

# Immunomodulatory effects of turmeric, *Curcuma longa* (Magnoliophyta, Zingiberaceae) on *Macrobrachium rosenbergii* (Crustacea, Palaemonidae) against *Vibrio alginolyticus* (Proteobacteria, Vibrionaceae)

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**Abstract.** *Macrobrachium rosenbergii* is being favored for farming due to its large size. However, like other crustaceans, it is also prone to bacterial and viral infections. Turmeric, a derivative of the plant *Curcuma longa*, is a spice commonly used in Middle East and Asia as an herbal remedy. Immuno-modulatory effects of turmeric powder on hemocyte population and expression of antimicrobial peptides (AMPs) of *M. rosenbergii* challenged with *Vibrio alginolyticus* was investigated. Eighty (80) juveniles of *M. rosenbergii* were divided into three groups labeled as D0, D1 and D7 (Day 0, 1 and 7 respectively). D0 were fed with commercial feeds while D1 and D7 were fed with turmeric-incorporated (enhanced) feeds for one and seven days respectively. The total hemocyte count (THC) of D0 remained constant and a significant increase was observed from D1 to D7 treatment. Prawns were challenged with *V. alginolyticus* and total RNA was isolated and synthesized into cDNAs from hepatopancreas. RT-PCR was performed with crustin and lysozyme as target genes and EF-1 $\alpha$  as the reference gene. PCR products were run through 1% agarose gel electrophoresis. Semi-quantitative RT-PCR revealed an increasing expression of crustin and lysozyme PCR relative to duration of feeding, indicating a remarkable increase in the expression of AMPs (antimicrobial peptides). Challenged prawns fed with enhanced feeds also had an induced expression of AMPs. It is noteworthy to mention that this is the first report on AMPs expression in *M. rosenbergii*.

**Key Words:** shrimp biotechnology, *Curcuma longa*, *Macrobrachium rosenbergii*, crustin, lysozyme.

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## Introduction

The Philippines and other Asia-Pacific countries play a major role in the world aquaculture shrimp trade industry. Shrimp farming has been promoted because of its economic importance but, in the past few years, the industry has been plagued with environmental pollution, degradation and pathogen proliferation, resulting in poor production and mass mortality due to diseases. One of the important species being cultured nowadays is *Macrobrachium rosenbergii* (De Man, 1879) or giant freshwater prawn (Kennedy *et al* 2006). It is the largest species in its genus, thus being favored for farming and suited for inclusion in polyculture systems, and in integrated aquaculture–agriculture (Nhan *et al* 2009). However, this species like any other commercially farmed shrimps is also prone to viral and bacterial infection. *Vibrio alginolyticus* Sakazaki, 1968 is

an opportunistic pathogen that causes mortality in shrimp especially under poor environmental conditions (Tseng & Chen 2004; Chen & Hou 2005). Penaeid shrimps have been found to have antimicrobial peptides (AMPs) which are key factors of their innate immunity (Okumura 2007). These are considered as the front line host defense against pathogens. While several AMPs have been reported in different species of shrimp (Zhao & Wang 2008), this is the first report on the expression of these molecules in a freshwater shrimp species.

Other innate immune response of crustaceans include melanization, cytotoxic reactions, cell adhesion, encapsulation, nodule and capsule formation, hemocyte locomotion and phagocytosis (Lee & Soderhall 2002). Among these responses, crustacean

hemocytes are also known for their significant function in foreign body removal (Burge et al 2007). In this study, the total hemocyte count (THC) of *M. rosenbergii* is observed before and after bacterial challenge with *V. alginolyticus*.

Turmeric, a derivative of the plant *Curcuma longa* Linnaeus, 1758 is a spice commonly used in Middle East and Asia as an herbal remedy (Tayyem et al 2006). Its main yellow bioactive substances are due to curcumin and two related demethoxy compounds, demethoxycurcumin and bisdemethoxycurcumin (Pothitirat & Gritsanapan 2006). Several studies found turmeric to have anticarcinogenic, antioxidant, anti-inflammatory, anti-allergy, anti-mutagenic (Suresh et al 2006), immunomodulatory properties (Varalakshmi et al 2008; Yue et al 2010), antiviral and antibacterial activities (Singh et al 2010).

This paper accounts for the first time in freshwater shrimp, the immuno-modulatory effects of dried turmeric powder on the hemocyte population and expression of AMPs in *M. rosenbergii* against *V. alginolyticus* by incorporating them on commercial shrimp feeds.

## Material and Methods

Eighty juvenile of *M. rosenbergii* obtained from Southeast Asian Fisheries Development Center (SEAFDEC), Binangonan, Rizal (14° 24' 48.84" N 121° 13' 2.93" E) (SEAFDEC/AQD-BFS [map]) were acclimated for seven days (August, 2010) in a 122 L aquarium for 24 h with water temperature ranging 26–29°C. Shrimps were fed with commercial feeds prior to all experimental procedure. Then healthy shrimps were chosen for the entire experiment.

Eight kg of *C. longa* purchased from Pasig Public Market were sent to the UST Herbarium for proper identification. These were cut into smaller pieces, oven-dried for three days and pulverized. Three concentrations: 10%, 25%, and 50% turmeric powder were incorporated into commercial feeds with 2% starch and the mixture was molded into strips, oven-dried at 40°C for two days to produce pellets. The enhanced feeds produced were fed separately to three different sets of prawns to determine the optimum concentration to be used for the entire experiment (data not shown). The 25% turmeric incorporated feed (TF) was observed to be preferred by the prawns, thus, it was used for the treatment. One set of shrimps were fed with commercial feed only (D0) while two different sets were fed with TF for only a day (D1) and for seven days (D7).

### Total hemocyte count

On the 1st and 7th day of feeding, 0.1 mL hemolymph sample from each prawn (3 prawns per sampling) were extracted from the ventral side of tergum using a 1.0 mL syringe containing 0.9 mL anticoagulant sodium citrate and was then transferred to a 1.5 mL microcentrifuge tube. The extracted hemolymph was centrifuged at 800 rpm for 10 min at 4°C. The supernatant was discarded and the pellets containing the hemocytes were diluted with 200 µL of 0.9% NaCl. One hundred µL of the hemocyte solution was stained with 100 µL Giemsa stain and was mixed thoroughly using the vortex. The hemocytes were counted using a Neubauer hemocytometer under a light microscope using

standard procedures. The total number of cells counted in the four corner squares was calculated using the formula: Number of cells/mL = total hemocyte count x 104) in determining the final cell count per mL (Veile 1990).

### Bacterial challenge

To determine the immune-modulatory effect of turmeric, prawns fed with the TF were challenged with *V. alginolyticus* obtained from the Philippine National Collection of Microorganisms (PNCM) of the University of the Philippines, Los Baños (UPLB). Bacterial cells were prepared by growing the cultures in nutrient agar medium overnight at 37°C. A single colony of *V. alginolyticus* was transferred from nutrient agar plate to 2 mL of nutrient broth (NB) with 3% NaCl, and was incubated overnight to produce a bacterial stock. One mL bacterial stock was diluted to 1 L NB with 3% NaCl. This was incubated overnight on a horizontal shaker. After incubation, the culture suspension was serially diluted 10-fold with phosphate buffer solution (PBS). Bacterial concentration was found to have 8.9 x 10<sup>6</sup> CFU/mL (colony forming units per mL) through pour plate method of colony counting. One liter of bacterial solution was then placed in tanks with 4 L water. The tank was divided into three chambers to separate the shrimps according to the number of days they were fed with enhanced feed. After three hours of bacterial exposure, the shrimps were washed in a tank with dechlorinated water, and placed on separate tanks according to their grouping. Cumulative mortality for each group was observed for seven days.

### Gene expression

Relevant tissues (gills, heart, hemocyte, hepatopancreas, and muscle) were dissected from three shrimp samples from each set (D0, D1 and D7). Total RNA was isolated using Trizol (Invitrogen, USA) following the manufacturer's instructions and was quantified through UV spectrophotometry. One µg of each sample was reverse transcribed to produce cDNA with M-MLV transcriptase (Invitrogen, USA) and 10 units of RNase inhibitor (Invitrogen, USA) following the manufacturer's recommendation. RT-PCR was performed using gene specific primers for crustin, lysozyme and EF-1α (Elongation-Factor 1 α) as standard control (Table 1).

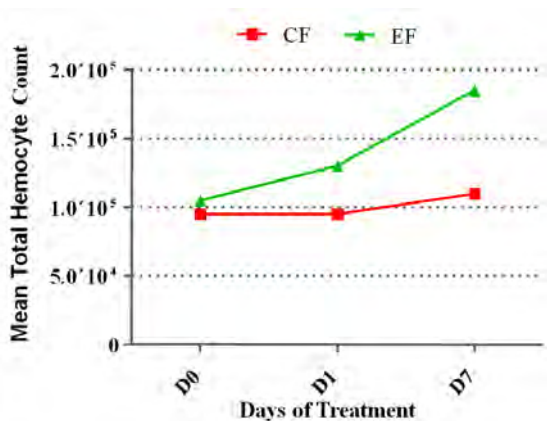
Transcripts were visualized and analyzed using AlphaImager MINI gel documentation system. Semi-quantitative RT-PCR for crustin and lysozyme is calculated as the ratio of crustin and lysozyme to EF-1α level of expression.

### Statistical analysis

All data were analyzed using one-way analysis of variance (ANOVA) using the statistical software GraphPad Prism 5.

**Table 1.** Primers used for RT-PCR

Primer	Sequence	Expected length
Crustin	F 5'-TGTTCCACGACTTCAAGTGTGC-3' R 5'-CAAAGATTCAACTAAATAAACAG-3'	299 bp
Lysozyme	F 5'-ATGTGGAATCTGAAGGACTTGT-3' R 5'-CCAGTATCCAATGGTGTAGGG-3'	368 bp
EF-1α	F 5'-ATGGTTGTCAACTTTGCCCC-3' R 5'-TTGACCTCCTTGATCACACC-3'	500 bp



**Figure 1.** Mean total hemocyte count (THC) of prawns fed with the commercial feed (CF) and with the turmeric-incorporated feed (TF) for Days 0 to 7.

## Results and Discussion

The invertebrate immune system must rely on non-self-recognition molecules to ensure efficient defense responses against infectious pathogens that continuously threaten their survival. In the last three decades, the country's shrimp aquaculture industry has been in quest to improve feed meal in the hope to enhance the shrimp immune system. It is in this light, that *C. longa* crude powder extract was evaluated as feed additive by THC, and expression of two AMPs, crustin and lysozyme against bacterial infection.

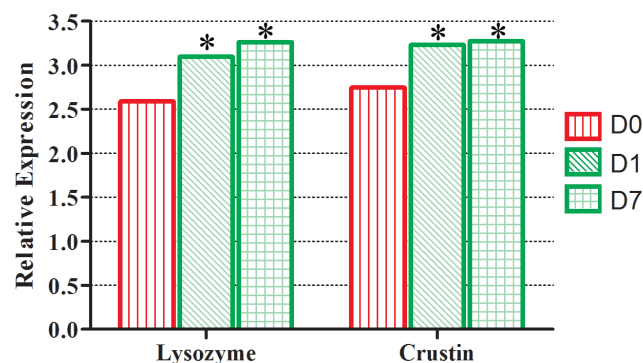
THC can be considered an indicator of shrimp health status (Perazzolo *et al* 2002; Sritunyalucksana *et al* 2005) because hemocytes contain several proteins that are involved in the defense responses of the crustacean system (Johansson *et al* 2000). The mean THC of prawns fed with only commercial feeds (D0) remained constant from Day 0 to 7. Meanwhile, the mean THC of prawns fed with TF for seven days (D7) gradually increased from Day 0 to 1 and there was a considerable increase from Day 1 to 7 (Figure 1). This finding corroborates the recent study of Lawhavinit *et al* (2011) where ethanol turmeric extract was found to increase THC count in *Litopenaeus vannamei* (Boone, 1931).

Production of AMPs is a first-line host defense mechanism of innate immunity. These are widely distributed in the whole living kingdom, and they are thought to be essential for organisms lacking adaptive immunity (Amparyup *et al* 2008). Studies concerning their expression in crustaceans during bacterial challenge are therefore essential to better understand the host defense responses (Sperstad *et al* 2010). During the past years, a number of AMPs have been identified and characterized from crustaceans (Pisuttharachai *et al* 2009). However, there is dearth of information on AMPs in the freshwater shrimp *M. rosenbergii*. Total RNAs from the hepatopancreas of three shrimps from each set (D0, D1 and D7) were used for the entire experiment since it exhibited the best quality after quantifying through UV spectrophotometry. Semi quantitative RT-PCR showed that there is an increasing expression of crustin and lysozyme expression (as measured by AlphaImager MINI gel documentation software) with respect to the length of feeding time (Figure 2), indicating that shrimps fed with TF after bacterial challenge induced

expression of the mentioned AMPs. Furthermore, a significant increase in the expression of the two AMPs was observed between D0 and D1 ( $p < 0.05$ ), and D0 and D7 ( $p < 0.05$ ). Data showed that dried turmeric powder induced the expression of antimicrobial peptides, particularly crustin and lysozyme, of *M. rosenbergii* when challenged with a pathogen such as *V. alginolyticus*. The findings also support other studies regarding the beneficial effects of turmeric extracts on immune cells (Harikrishnan *et al* 2011; Luo *et al* 2011).

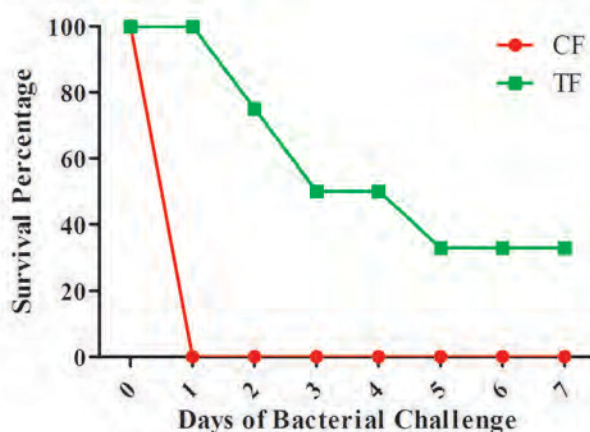
Crustins has been highlighted to have antimicrobial activity against Gram-positive bacteria according to Brockton *et al* (2007) and absence of which increased mortality in *L. vannamei* challenged with *Vibrio penaeicida* (Ishimaru, Akagawa-Matsushita & Muroga, 1995) according to Shockey *et al* (2009). Among the various AMPs, lysozyme is the most well-known and has been described in numerous phylogenetically diverse organisms such as bacteria, bacteriophages, fungi, plants and animals (Supungul *et al* 2010). It has been widely accepted as a crucial biodefense reactor during innate immunity (Zhao *et al* 2007). Increasing evidences have also demonstrated that lysozyme is highly relevant in shrimp's bacterial defense response.

After exposure to *V. alginolyticus*, all prawns fed with only commercial feeds (D0) died immediately. Hemocytopenia might have occurred, a condition where hemocytes are depleted which is also observed in other crustaceans and occurs within 12, 24 and 48 hours of infection by a pathogen (Li *et al* 2008). Meanwhile, prawns fed with the enhanced feed for seven days (D7) withstood the bacterial infection after Day 1 with 100% survival. The survival rate decreased but remained constant at Day 3 and Day 4 (50%). Moreover, 15% of the prawns survived for seven days (Figure 3). This is in conjunction with the study of Yano *et al* (2006) which listed turmeric as a spice that exhibits antibacterial activities against *Vibrio parahaemolyticus* (Sakazaki, Iwanami & Fukumi, 1963). A recent study reported that ethanol and hexane turmeric extract showed inhibitory effects against 13 bacteria, including *V. alginolyticus*, isolated from shrimp and chicken (Lawhavinit *et al* 2010). Meanwhile, studies regarding the immunomodulatory activity of turmeric extracts are slowly



**Figure 2.** Semi-quantitative RT-PCR of lysozyme and crustin in shrimp fed with commercial feed (D0) and turmeric-incorporated feed for one day (D1) and for seven days (D2) (\* $p < 0.05$ ).





**Figure 3.** Percentage of *M. rosenbergii* fed for seven days (D7) with enhanced feeds (EF) and with turmeric incorporated feed (TF) that survived after bacterial challenge for seven days.

emerging. Immunomodulation means that one can modulate or regulate immunity using various substances whether of natural or synthetic origin (Wybran 1988). Varalakshmi (2008) identified that curcumin from turmeric extracts provides the immune cells with an improved proliferative capacity that results in a stronger immune response under pathological conditions. Furthermore, curcumin has been shown to be a potent immunomodulatory agent that can modulate the activation of T cells, B cells, macrophages, neutrophils, natural killer cells, and dendritic cells (Allam 2009).

## Conclusions

Taken all together our findings indicate that dried turmeric powder has the capacity to modulate the antimicrobial peptides, particularly crustin and lysozyme, of the shrimp *M. rosenbergii* when challenged with *V. alginolyticus*. Addition of turmeric extract into commercial feeds also increased survival rate of shrimps challenged with a bacterial pathogen. Protective effect of the turmeric against viral infection will be worth pursuing and the plant can be exploited by the shrimp aquaculture industry for shrimp feed formulation.

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