International Journal of Agricultural Technology 2024 Vol. 20(1):427-440
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Effects of Bacillus probiotics, *Bacillus subtilis* and *Bacillus cereus* dietary additional to controlling Vibriosis infection of white shrimp (*Litopenaeus vannamei*)

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Wongpracha, P., Kanthawong, P., Arun, J., Jeyaraj, G. P., Jongput, B., Srijad, S. and Kitikiew, S. (2024). Effects of Bacillus probiotics, *Bacillus subtilis* and *Bacillus cereus* dietary additional to controlling Vibriosis infected of white shrimp (*Litopenaeus vannamei*). International Journal of Agricultural Technology 20(1):427-440.

Abstract The effects of the *Bacillus* probiotics powder, *Bacillus subtilis* 7 x 10^6 cfu g^{-1} and *Bacillus cereus* 2.5 x 10^7 cfu g^{-1} via dietary additional to control vibriosis disease in white shrimp culture for 14 days were evaluated. The results indicated that good recovery in shrimp fed with probiotic (T4) and the mortality reduced (86.7%), in comparison with the group without probiotics and with pathogen, *Vibrio* spp. (T3). In addition, it presented lower counts of *Vibrio* spp. than group fed without the probiotic causing a significantly reduced pathogen. *Bacillus* probiotics, *B. subtilis* and *B. cereus* had a better effect on hematological shown in the total haemocyte counts (THC) of the group with probiotic and with pathogen, *Vibrio* spp. (T4) had the highest THC every day throughout the experiment experiment followed by the group with probiotic and without pathogen (T2) respectively. Therefore, *B. subtilis* and *B. cereus* significantly enhanced the shrimps' ability to recover from *Vibrio* spp. infections and enhanced the quality of their cultures.

Keywords: *Bacillus subtilis*, *Bacillus cereus*, *Vibrio* spp., White Shrimp

Introduction

The white shrimp, (*Litopenaeus vannamei*) is an important economical species in Thailand aquaculture industry. In 2011, the Fisheries Department permitted the importation of this shrimp species. Because it is a fast-growing shrimp and significantly due to the increased global market demand (Harlioglu and Farhadi, 2017). Shrimp disease outbreaks are widespread in the rapidly expanding shrimp farming, resulting in significant economic losses (Shinn *et al.*, 2018). According to FAO (2019) reports that from 2009 to 2018, shrimp

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infections in Asia caused an annual loss of over $4 billion for the shrimp industry. In addition, shrimp rearing quality, and water quality are essential to the success of white shrimp culture. In case of the water in the aquaculture pond is poor quality, the shrimp will grow slowly, get feeble, and finally become a disease outbreak. Especially, vibriosis has been discovered as a major disease concerned in shrimp farm. Many countries have reported mass mortalities in shrimp hatcheries and grow out ponds caused by outbreaks of vibriosis (Jayasree *et al*., 2006). According to Abdel-Latif *et al*. (2022) vibriosis is caused by a variety of Vibrio species: gram-negative, motile, facultative anaerobic bacteria belonging to the Vibrionaceae family, and is a major risk to shrimp farming. Isolated and identified from infected shrimp include representatives of the genus *Vibrio, include* the species *V. harveyi, V. parahaemolyticus, V. alginolyticus, V. campbellii, V. vulnificus, V. anguillarum, and V. splendidus* (Chatterjee and Haldar, 2012; Kumar and Roy, 2017). Vibrio infection cause several kinds of syndromes in shrimp including shell disease, tail necrosis, red disease, luminous vibriosis, loose shell syndrome, acute hepatopancreatic necrosis disease or early mortality syndrome, vibrio septicaemia, septic hepatopancreatic necrosis disease, and white gut disease (Jayasree *et al*., 2006; Amatul-Samahah *et al*., 2020).

Shrimp have a less developed immune system than vertebrates, resulting in a less specific response to infection. The shrimp has a shell to prevent antigen from reaching the organism. That able to enter the interior of the crustacean or become infected through ingestion or infection of the gills. In cases, while the shrimp exhibit weakness or have undergone molting, the bacteria could infect the shrimp. Moreover, shrimp possess an immune system. Cellular and humoral immune responses to eliminate antigens. Haemocytes are the immune system of crustacean; the shrimp internal circulatory system is exposed. The haemolymph of crustacean is composed of three haemocyte types: the smallest cells are hyaline cells, contributes to blood coagulation and molting. Likewise, these are Phagocyte cells. Semi-granular cells are multi-shaped cells composed of numerous Eosinophilic granular cells, they eliminate action and facilitate numerous immune processes. They stored the enzymes ProPO, AMPs along with protease inhibitors. To enhance the shrimp immunity, many aquaculturists try to use chemicals and drug especially antibiotics, are frequently utilized for treating diseases from the Vibriosis. But many countries have prohibited the application of using antibiotics in aquaculture for human consumption because of concerns about antibiotic resistance, negative effects on human health, decreased effectiveness due to overuse, and environmental contamination (Alderman and Hastings, 1998; Cabello, 2006; Smith, 2008; Limbu *et al*., 2018). Therefore, it is crucial to discover alternative ways to avoid antibiotic

utilization that are feasible, effective, relatively cost-efficient, and ecologically friendly.

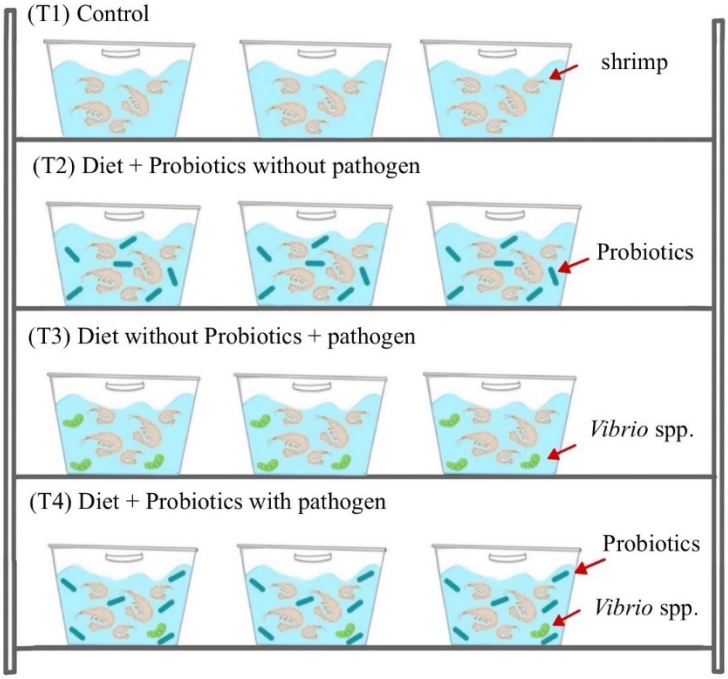
Probiotics have a crucial role with their versatile potential in preventing pathogens by competition, antagonistic functions, promoting shrimp health, and growth with gut activity and immunomodulatory effects (Van Hai and Fotedar, 2010; Zorriehzahra *et al*., 2016; Pérez-Sánchez *et al*., 2018; Madhana *et al*., 2021). Several studies have investigated the utilization of different gram-positive and gram-negative probiotic bacterial species, including Bacillus species, Vibrio species, lactic acid bacteria, and other bacterial species, as probiotics in the environment of shrimp farming (Hoseinifar *et al.,* 2018; Abdel-Tawwab *et al*., 2020; Mohammadi *et al*., 2021; Ringø *et al*., 2020; Ringø, 2020). The most effective probiotic are Bacillus strains. Pattukumar *et al*. (2013) and Wang *et al*. (2019) reported that the *Bacillus subtilis* has ability to enhance the immune responses of treated shrimps and increased their resistance against *Vibrio* sp. infections. *B. cereus* improved water quality parameters in the rearing ponds of shrimp via controlling the pathogenic bacteria in water (Cai *et al.,* 2019), water quality parameters (Zokaeifar *et al.,* 2014). In this study, *B. subtilis* and *B. cereus* were used in addition to diet, examined by haemocyte count, survival rate, clearance efficiency and water quality within 14 days to evaluate the efficacy of probiotics.

Materials and methods

Experimental design

The white shrimp were raised in saltwater with a salinity of 15 ppt, pH 7.5-8.0, alkalinity 100-200 mg l^{-1}. The saltwater was disinfected with 20 mg l^{-1} of chlorine powder for 24 hours. Thereafter turned on the aerator to remove the chlorine before rearing. The experiment was designed through a Completely Randomized Design (CRD). This study was divided into four different groups of experiments, with three replicates in each group (Figure 1).

Treatments were T1 – control, T2 – with probiotic and without pathogen, T3 – without probiotic and with pathogen, *Vibrio* spp. And T4 – with probiotic and with pathogen, *Vibrio* spp.



* Probiotics contain bacteria *Bacillus subtills* (ABPL 154) 7 x 10^6 cfu g^{-1} and *Bacillus cereus* (ABPL 155) 2.5 x 10^7 cfu g^{-1}

Figure 1. Experimental group designed by control (T1), feed added with probiotic, *Bacillus subtilis* and *Bacillus cereus* and without challenge *Vibrio* spp. (T2), normal feed and challenge with *Vibrio* spp. (T3) feed added with probiotic, *Bacillus subtilis* and *Bacillus cereus* and challenge *Vibrio* spp. (T4)

Animal

White shrimps were obtained from a private farm in Chachoengsao Province, Thailand. Before examination, the white shrimp were acclimated in seawater salinity of 15 ppt for two weeks. After acclimatization the white shrimp utilized in the experiment had an average weight of 6 grams. Then placed in a plastic tank sized 12x10x12 inches, of 20 L. There were twelve tanks were stocked at a density of 15 shrimps per tank. During acclimatization and experiment the white shrimp were fed with commercial feed (GF555, Krungthai Food Public CO., LTD.) at 2% of bodyweight, 2 times per day. Aeration was always available.

Preparing probiotics to add to the white shrimp dietary

The experimental groups of T2 and T4 were used commercial white shrimp feed which contains 40% protein, 8% fat mixed with a probiotic product of the R&D facility of Tablets (India) Limited, Chennai, India containing *Bacillus subtills* (ABPL 154) 7 x 10^6 CFU g^{-1} and *Bacillus cereus* (ABPL 155) 2.5 x 10^7 CFU g^{-1} in powder form. The probiotic 10 g was mixed per Kg of white shrimp feed and fed at 2% of body weight 2 times per day for 14 days.

Preparation of Vibrio spp.

Vibrio spp. were taken from infected shrimp which isolated in selective media thiosulfate citrate bile salts sucrose agar (TCBS) to identify *Vibrio* spp. which were cultured in nutrient broth of 20 ppt at 30 ºC for 16 hr. *Vibrio* spp. pellets were isolated from Nutrient broth (NaCl 20 ppt) by centrifuging at 3,000 rpm at temperature 4 ºC for 15 minutes, 3 times and re-suspended in saline (0.85% NaCl) for challenge test at 10^8 CFU ml^{-1}.

Water quality parameter

Water quality parameters dissolved oxygen (DO) and temperature were measured using a meter (YSI MODEL 57), the pH value was measured using a pH meter (H198128), salinity was measured by refractometer, and ammonia was measured by phenate method at 630 nm with a spectrophotometer (AQUA-VBC).

Survival rate

Survival rate (%), the surviving shrimp of each replicate in the experimental set was counted every day from the start of the experiment for 14 days after which the survival rate was assessed by analysis.

$$Sur(\%) = \frac{FNWS}{SNWS} x100$$

Sur (%) is the survival rate. *FNWS* is the final number of white shrimps. *SNWS* is the starting number of white shrimps (Vidal *et al.*, 2018).

Microbiological analyses

Water samples from each set of experiments were collected to test *Vibrio* spp. in thiosulfate citrate sucrose agar (TCBS). Water samples were collected and diluted with 0.85% saline dilute 10X. Water samples were spread after dilution, 100 µl of each set of experiments was spread on the culture medium. The TCBS was spread and incubated at 30 ºC for 16 h to determine *V. parahaemolyticus* and *V. alginolyticus.* Water samples were collected on the days of 0^{th}, 1^{st}, 4^{th}, 7^{th}, 11^{th}, and 14^{th} throughout the experiment.

Clearance efficiency

Haemolymph was collected from 3 shrimps from each treatment to test *Vibrio* spp. with Thiosulfate citrate bile salts sucrose agar (TCBS) by using 1 ml syringe, collected 10µl of haemolymph and diluted with 0.85% saline 90 µl to dilute the concentration in a 1. 5ml Eppendorf™ tube. 100µl haemolymph sample was taken and spread to the culture medium. The petri dishes were incubated at 30 ºC for 16 hours and collected data at 0^{th}, 1^{st}, 4^{th}, 7^{th}, 11^{th} and 14^{th} of experimental days.

Total haemocyte count and differential haemocyte count

Hemolymph samples were collected from 3 shrimps for each treatment. Using a 1 ml syringe with anticoagulant of Formalin 10% in NaCl 0.85% at a ratio of 1:1 from the hemolymph. The total haemocyte count (THC) were directly counted the different haemocyte count (DHC) according to the method of Moullac *et al*. (1997) by dropping 10 µl of hemolymph onto the hemocytometer. Counts were performed under a microscope Nikon ECLIPS E200 at 40x magnification. Total haemocyte count and differential haemocyte count density was calculated with a result of 10^6 cell ml^{-1}.

Histopathological analysis

At 14^{th} day of the collected shrimp samples from each experimental set. Data were collected from tissue pathological analysis, the hepatopancreas and intestines were fixed in Davidson's Fixative. Shrimp's tissues were placed on embedding dehydrated tissue by processer (Leica Modell TP 1020-1-1). Section the tissues were done by microtome (Microm GMbH HM 336E) to meet a size of 5µl. The slides were dried at 30 °C and left for at least 3 hours to allow the tissue to adhere to the slides. Sample slides were stained with

hematoxylin and eosin, then made into permanent slides (Permount). The tissues were examined for pathological characteristics with a microscope (Olympus CX33) according to the method of (Bell and Lightner, 1988) and photographed using image via Olympus EP 50EP view application.

Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA). The multiple comparisons (Tukey's) test was used to compare the significant differences among treatments using the SPSS computer software (IBM SPSS Statistic, V.28). statistically significant differences required that *p* values less than 0.05.

Results

Water quality

The water quality parameters: salinity, temperature, pH, dissolved oxygen, and ammonia were not significantly differed ($P > 0.05$) during the day of experiment (Table 1).

Survival rate

No mortality was observed in the control group and the group with probiotic and without pathogen (T2) over 14 days. In contract, the onset of mortality occurred at 2nd day in the group without probiotics and with pathogen, *Vibrio* spp. (T3). For the group with probiotic and with pathogen *Vibrio* spp. (T4), the survival rate shown 86.7 % was significate higher than the group without probiotics and with pathogen, *Vibrio* spp. (T3) 26.7 % at the end of experiment, respectively (Figure 2).

Vibrio spp. in reared water

At the end of experiment, the group with probiotic and with pathogen, *Vibrio* spp. (T4) had significant decrease the population of *Vibrio* spp. 1 x 10^6 CFU ml^{-1} in water than the group without probiotic and with pathogen, *Vibrio* spp. (T3) 5 x 10^6 CFU ml^{-1} (Figure 3).

Total haemocyte count and differential haemocyte counts

Total haemocyte counts (THC) of the group with probiotic and with pathogen, *Vibrio* spp. (T4) was 12.55 x 10^6 Cell ml^{-1} had the highest THC every day of experiment followed by the group with probiotic and without pathogen (T2) was 10.78 x 10^6 Cell ml^{-1} which had higher THC than the group without probiotic and with pathogen, *Vibrio* spp. (T3) was 8.08 x 10^6 Cell ml^{-1} and the control group (T1) was 5.97 x 10^6 Cell ml^{-1}, respectively (Figure 4C). Hyaline cell of the group with probiotic and with pathogen, *Vibrio* spp. (T4), had the highest number of hyaline cells every day throughout the experiment (Figure 4B). Moreover, granular cells of every group had not significantly differed between groups (Figure 4A).

Clearance efficiency

The clearance efficiency in the group without probiotic and with pathogen, *Vibrio* spp. (T3) had the lowest clearance efficiency 52.81%. Moreover, group with probiotic and with pathogen, *Vibrio* spp. (T4), group with probiotic and without pathogen (T2) had not significantly differed each other but significantly differed higher clearance efficiency than in the group without probiotic and with pathogen, *Vibrio* spp. (T3) (Figure 5).

Histopathological

The experiment was carried out for 14 days. Found that the control group (T1), group with probiotic and without pathogen (T2), and group with probiotic and with pathogen, *Vibrio* spp. (T4) were not detecting the different or abnormal cell from this group.

Table 1. Water quality parameters

	Experimental group			
	T1	T2	T3	T4
Salinity (ppt)	$15^a \pm 0.00$	$15^a \pm 0.00$	$15^a \pm 0.00$	$15^a \pm 0.00$
Temperature (°C)	$28.26^a \pm 0.36$	$28.44^a \pm 0.28$	$28.13^a \pm 0.31$	$28.13^a \pm 0.36$
pH	$7.81^a \pm 0.25$	$7.77^a \pm 0.28$	$7.77^a \pm 0.27$	$7.74^a \pm 0.27$
Dissolved oxygen (ppm)	$6.25^a \pm 0.33$	$6.20^a \pm 0.25$	$6.38^a \pm 0.28$	$6.32^a \pm 0.22$
Ammonia (ppm)	$0.0296^a \pm 0.006$	$0.0201^a \pm 0.010$	$0.0284^a \pm 0.007$	$0.0224^a \pm 0.008$

Note: Mean values with same superscripts are non-significantly different (P > 0.05)

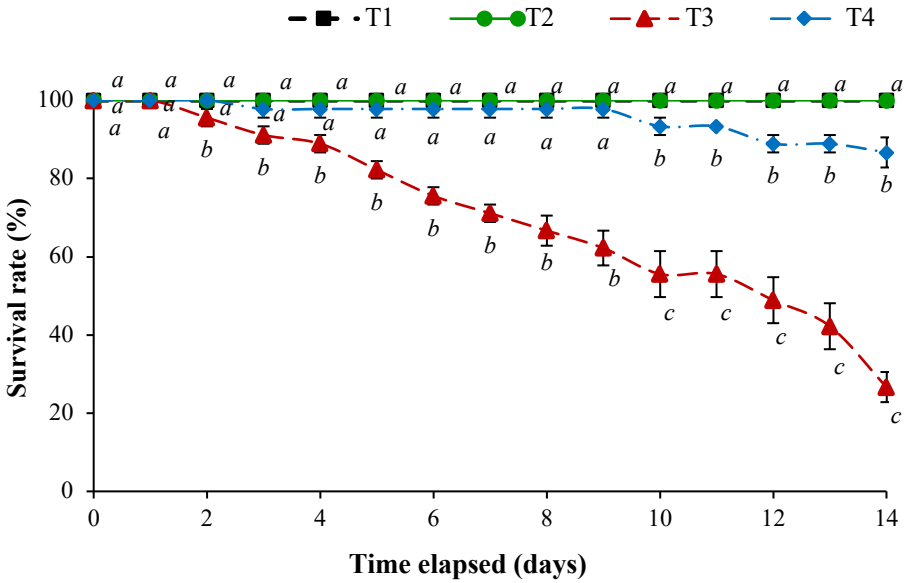


Figure 2. The survival rate of white shrimp

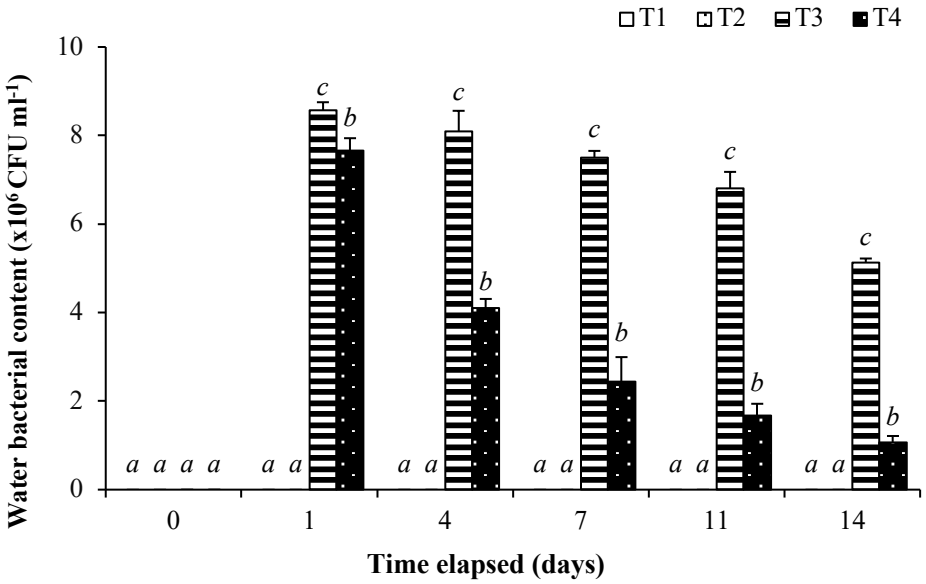


Figure 3. Water bacterial content

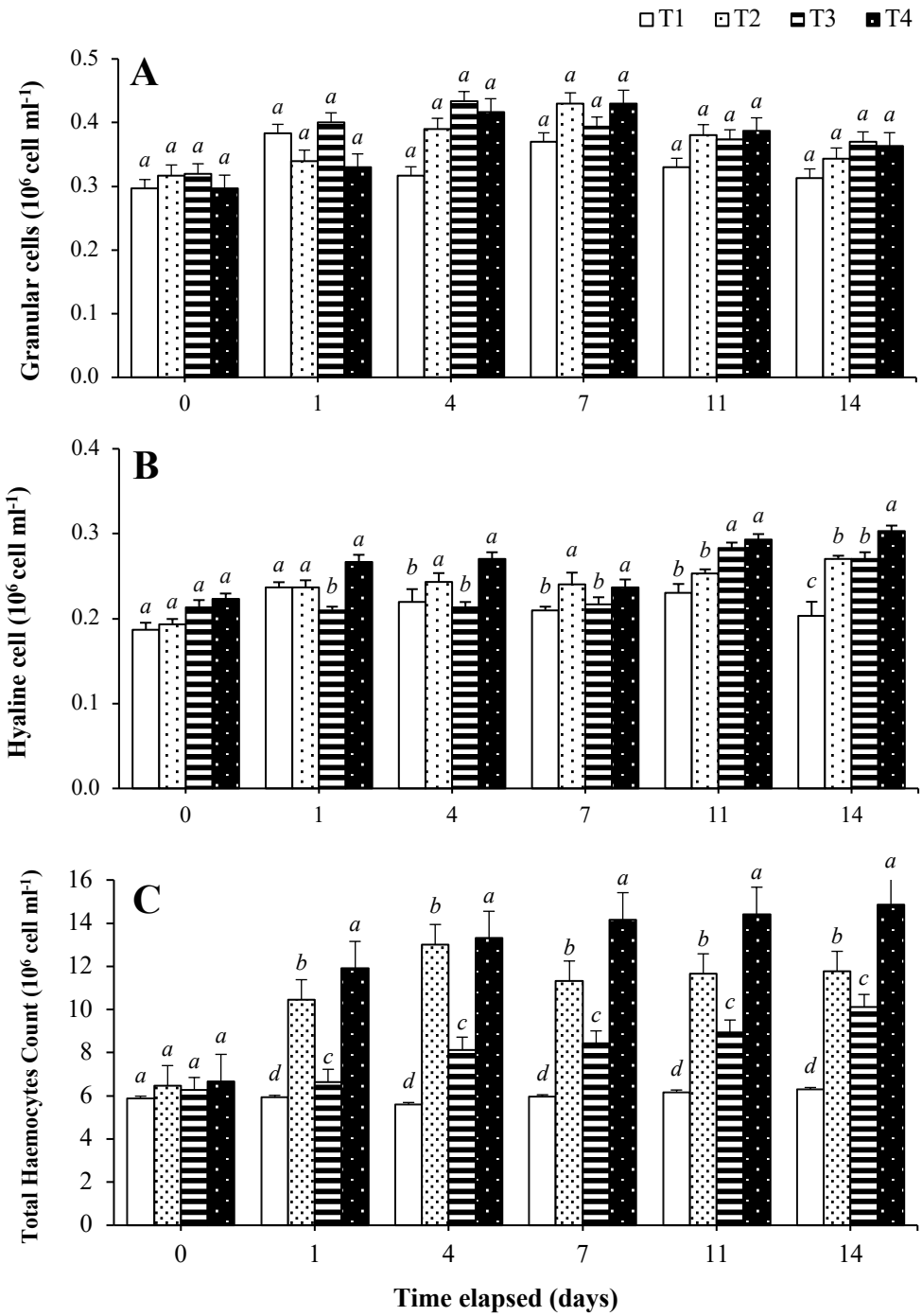


Figure 4. Granular cells (A), Hyaline cell (B) and Total Haemocyte Count: THC (C)

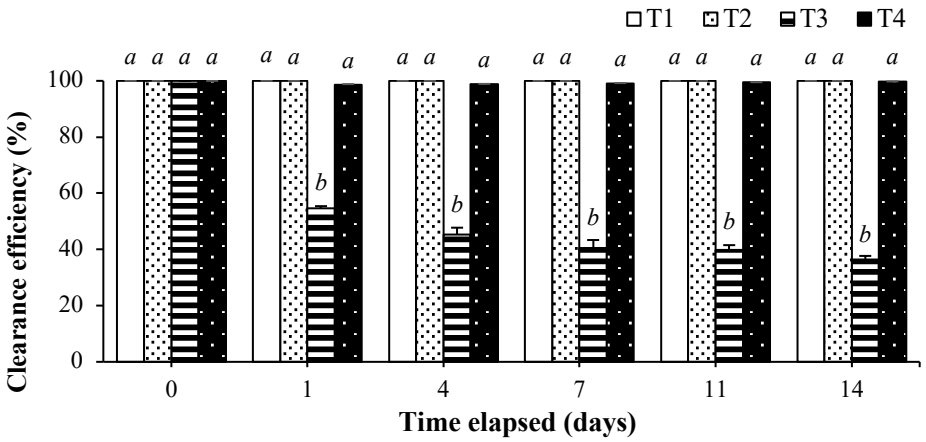


Figure 5. Clearance efficiency

Discussion

In water quality parameters of this study were conducted the salinity, temperature, pH, dissolved oxygen, and ammonia was not differed between the groups. Huisakul *et al*. (2007), studied on application of mixed probiotics to diet for black tiger shrimps farming ponds. The water quality parameters among ponds fed with probiotics was within the appropriate criteria for shrimp cultured. The result of population of *Vibrio* spp. of the end of experiment were decreasing after feed probiotic *B. subtilis* and *B. cereus*. According to George *et al.* (2018), the application of probiotics *Bacillus* strains of NOVIB^{TM} was able to control the *Vibrio* spp. in shrimp rearing water. According to Heyman and Menard (2002) and Verschuere *et al.* (2000), the probiotics have a mechanism for destroying pathogenic bacteria. Therefore, in the present study application of probiotics *B. subtilis* and *B. cereus* did not affect to reared water parameters but able to control the *Vibrio* spp. content in reared water.

The survival rate of the group that received probiotics mixed to diet was higher than the challenged group and without probiotics. Because of probiotics able to inhibit disease-causing bacteria, especially *Vibrio* spp. According to the experiment of Vaseeharan and Ramasamy (2003), application of the probiotic *B. subtilis* in rearing black tiger shrimps, the rate of bacterial infection (*V. harveyii*) was decreased and significantly increased the survival rate of shrimps. According to Balcázar and Rojas-Luna (2007), study of application probiotics. The *B. subtilis* UTM 126 was mixed into diet. The survival rate of shrimp which received probiotic had increased. Moreover, mixed *B. cereus* to shrimp diet. The group with probiotics had the highest survival rate. While the group infected with *Vibrio* spp. and did not received probiotic had the lowest survival rate in the experiment (Vidal *et al*., 2018). In addition, the experiment

of Songsuk *et al*. (2019), the content of *Vibrio* spp. bacteria in the group with probiotic was significantly lower than the control group. Therefore, the application of probiotics *B. subtilis* and *B. cereus* to shrimp rearing had an efficacy to increase the survival rate.

The immune parameters, clearance efficiency in the pesent study. The group without probiotics and with pathogen, *Vibrio* spp. (T3) had the lowest of clearance efficiency. According to Sritunyalucksana *et al.* (2005), the shrimp were fed feed coated with yeast extract and inject *Vibrio* spp. into the muscle. The shrimps fed with feed coated with yeast extract 4%, had the highest clearance efficiency. In addition, a study on haemocytes of shrimp. Haemocytes, including hyaline cell, granular cell, and semi-granular cell. The haemocyte has an ability to eliminate bacteria or microorganisms from the shrimp circulatory system. That as an indicate for the efficiency of the immune system (Moullac *et al*., 1997). The group with probiotic and with pathogen, *Vibrio* spp. (T4) had the highest of total haemocyte count (THC) and hyaline cell. According to Rodrýgez and Moullac (2000) studies of diets with yeast had an increased their ability to remove bacteria from the haemolymph system. The results of immunity able to imply, the application of *B. subtilis* and *B. cereus* able to enhance immune response.

In summary, probiotics are the most important component of the preventative measures required to prevent the shrimp and the pond from the infections and disease. It is further recommended that, preventative dosages must be provided so that the shrimps could be cultured with minimal infection, which would promote the development and promote a successful culture.

Acknowledgements

The authors gratefully acknowledge the TIL Biosciences (The Animal Health Division) of Tablets (India) Limited, Chennai, India for provision of probiotics and support.

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(Received: 27 September 2023, Revised: 21 December 2023, Accepted: 1 January 2024)