the existence of TNF genes in kuruma shrimp and suggest that MjTNF might play a role in the innate immune defence of shrimp.

In conclusion, the obtained results suggest that quantification of the expression levels of MjCrus, MjLyz, MjPEN, MjToll, and MjTNF is useful for evaluating the immune status of *M. japonicus*. Our understanding of the innate immune response of shrimp when stimulated with allicin remains in its early stages. On the basis of these results, it will be of great interest to determine the expression profiles of these innate immune-related genes in *M. japonicus* tissues in response to *in vivo* stimulation with allicin and the subsequent resistance to *V. nigripulchritudo*. The use of garlic in aquaculture can augment growth and antimicrobial capabilities, act as a tonic for the immune system, stimulate appetite and improve anti-stress protection. Further, the use of garlic will reduce the side effects and costs associated with the application of synthetic antibiotics and will also be an eco-friendly measure. Alternative garlic bio-medicines could enhance environmentally friendly production and sustain-

able development in shrimp culture. However, the effect of allicin on shrimp is still not completely characterised and further experiments directed at clarifying its antioxidative and immunostimulatory effects are currently ongoing.

Acknowledgement

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