Effect of *Bacillus* sp. as probiotic on the treatment of environment in brackish water shrimp aquaculture

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Abstract. In the face of increasing consumers demand for aquatic food products is to be safe and quality, requiring aquaculture farmers to strengthen preventive measures to limit the residues of chemicals and antibiotics in aquatic products, especially in the international markets. Therefore, the application of probiotics is considered to be the key to the success of aquaculture. In this work, we have studied probiotics produced from *Bacillus*, capable of treating environment in brackish water shrimp aquaculture. Apply 1x10^5 CFU/mL water treatment concentration, 5 days/time. Shrimp with mean initial weight of 0.034 g. There were two treatments in triplicates including 1) the control (without supplementing bacteria); 2) supplementing *Bacillus subtilis*. After 60 days, water quality parameters (DO, COD, TAN, NH₃ and TSS) indicated that in the supplemental probiotic treatments had better decomposition of organic substances and lower *Vibrio* density than in the control treatments. Growth rate of shrimp in terms of daily weight gain and daily length gain were highest in treatment 2 (0.098±0.012 g/day) and 0.158±0.011 cm/day), and lowest in the control (0.078±0.009g/day) and 0.121±0.012 cm/day). These results indicated that supplementation of these bacteria in the culture medium could promote a better decomposition of organic matter, help improve survival and growth rate of shrimp.

1. Introduction

Environmental pollution is happening seriously in water bodies in general and aquaculture ponds in particular. In aquaculture ponds, water pollution is mainly caused by the use of feed from domestic waste, animal wastes, animal waste products and leftovers. In addition to the above wastes, a large amount of organic matter comes from dead bodies of aquatic plants in deposition ponds, drugs and chemicals used in the process of environmental management and aquatic disease treatment production in the rearing ponds.
The formation of the pond bottom mud due to long-term accumulation of organic matter deposition is inhabited by rotten microorganisms, toxic gases such as ammonia (NH$_3$), pathogenic microorganisms such as *Vibrio, Aeromonas sp.*, *Pseudomonas sp.*, *Edwardsiella sp.*, ... The majority of pathogenic microorganisms for aquatic animals are opportunistic agents, they only cause disease when farmed animals are stressed due to environmental problems or other reasons, then pathogenic microorganisms proliferate, increase virulence and generate disease. Pathogenic microorganisms can also be controlled through control of culture environment by the use of useful microorganisms through a mechanism of nutrient competition, cleaning the culture environment and some pathogenic microorganisms. Capable of secreting antibacterial agents to inhibit the growth of harmful microorganisms. In addition, the pond environment is polluted if only for a short time, with the level of not serious can only cause growth reduction due to reduced ability to digest, absorb food. But if the pollution of the aquaculture environment is serious, it can be toxic to the aquatic animals, which can cause a concurrent death in a short time, then it causes risks to the farmers and adversely affects the environment. The wastes in ponds often contain large amounts of easily biodegradable organic matter so that the effect of beneficial microorganisms can impact on cleaning the culture environment to bring high efficiency for farmers.

Currently, the commonly used useful microorganism is a positive solution, with great prospects for managing microorganisms in ponds, limiting the use of antibiotics and reducing the amount of organic waste discharged. The environment contributes to the sustainable development of aquaculture. Recent research has isolated, identified and evaluated the water treatment efficiency of some *Bacillus* strains originating from intensive shrimp ponds in Soc Trang Province [1]. The study was conducted with the purpose of using probiotics produced from *Bacillus* to improve water quality, enhance productivity and survival in brackish water Shrimp.

2. Materials and Methods

2.1 Materials
Probiotic: Isolation and selection of bacterial strains of genus *Bacillus sp* from shrimp ponds in Tran De district, Soc Trang province [1]. Propagation and culture to obtain the biomass of selected bacterial strains; and mix and create products. Whiteleg shrimp (PL15) were purchased from the farm varieties in Can Gio. Shrimp are measured in length and weight before experiment arrangement 0.034 ± 0.016 g/ind. and 1.75 ± 0.19 cm/ind. Shrimp treated with formol at a concentration of 30 mg/L approx 15-30 minutes before layout. Aeration tank and salinity of 15 mg/L.

2.2 Experimental arrangement
The experiment consists of 2 treatments, each solution the formula is repeated 3 times and arranged completely random. Treatment 1 (T1-In comparison): No additional bacteria, treatment 2 (T2-Probiotic): Complement supplement *Bacillus*. Density after adding to the water environment farming reached $10^5$ CFU/mL and bacteria replenishment cycles into the tank is 5 days/times. Experiments are arranged in 12 tanks, 120 L/tank were disinfected with chlorine before the experiment arrangement. Stocking density of shrimp 0.5 ind./L. Shrimp were fed 4 times/day with GrowFeed industrial feed for shrimp at 06, 11, 16 and 21 hours, the dosage according to the manufacturer's instructions. Time experiment is 60 days.

2.3 Data collection
- pH and temperature are checked 2 times/day (8 hours and 14 hours),
- Water quality parameters (DO, COD, TAN, NH$_3$, TSS)
- Bacterial density was monitored every 5 days. Bacterial samples were collected before adding bacteria. Shrimp growth and survival were determined at the end of the experiment.
Water samples: Water samples were collected about 20-30 cm from the water surface. All environmental indicators were analyzed using standard methods (APHA, 1995). NH$_3$ content is calculated based on NH$_4^+$ content and percentage of NH$_3$ according to pH and temperature.

Bacterial density: Total bacteria and Vibrio following the method of spreading samples on TCBS agar (Thiosulphate Citrate Bile Sucrose Agar) plates: One gram of sludge sample is transferred to a test tube contain 9mL of sterile physiological saline, mix well by Vortex about 1 minutes, given a $10^{-1}$ dilution. Continue dilution until a dilution is reached appropriate, start at $10^{-2}$ dilution only shake 30 seconds and allow to settle for 15 seconds. Later, 3 suitable dilutions have been selected for densities of total bacteria and bacteria *Vibrio* bacteria. From each diluted concentration, 100 μL of a water sample was sucked out and place into dishes TSA+ and TCBS medium, spread evenly with a glass rod. Each concentration dilution is repeated 3 times. After canopy media plates are incubated at 30°C for 24-28 hours and bacteria density were determined after incubation.

- Survival rate (%) = number of last individuals / first individuals * 100

- Absolute growth rate: DWG (g/day) = ($W_t - W_0$)/t

- Specific growth rate: SGR (% /day) = ((ln$W_t$ – ln$W_0$) / (t-t0)) * 100 (%/day)

- Absolute growth in length: DLG (cm/day) = ($L_t - L_0$)/t

- Relative growth in length: (%) = ((ln$L_t$ – ln$L_0$) / (t-t0)) * 100 (%/day)

   *In which:*

   $L_0$: The initial length of shrimp, $L_t$: Length of shrimp at time t, $W_0$: The initial weight of shrimp, $W_t$: Weight of shrimp at time t, t: Time to raise.

3. Results and Discussions

3.1 Temperature, pH and salinity

The temperature in the rearing tanks ranges from 26-28°C in the morning and 27-30°C in the pm. According to Whetstone et al. (2002), the temperature is appropriate for the development of vannamei shrimp about 23-34°C and according to Boyd et al., (2002) difference temperature deviation of day and night not more than 5°C during the day is considered optimal for the culture shrimp. During the experiment, the pH was at the treatments did not change significantly over time sampling times from 7.8-8.4. Briggs et al. (1994) [2] for that water with a pH of 7.5-8.5 is a dark condition optimal for nitrifying bacteria to grow. Salinity well maintained 15-17 ‰. Thus, the factors temperature, pH and salinity during the experiment is in the right range for development and shrimp growth.

3.2 Dissolved oxygen (DO)

Dissolved oxygen content ranges from 5.60 to 7.46 mg/L and tends to decrease gradually at the end of the experiment experience. DO content in T2 is highest (6.34 ± 0.07 mg/L) and lowest in T1, without supplementation bacterial supplement (5.65 ± 0.10 mg/L). According to Whetone et al. (2002), dissolved oxygen in water is ideal for Shrimp is above 5 mg/L and does not exceed 15 mg/L. Near the end of the experiment decomposition of matter The organic matter is strong, so we have used a lot of The oxygen dissolves more, making the amount of oxygen of T1 low than the additional treatment of B. subtilis bacteria. Thus, the content of dissolved oxygen in the experiment is in accordance with the development of shrimp.

3.3 Chemical oxygen demand (COD)

Figure 1 shows that the COD content is inversely opposite to the DO content increasing with the culture period. The COD content at T1 was the highest (32 ± 0.3 mg/L), which was statistically significant (p <0.05) compared with treatment T2 of 17.5 ± 4.1 mg/L. This result is similar to the study of Pham Thi Tuyet Ngan (2012) when arranging black tiger shrimp with the density of 30 shrimp / m2 in 500 L tank supplemented with bacteria B67, B41 (106 CFU/mL), the COD content in Treatment B41 (12.8 ± 4.0
mg/L) and B67 (12.9 ± 4.4 mg/L) were much lower than the control (14.0 ± 4.0 mg/L). COD of pond water may fluctuations range from 10-20 mg/L, normally it ranges from 40-80 mg/L and COD is suitable for aquaculture in 15-20 mg/L. Thus, the addition of *Bacillus subtilis* bacteria to the shrimp culture tank helped reduce organic pollution compared to the control tank, preferably the addition of *Bacillus.sp* bacteria.

![Figure 1. The fluctuation of COD during the experiment](image)

3.4 Total Ammonia Nitrogen (TAN)
The highest TAN content in T2 (6.02 ± 0.10 mg/L) and lowest in the treatment T1 formula (4.48 ± 0.10 mg/L). According to Whetstone et al. (2002) suggested that shrimp could exist and developed at TAN content ranging from 0.02-2 mg/L and according to Boyd et al. (2002), TAN in the lips pond field must be less than or equal to 3 mg/L. Probable cause is organic protein metabolism should create more NH₄⁺ and NH₃ by *Bacillus sp*. Thus, *Bacillus sp*. bacterias have participated process of decomposing organic material in rearing tanks better.

![Figure 2. The fluctuation of TAN during the experiment](image)
3.5. \textit{NH}_3
In this experiment, \text{NH}_3 \text{ ranged from 0 to 0.198 mg/L (Figure 3). NH}_3 \text{ content was highest in T2 (0.198 mg/L) and lowest in DC treatment (0.067 mg/L). NH}_3 \text{ in water bodies is provided by the breakdown of proteins, debris of zooplankton, animal waste products or from organic and inorganic fertilizers. Therefore, thanks to the activity of bacteria, this content in the supplemented bacteria tends to increase higher than the control. As the temperature and pH of water increase, NH}_3 \text{ content will increase and vice versa. According to Cold and Armstrong (1981), pH> 7 did not affect shrimp, but increased NH}_3 \text{ and NH}_3 \text{ were considered harmful to shrimp.}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{The fluctuation of \text{NH}_3 \text{ during the experiment}}
\end{figure}

3.6 Total Suspended Solids (TSS)
We can see that the total material suspended in the control pond is higher than the pond with probiotic. In the control ponds the total suspended matter increased rapidly at the time of 7 sampling onwards. In the time of 12 sample collection, total suspended matter was up to 4.6 mg/L, while in ponds with \textit{Bacillus} only 1.2 mg/L.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{The fluctuation of \text{NH}_3 \text{ during the experiment}}
\end{figure}
3.7 Fluctuation of total bacterial density
For ponds with microbiological supplement, the concentration of *Bacillus* is about $0.5 \times 10^3$ CFU/mL - $4.6 \times 10^3$ CFU/mL and higher than the control pond by 2-3 Log units and kept at $10^3$ to $10^4$ CFU/mL. The total density of bacteria in the control was lower than in the supplement *Bacillus* at the beginning of the experiment because there was no bacterial supplement every 5 days. The increase in total bacteria in the control pond was explained by the accumulation of organic matter which quickly increases the number of bacteria living in the culture environment, and also increases the density of the microorganisms. The bacteria cause disease over time. According to Anderson (1993), the total bacteria in clean water is usually less than $10^3$ CFU/mL and greater than $10^7$ CFU/mL in contaminated water.

![Figure 5. The fluctuation of total bacterial density during the experiment](image)

3.8 Fluctuation of total Vibrio
In this experiment (Figure 6), the vibrio density in the treatments ranged from $1-4.2 \times 10^3$ CFU/mL. Treatment T1 had the highest Vibrio density ($4.27 \times 10^3 \pm 404$ CFU/mL) compared to T2 with $1.38 \times 10^2 \pm 12.5$ CFU/mL. According to Aftabuddin et al. (2013), the density of Vibrio control treatment was significantly higher than that of *Bacillus*. The reason for the high increase in T1 treatment was the accumulation of waste of shrimp and leftovers during the experiment to create favorable conditions for Vibrio to develop. This result shows that the addition of *Bacillus sb*. bacteria during the experiment reduced the vibrio concentration in the culture system. This is consistent according to Moriarty (1998), *Bacillus* supplementation can control Vibrio, increase shrimp survival rate, limit pathogens caused by Vibrio sp. domestic. Usually the density of Vibrio in tanks or shrimp ponds without water exchange can reach $> 10^5$ CFU/mL.
3.9 Shrimp growth after 60 days

3.9.1 Growth in weight With an average initial shrimp weight of 0.034 g, after 60 days of culture, the average shrimp weight was 4.55-6.55 g, corresponding to the absolute growth rate (DWG) of 0.078-0.098 g/day and the relative growth rate (SGR) is 0.17-0.19 %/day (Table 1). Statistical results showed that the control treatment did not add bacteria to the shrimp pond with the lowest weight and growth rate and significant difference (p <0.05) compared to the treatments with adding bacteria to the rearing tank.

Table 1. Growth in weight of shrimp after 60 days of culture

| Pond | $W_0$ (g)     | $W_{60}$ (g)  | DWG (g/day) | SGRw (%/day) |
|------|---------------|---------------|-------------|--------------|
| T1   | 0.034±0.015   | 4.55±0.17     | 0.078±0.009 | 8.10±0.016   |
| T2   | 0.034±0.015   | 6.55±0.29     | 0.098±0.012 | 8.62±0.010   |

3.9.2 Growth in length Table 2 shows that the shrimp had an average initial length of 1.78 cm and at the end of the experiment the average length of the treatments was 9.50-11.20 cm, the absolute length growth (DLG) reached 0.121-0.158 cm/day, the relative growth rate reached 2.78-3.14 %/day. The statistical analysis showed that the length and DLG in the control treatment were significantly lower than the other treatments.

Table 2. Growth in weight of shrimp after 60 days of culture

| Pond | $L_0$ (cm) | $L_{60}$ (cm) | DLG (cm/day) | SGR$_L$ (%/day) |
|------|------------|---------------|--------------|-----------------|
| T1   | 1.78±0.19  | 9.50±0.75     | 0.121±0.012  | 2.78±0.011      |
| T2   | 1.78±0.19  | 11.20±0.63    | 0.158±0.011  | 3.14±0.010      |
4. Conclusions
The addition of Bacillus subtilis bacteria helps to accelerate the decomposition of organic matter in the shrimp culture environment, helps reduce organic pollution of COD, TAN, NH₃ and TSS in the tank environment very well. Total Vibrio bacteria of the added bacteria treatment was always lower than the test control formula. Shrimp in the treatments supplemented with beneficial bacteria had growth and rate survival was significantly higher than shrimp in the control treatment. The use of Bacillus sp. as probiotics has high scientific and practical value, bringing many environmental and economic benefits.

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