

Influences of Cultivation Conditions on Microbial Profiles of Pacific White Shrimp (*Litopenaeus vannamei*) Harvested from Eastern and Central Thailand*

Matichon Lokkhumlue and Cheunjit Prakitchaiwattana*

Department of Food Technology, Faculty of Science, Chulalongkorn University, Bangkok 10300, Thailand

*Corresponding author. E-mail: pcheunjit@chula.ac.th

ABSTRACT

*This study investigated influences of cultivation conditions on microbial profiles of Pacific White Shrimp (*Litopenaeus vannamei*) from three different cultivation environments: location, season and cultivation system (sanitation control farm and semi-natural farm). The first shrimp samples were collected from a sanitation control farm in Rayong (Eastern Thailand) in January 2011 (S1). Average temperature of water in the ponds was 27.1 ± 0.2 °C with salinity 22.8 ± 2.5 ‰ and pH 8.50 ± 0.16 . After harvesting, the samples were immediately shocked by ice before subjecting to microbiological analysis. Under these conditions, total plate counts (TPCs) of shrimps were generally below 4.00 Log CFU/g. *Vibrio* spp. was observed in 25 g of all samples. *Vibrio parahaemolyticus* was observed in terms of most probable number (MPN) values ranging from 3.6 to 11 MPN/g. The second shrimp samples were collected from the same farm in June 2011 (S2). Average temperature and salinity of waters were significantly higher than S1 (30.6 ± 2.1 °C and 30.4 ± 0.4 ‰, respectively), whereas pH value was not significantly different (8.37 ± 0.17). The TPCs and occurrence of *Vibrio* spp. of this batch were also similar to S1, whereas MPN values of *V. parahaemolyticus* were significantly higher (240 to >1100 MPN/g). The third shrimp samples were collected from a semi-natural farm in Samut Songkram (Central Thailand) in August 2011 (S3). In this farm, salinity was dramatically lower than the sanitation control farms (0.6 ± 0.5 ‰), whereas the temperature and pH value were relatively similar (29.1 ± 0.3 °C and 8.27 ± 0.27 , respectively). TPCs observed were significantly higher than the control system farms (5.14 to 5.86 Log CFU/g). *Vibrio* spp. was detected in all samples. Interestingly, MPN values of *Vibrio parahaemolyticus* were significantly lower than the first two samples (<0.3 to 3.6). According to the results, microbial load on shrimp could*

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be influenced by salinity and farming operation systems. The V. parahaemolyticus populations correlated positively with increasing temperature. In addition, salinity increase seems to be a key factor influencing contamination levels of V. parahaemolyticus on shrimps.

Keywords: *Vibrio Parahaemolyticus*, Salinity, Temperature, Shrimp, Cultivation

INTRODUCTION

In Thailand, the Pacific White Shrimp (*Litopenaeus vannamei*) is an important aquaculture product that is exported to several countries, including the United States, Japan, Canada, European Union, Australia and Korea (OAE, 2010). The average annual production was 153,737 tons and average annual export income was over THB 34,000 million, respectively, over the 5-year period from 2006 to 2011 (OAE, 2011). Cultivation farms are mainly located in coastal areas and estuaries bordering the Gulf of Thailand and Andaman Sea, including Rayong and Samut Songkhram provinces (OAE, 2011).

The Genus *Vibrio* is a gram-negative curved rod and facultative anaerobic bacteria that are prevalent in estuaries and marine environments (Jakšić et al., 2002; Hosseini et al., 2004; Thongchankaew et al., 2011). Some *Vibrio* spp. are halophilic bacteria (tolerance up to 10% NaCl), with sodium ions stimulating growth. The pH and temperature conditions for their growth range from 8 to 8.80 and 20 to 37°C, respectively (Bhunja, 2008). Several *Vibrio* spp., including *Vibrio parahaemolyticus*, are reported pathogens, mainly found in seafood imported from Asian countries (Wong et al., 1999; Nishibuchi, 2003). *V. parahaemolyticus* infection from consuming raw and undercooked seafood causes acute gastroenteritis (Nolan et al., 1984; Lozano-León et al., 2003; Harth et al., 2009).

Vibrio spp. and *V. parahaemolyticus* in seafood are most common in summer, followed by spring, autumn and winter, respectively (Zarei et al., 2012). Environmental conditions – including temperature, pH, dissolved oxygen, turbidity and salinity – affect microbial diversity and population in sediment and seawater (Parveen et al., 2008). In particular, warm temperatures (above 20°C) were found to be a primary factor that increased the prevalence of *Vibrio* spp. (Maeda et al., 2003; Thompson et al., 2004). The combination of high temperature and salinity was reported as an important factor to support growth of the bacteria, particularly *Vibrio* spp. and *V. parahaemolyticus* (DePaola et al., 2003; del Refugio Castañeda Chávez et al., 2005; Parveen et al., 2008; Thongchankaew et al., 2011).

The microbiological quality of seafood products for export must meet the standards of exporting countries. Contamination levels of *V. parahaemolyticus* in frozen and chilled crustacean products for export to the United States (fresh-raw: for consumption without further cooking), Australia and New Zealand (cooked-ready to eat: for consumption without further cooking) and the EU (cooked-ready to eat: for consumption without further cooking) must be less than 10^4 , 10^3 and 3 MPN/g, respectively. For export to Japan (fresh-raw: for consumption without

further cooking), China (fresh: cooked before consumption) and Korea (cooked: food that is consumed without cooking), *V. parahaemolyticus* must not be detected in any 25 grams of product (FIQD, 2011). However, seafood manufacturers, particularly frozen Pacific White Shrimp industries (a top-five export product of Thailand), still experience problems with *V. parahaemolyticus* contamination. Contamination with this bacteria is difficult to control with levels varying, depending upon where cultivated.

This study therefore aims to investigate the influences of cultivation conditions on the microbiological profile of Pacific White Shrimp (*Litopenaeus vannamei*) from the main cultivation areas of Eastern and Central Thailand.

MATERIALS AND METHODS

Sampling sites

The first batch of Pacific White Shrimp (*Litopenaeus vannamei*) samples was collected from five cultivation ponds of a sanitation control farm located in Rayong, Eastern Thailand in January 2011 (S1). In sanitation control farms, personal hygiene and equipment sanitation are controlled and aquaculture chemicals applied. The second batch of samples was collected from the same farm in June 2011 (S2). The third batch of samples was collected from a semi-natural farm located in Samut Songkhram, Central Thailand in August 2011 (S3). This farm used a semi-natural cultivation technique in which only commercial feed was used.

Samples collection and preparation

Approximately 500 g of shrimp were collected from each pond as samples. Shrimp were then placed in a sterile plastic bag, which was immediately shocked by sterile ice, before subjecting to microbiological analysis on location.

Water samples were collected along with the shrimp from the five cultivation ponds. Two hundred and fifty milliliter water samples were collected at approximately 50 cm below the surface of the water and transferred into sterile 250 ml screw cap bottles. The water samples were transported to a laboratory for further physical analysis.

Microbiological analysis of shrimp

The total plate counts (TPCs) and *Vibrio* spp. was determined by following the methods of the Bacteriological Analytical Manual (USFDA, 2004).

Vibrio parahaemolyticus was determined by use of the most probable number method (MPN) with partial modification from the Bacteriological Analytical Manual (USFDA, 2004). Twenty-five grams of samples were placed in stomacher bag with 225 ml of alkaline peptone water (APW) (Himedia, India) then shaken for 30 s. The APW was then serially diluted and incubated at $35\pm 2^{\circ}\text{C}$ 24 hr before streaking onto Thiosulfate Citrate Bile Sucrose agar (TCBS agar) (Merck, Germany) plate and incubated at $35\pm 2^{\circ}\text{C}$ 24 hr. The suspect colonies of *V. parahaemolyticus* were evaluated with screening and confirmation test follow-

ing the Bacteriological Analytical Manual (USFDA, 2004).

The physical and chemical properties analysis of water

The temperature of pond water at three sites (1 m from the edge of the pond and 50 cm below the surface) was measured with a thermometer. The salinity was also measured on location with a reflecto-salinometer (N.O.W., Japan). The pH of the water was further determined at the laboratory using a pH meter (Cyberscan, USA).

RESULTS

Pond water properties and microbial profiles of shrimp from S1

The conditions of water collected from three different environments are shown in Table 1.

Table 1. Chemical and physical properties of pond water of Pacific White Shrimp cultivation farms located in Rayong determined in January 2011 (S1) and June 2011 (S2) and Samut Songkhram in August 2011 (S3).

| Locations | Pond water properties ^A | | |
|-----------|------------------------------------|--------------------------|---------------------------|
| | Water temperature (°C) | Salinity (‰) | pH |
| S1 | 27.1 ^[a] ±0.2 | 22.8 ^[b] ±2.5 | 8.50 ^[a] ±0.16 |
| S2 | 30.6 ^[b] ±2.1 | 30.4 ^[c] ±0.4 | 8.37 ^[a] ±0.17 |
| S3 | 29.1 ^[b] ±0.3 | 0.6 ^[a] ±0.5 | 8.27 ^[a] ±0.27 |

Note: ^AThe values are means ± SD of three replications. [a], [b], [c] The mean values with significant difference at $P \leq 0.05$ in each column are indicated by superscript letter.

The average temperature of S1 water at sampling time was 27.1±0.2°C with salinity 22.8±2.5 ‰ and pH 8.50±0.16. The microbial counts in TPC values are displayed in Table 2. The TPC values of S1 samples ranged from 2.78 to 4.03 Log CFU/g. The detection of *Vibrio* spp. and the MPN/g values of *V. parahaemolyticus* are shown in Table 3. *Vibrio* spp. was observed in every S1 sample. The contamination level of *V. parahaemolyticus* as indicated by MPN values ranged from 3.6 to 11 MPN/g.

Table 2. Total plate counts (TPCs) of Pacific White Shrimp collected from Rayong in January (S1) and June 2011 (S2) and Samut Songkhram in August 2011 (S3).

| Cultivation ponds | Total plate counts; TPCs (Log CFU/g) | | |
|-------------------|--------------------------------------|------|------|
| | S1 | S2 | S3 |
| 1 | 4.03 | 3.50 | 5.59 |
| 2 | 3.21 | 3.00 | 5.45 |
| 3 | 3.55 | 3.16 | 5.34 |
| 4 | 3.58 | 3.28 | 5.86 |
| 5 | 2.78 | 2.93 | 5.14 |

Table 3. The *Vibrio* spp. and MPN/g values of *Vibrio parahaemolyticus* of Pacific White Shrimp collected from Rayong in January (S1) and June 2011 (S2) and Samut Songkhram in August 2011 (S3).

| Cultivation ponds | S1 | | S2 | | S3 | |
|-------------------|---------------------------------|------------------------------------|---------------------------------|------------------------------------|---------------------------------|------------------------------------|
| | <i>Vibrio</i> spp. ^a | <i>V. parahaemolyticus</i> (MPN/g) | <i>Vibrio</i> spp. ^a | <i>V. parahaemolyticus</i> (MPN/g) | <i>Vibrio</i> spp. ^a | <i>V. parahaemolyticus</i> (MPN/g) |
| 1 | + | 3.6 | + | > 1100 | + | < 0.3 |
| 2 | + | 7.4 | + | 1100 | + | < 0.3 |
| 3 | + | 11.0 | + | 460 | + | < 0.3 |
| 4 | + | 7.4 | + | 240 | + | 3.6 |
| 5 | + | 11.0 | + | 240 | + | < 0.3 |

Note: ^a + = Detected, - = Not detected.

Pond water properties and microbial profiles of shrimp from S2

The average temperature, salinity and pH value of S2 water at sampling time were 30.6±2.1°C, 30.4±0.4‰ and 8.37±0.17, respectively (Table 1). The microbial counts in TPC values of S2 samples ranged from 2.93 to 3.50 Log CFU/g (Table 2). *Vibrio* spp. was detected in all S2 samples. The contamination levels of *V. parahaemolyticus* ranged from 240 to >1100 MPN/g (Table 3).

Pond water properties and microbial profiles of shrimp from S3

The average temperature of S3 water at sampling time was 29.1±0.3°C with salinity 0.6±0.5 ‰ and pH 8.27±0.27. The TPCs values shown in Table 2 ranged from 5.14 to 5.86 Log CFU/g. In Table 3, *Vibrio* spp. was observed in all S3 samples and the contamination level of *V. parahaemolyticus* ranged from <0.3 to 3.6 MPN/g.

DISCUSSION

According to the results in Table 1, the significant differences of pond water conditions are noted. These variations, such as temperature and salinity level, are significantly related to seasonal and geographical factors as demonstrated in several previous observations (DePaola et al., 2003; Parveen et al., 2008; Thongchankaew et al., 2011). From the Annual Weather Summary of Thailand in 2011 (TMD, 2011), mean area temperature and rainfall in Eastern Thailand in January 2011 (S1 sample collection period) were 26.4°C and 0.0 mm, respectively. In June 2011 (S2 sample collection period), they were 28.4°C and 277.5 mm, respectively. For S3 farm, in August 2011, mean temperature was approximately 28.1°C and rainfall was around 211.8 mm. Interestingly, temperature of shrimp cultivation water of each farm as observed in this study was relatively close to the area temperature. Thus, water temperature of S1 collected in winter was accordingly lower (27.1±0.2°C) than S2 and S3 (30.6±2.1 and 29.1±0.3, respectively). In the same way, salinity levels of pond water from different parts of Thailand were also notably different, which could be affected by geographical factors. Such factors can relate

to particular physical and chemical conditions of the soil and water in the ponds. Consequently, these different conditions could affect the occurrence of microbes in the shrimp cultivation ecosystems (DePaola et al., 2003; Maeda et al., 2003; Parveen et al., 2008; Thongchankaew et al., 2011).

As observed in this study (Tables 2 and 3), the TPC occurrence of *Vibrio* spp. and *V. parahaemolyticus* in shrimp from three different conditions were relatively different. In particular, the contamination levels (MPN/g) of *V. parahaemolyticus* in shrimps were found to be significantly different in samples from Rayong (S1 and S2) and Samut Songkhram (S3). This could be an effect of the high salinity of the pond water. Based on the nature of *V. parahaemolyticus*, this microorganism needs salt (NaCl) for growth (Bhunja, 2008). Salt concentrations of 0.5 to 8% stimulates the growth of *V. parahaemolyticus*, with an optimum concentration of approximately 3% (Lake et al., 2003). As shown in Table 1, the salt concentration of S3 water (0.06%) was lower than the minimum threshold (0.5%) for *V. parahaemolyticus* growth, whereas the salt levels in S1 and S2 were in an appropriate range (2 and 3%, respectively). In accordance with these salinity values, the MPN levels were significantly higher in S1 and S2 shrimp compared to S3. This observation reflected the significant influence of salinity on the occurrence of *V. parahaemolyticus* in shrimp.

However, although the salinity values of S1 and S2 waters were relatively similar, the MPN levels of S2 (240 to >1,100 MPN/g) observed were higher than S1 (3.6 to 11 MPN/g). Other factors, in particular temperature, might also be influencing the occurrence of *V. parahaemolyticus* in combination with salinity. *V. parahaemolyticus* is a mesophilic bacteria preferring to grow in temperatures of 10 to 44°C, with an optimum temperature of 37°C (Nishibuchi, 2003). The temperature of S2 water (30.6°C), as shown in Table 1, was close to the optimum temperature. At this temperature, *V. parahaemolyticus* would grow well, and consequently were more prevalent in S2 shrimp. This observation also corresponds with previous reports, which demonstrated that the occurrence of *V. parahaemolyticus* tended to increase at temperatures close to 30°C (DePaola et al., 2003; Parveen et al., 2008).

In this study, *Vibrio* spp. was found in all shrimp samples. The occurrence of these bacteria do not appear to be related to any cultivation condition, since *Vibrios* are marine bacteria which occur naturally in the estuaries, coasts and marine environments. Thus, they are commonly found in seafood, especially in shrimp, regardless of season (Zarei et al., 2012).

Total plate count (TPC) is an important criterion for indicating microbiological quality of seafood. The International Commission on Microbiological Specifications for Foods (ICMSF) recommends that the TPC value of frozen raw crustaceans should be not over 10^6 CFU per gram (ICMSF, 1986). From the results in Table 2, TPCs of shrimp cultivated under different conditions also differed. The TPCs of shrimp cultivated in higher salinity water (S1 and S2) were lower than in lower salinity water (S3). In this case, salt could adversely affect microorganisms growing in pond ecosystems (Hotchkiss, 1923).

However, as found in this study, farming operation systems seemed to be another influential factor. According to the results, the TPCs of shrimps from the sanitation control farm were significantly lower than the semi-natural farm (Table 2). This demonstrated that sanitation controls and/or the addition of aquaculture chemicals in the sanitation control farm helped reduce microbial contamination. Consequently, shrimp from the sanitation control farm showed relatively better microbiological quality in terms of TPC level.

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