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Original Article

Evaluation of *Pseudomonas stutzeri* AM1 and *Pseudomonas oleovorans* ST1.1 isolated from shrimp pond sediments as probiotics for whiteleg shrimp, *Litopenaeus vannamei* culture

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Abstract: This study aimed to isolate the probiotic potential of nitrifying bacterial strains and to evaluate their effects on water quality and growth performance of the whiteleg shrimp, *Litopenaeus vannamei*. Based on an initial screening of 100 isolates identified from sediment samples, 12 strains could remove nitrogen compounds and two strains (*Pseudomonas stutzeri* AM1 and *P. oleovorans* ST1.1) showed highly efficient nitrogen removal ability. Within 96 h, total ammonia nitrogen (TAN) removal efficiency in the two strains was 28.0-31.6% and 21.5-24.9%, respectively. The water addition of 10^3 CFUmL^{-1} of *P. stutzeri* AM1 (T1) and *P. oleovorans* ST1.1 (T2) effectively reduced TAN, nitrite, nitrate, and total sulfide and increased the survival rate and biomass of shrimp. However, no significant differences were found between the control (T0) and treatment groups (T1 and T2) in the final weight, weight gain and specific growth rate of shrimp. Overall, *P. stutzeri* AM1 (T1) and *P. oleovorans* ST1.1 used as water supplements improved water quality and the survival rate of whiteleg shrimp.

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Introduction

Shrimp farming is one of the fastest-growing aquaculture sectors in many countries worldwide. In Vietnam, the whiteleg shrimp, *Litopenaeus vannamei* is a hugely popular farmed shrimp; in 2020, this species's farming area and production reached 113,418 ha and 900,000 tons, respectively, increasing by 12% over the same period in 2019 (MARD, 2021). In recent years, environmental problems caused by poor water quality are one of the threats to shrimp production (Wang et al., 2018). Through several studies, probiotics have demonstrated significant potential as therapeutic options for improving water quality (Cai et al., 2019; Nimrat et al., 2019; Kim et al., 2021). Probiotics in aquaculture can be administered through water additives or feed supplements during the rearing of aquatic species (Zhou et al., 2009; Leyva-Madrigal et al., 2011). A variety of bacteria (*Bacillus*, *Lactobacillus*, *Lactococcus,* *Entercoccus*, *Clsotridium*, *Aeromonas*, and *Pseudomonas*), yeasts, and unicellular algae have been studied for use as probiotics in aquaculture (Irianto and Austin, 2002; Vijayan et al., 2005).

Members of the genus *Pseudomonas* are commonly found in aquatic environments, including shrimp culture ponds (Preetha et al., 2015). The members of this genus are Gram-negative rod with polar flagella providing motility, oxidase-positive, and catalase-positive, and it is obligate respiratory (Jurat-Fuentes and Jackson, 2012). Based on the results of *Pseudomonas* application in several studies, these species have been proven to control pathogens, enhance growth performance, immune system function, and improve environmental conditions in the culture systems favorable to the

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species being cultured (Preetha et al., 2015; Hai et al., 2009). In addition, Dou et al. (2021) suggested that *Pseudomonas* species possess great potential to remove nitrogen from wastewater based on aerobic autotrophic nitrification and anaerobic heterotrophic denitrification processes. For example, Jin et al. (2015) reported that *Pseudomonas* sp. ADN-42, isolated from soil, has good nitrogen removal performance. Similar results were found in *P. stutzeri* (Wen et al., 2010), *P. putida* Y-9 (Xu et al., 2017), and *P. putida* ZN1 (Zhang et al., 2018).

The present study aimed to isolate and identify efficacious probiotic *Pseudomonas* spp. from shrimp pond sediments and examine their efficacy to remove nitrogen and enhance growth performance, survival, and water quality in whiteleg shrimp *in vitro* and *in vivo* conditions. Two strains, *Pseudomonas stutzeri* AM1 and *P. oleovorans* ST1.1, obtained in this study, exhibits huge potential as a probiotic candidate, as they effectively improved shrimp survival and water quality. The present study's findings can be applied to enhance health status and water quality in the shrimp culture industry.

Materials and Methods

Isolation of candidate probiotic bacteria: Sediment samples were collected from extensive shrimp ponds in Soc Trang, Tra Vinh, and Kien Giang provinces, and *Pseudomonas* spp. were isolated from the collected samples using AOB and NOB media (Chankaew et al., 2017). *Pseudomonas* isolates were screened by applying the nitrogen removal tests (Yang et al., 2011). They were identified using 16S rRNA sequencing according to the manufacturer's instructions after PCR amplification using a 27F-CM primer (5′ - AGAGT TTGATCMTGG CTCAG - 3′) and a 1492R primer (5′ - TACGGYTACCTTGTT ACGACTT - 3′) (Frank et al., 2008). The purified products were sequenced by the Nam Khoa Biotek company, Ho Chi Minh City, Vietnam. Sequences were analyzed through http://www.ncbi.nlm.nih .gov/blast by comparing them with bacterial 16S rRNA sequences in Genbank to identify species.

Experimental design

Experiment 1: *In vitro* evaluation of nitrogen removal efficiency of *P. stutzeri* AM1 and *P. oleovorans* ST1.1: Wastewater was collected from shrimp pond and analyzed for initial concentrations of TAN, NO_2^--N, and NO_3^--N. A single colony of strains AM1 and ST1.1 were cultivated in a Luria Bertani broth medium and incubated with shaking until the stationary phase. The bacterial cells were harvested by centrifuging at 3000 rpm for 10 min at 4℃ and washed twice with 0.9% sterile NaCl solution. Then, the cells were re-suspended in sterile saline (0.9% NaCl) and adjusted to achieve a bacterial cell density of 1×10^8 CFU mL^{-1} used for experiments (Lami et al., 2020).

The two-candidate probiotic bacteria were tested on the Duran bottle scale for nitrogen removal efficiency. *Pseudomonas stutzeri* AM1 (T1, T2, and T3) and *P. oleovorans* ST1.1 (T4, T5, and T6) at different concentrations (10^3, 10^4, and 10^5 CFUmL^{-1}) were inoculated into 200 mL of wastewater in 250 mL Duran bottle shaken at 150 rpm, 30ºC for four days. Each treatment was run in triplicates. Water parameters, including TAN, NO_2^--N, NO_3^--N, pH, and temperature, were measured daily to calculate the nitrogen removal efficiency using the formula of Nitrogen removal (%) = $((C_i - C_t) / Ci) \times 100$, Where C_i is the initial concentration of nitrogen and C_t is the concentration of nitrogen after sampling time.

Experiment 2: Evaluation of *P. stutzeri* AM1 and *P. oleovorans* ST11 on water quality and growth performance of whiteleg shrimp: Based on experiment 1, *P. stutzeri* AM1 and *P. oleovorans* ST1.1 at a concentration of 10^3 CFUmL^{-1} were used for experiment 2. This experiment was carried out in the wet lab of the College of Aquaculture and Fisheries, Can Tho University. Shrimp were fed a commercial diet (40% crude protein, 6% crude lipids, and 4% ash) for 7 days. After acclimatization, 100 shrimp (0.66±0.01 g) were randomly stocked into each composite tank, with a capacity of 500 L. The groups were given three treatments: the control group (T0) was not supplemented with probiotics,

Table 1. Nitrogen removal performance of the isolated nitrifying bacteria.

AOB Strains	Ammonia removal (%)	NOB Strains	Nitrite removal (%)
CN2.1	69	AM1	76.6
CN6.1	66.3	CN6.2	74.7
CN8.1	69.8	CN7.1	74.7
ST1.1	73.2	TB3.2	72.1
TB7.2	64.6	TB7.1	70.5
TV3.1	62.1	TV4.2	71.3

and the two other experimental groups were cultured in water supplemented every three days with probiotics (10^3 $CFUmL^{-1}$) containing *P. stutzeri* AM1 (T1) and *P. oleovorans* ST1.1 (T2), respectively. Each group consisted of three replicates. The commercial feed was supplied to the shrimp three times daily at 3-5% body weight.

Experiments were performed with a 50% water exchange twice a week and lasted for 30 days. Each tank was supplied with constant aeration during the experimental period to maintain the dissolved oxygen concentration (>4 mgL^{-1}), and the salinity was maintained at 20 ppt. The water temperature and pH were 28.8-30.0ºC and 7.8-8.3, respectively. Total alkalinity, TAN, NO_2^--N, $NO_3^—$N, and total sulfide (S^{2-}) were analyzed two times a week following the procedures of the standard methods (APHA, 2017). At the end of the experiment, the final weight (FW), weight gain (WG), daily weight gain (DWG), specific growth rate (SGR), survival rate (SR), and shrimp biomass (B) for all groups were measured and calculated following the method described by Niu et al. (2015):

WG (g) = Final weight - Initial weight

DWG (g day^{-1}) = [Final weight - initial weight]/days of culture

SGR (% day^{-1}) = 100 × (Ln final mean weight - Ln initial mean weight)/days of culture

SR (%) = 100 × [number of final shrimp / number of initial shrimp]

B ($kg.m^{-3}$) = [final weight × final population]/volume of water

Statistical analysis: Statistical analysis was carried out using SPSS ver. 22 (SPSS Inc. Chicago, IL, USA), and the results were presented as mean ± standard error (SE). All data were subjected to one-way analysis of variance (ANOVA), and Tukey's multiple comparisons test was then used to identify significant differences between treatments. All statistical significance tests were at the $P<0.05$.

Results

Isolation of probiotic bacteria: Hundreds of microbial strains were isolated from the samples collected from the sediment of shrimp ponds. Six strains, CN2.1, CN6.1, CN8.1, ST1.1, TB7.2, and TV3.1 showed a high ammonium removal ability (AOB), whereas strains AM1, CN6.2, CN7.1, TB3.2, TB7.1, and TV4.1 demonstrated high nitrite removal efficiencies (NOB) (Table 1). The ST1.1 and AM1 strains had the highest nitrogen removal ability among the twelve bacterial strains and were selected for further study. After BLAST analysis, partial 16S rRNA gene sequences analysis revealed 99% homology of isolates ST1.1 and AM1 with *P. oleovorans* and *P. stutzeri*, respectively.

The nitrogen removal performance of strains AM1 and ST1.1: Temperature and pH ranged 28.8-29.5ºC and 7.5-7.8, respectively. The aerobic inorganic nitrogen removal capacity of the strains ST11 and AM1 was shown in Figure 1. In both strains ST1.1 and AM1, the removal efficiencies of TAN, NO_2^--N, and NO_3^- -N during 96 h were 21.5-31.6, 3.5-14.4, and 8.1-19.2%, respectively. Although TAN, NO_2^--N, and NO_3^--N were reduced in all treatments after 24 and 48h, no significant differences were found between treatments ($P>0.05$). After 96 h of incubation, TAN in the treatments supplemented with strains ST1.1 and AM1 at different concentrations (T1, T2, T3, T4, T5, and T6) was significantly reduced than the control treatment. There were no significant differences

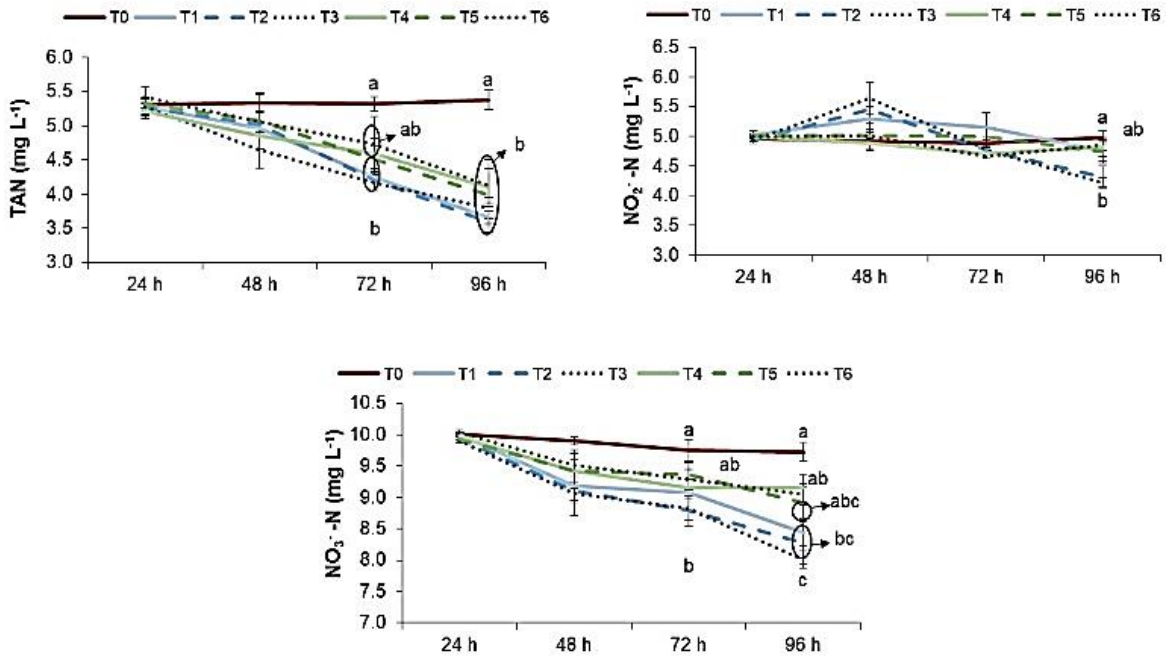

Figure 1. Nitrogen removal performances of strains AM1 and ST11 during the experimental period.

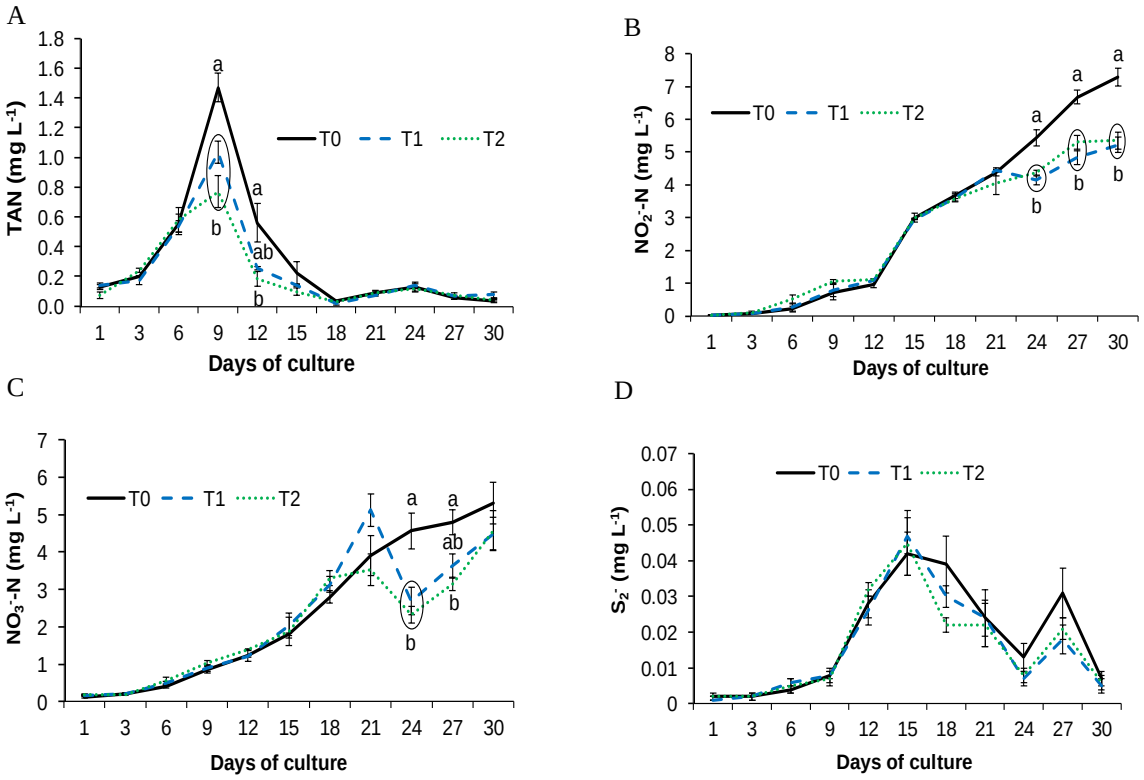

Figure 2. Concentrations of TAN, NO_2^--N, and NO_3^- -N in different treatments during the experimental period.

between T1, T2, T3, T4, T5, and T6 (P>0.05). The higher removal efficiency and faster removal rate of NH_4^+-N indicated that strains ST1.1 and AM1 possessed a higher potential for heterotrophic nitrification.

Effects of *P. stutzeri* AM1 and *P. oleovorans* ST1.1 on water quality parameters: Water temperature, pH, DO, and alkalinity in each group were in a

Table 2. Water temperature, pH, DO, and alkalinity during the experimental period.

Parameters	Treatments		
	T0	T1	T2
pH	7.87±0.05[a]	7.79±0.07[a]	7.8±0.08[a]
Temperature (°C)	28.1±0.3[a]	28.1±0.3[a]	28.1±0.3[a]
DO (mg L^{-1})	4.2±0.07[a]	4.21±0.08[a]	4.19±0.06[a]
Alkalinity (mg$CaCO_3$ L^{-1})	137.8±4.0[a]	137.5±4.1[a]	137.4±4.0[a]

Values shown are mean ± SE. Mean values within a row followed by the same letters show that there is no significant difference among the groups (P>0.05).

Table 3. Growth performance and survival rate of the whiteleg shrimp.

Parameters	Treatments		
	T0	T1	T2
Initial weight (g)	0.66±0.01[a]	0.67±0.01[a]	0.65±0.01[a]
Final weight (g)	5.28±0.09[a]	5.54±0.09[a]	5.45±0.14[a]
WG (g)	4.62±0.08[a]	4.87±0.08[a]	4.8±0.13[a]
DWG (g day^{-1})	0.154±0.003[a]	0.162±0.003[a]	0.16±0.004[a]
SGR (% day^{-1})	6.92±0.04[a]	7.06±0.06[a]	7.07±0.05[a]
Survival rate (%)	74.3±1.8[b]	88.7±3.5[a]	78.3±2.2[ab]
Biomass (kg m^{-3})	0.98±0.01[b]	1.23±0.03[a]	1.07±0.04[b]

Values shown are mean ± SE. Mean values within a row followed by the same letters show that there is no significant difference among the groups (P>0.05).

proper range for shrimp growth and survival from the beginning until the end of the experiment, and there were no significant differences between groups (Table 2). TAN in all groups reached the highest concentrations on day 9 of the experiment, and significant differences were found among treatments. TAN in the control group was significantly higher than the T1 and T2 (P<0.05). The TAN concentrations in all groups decreased later on day 12 and remained quite stable until the end of the experiment (Fig. 2A). The concentrations of NO_2^--N in all groups gradually increased during the experimental period, and reached a peak at the end of the experiment. NO_2^-N concentrations in two experimental groups inoculated with probiotics (T1 and T2) were significantly lower than those of the control group on days 24, 27, and 30, but no significant difference was found between the two probiotic treated groups (P>0.05) (Fig. 2B). The concentrations of NO_3^--N in all treatments varied from 10 to 8 mg L^{-1}. NO_3^--N in the T1 and T2 were significantly lower than those in the control group on days 21 and 24 (P<0.05). However, there were no significant differences among all treatments on the other sampling days (P>0.05) (Fig. 2C). Total sulfide (S^{2-}) in all treatments gradually increased from the beginning of the experiment until day 15 and decreased sharply later. No significant differences in total sulfide were found between treatments (Fig. 2D).

Effects of *P. stutzeri* AM1 and *P. oleovorans* ST1.1 on growth performance of the whiteleg shrimp: Table 3 shows the effects of inoculating *P. stutzeri* AM1 and *P. oleovorans* ST1.1 into the experimental tanks' water on the survival and growth performance of whiteleg shrimp. The results showed that the experimental group inoculated *P. stutzeri* AM1 (T1) showed a significantly higher survival rate and biomass compared with the control group (P<0.05). However, no significant differences were found in these parameters between T2 and the control groups. Similarly, there were no significant differences in the FW, WG, and SGR between all groups (P>0.05).

Discussion

The role of probiotics in controlling the colonization

and overgrowth of pathogens has been documented in many studies (Moriarty, 1999). In addition, the application of probiotics in aquaculture also enhances innate immunity (Chauhan and Singh, 2019), prevents microbial diseases (Meidong et al., 2018), promotes growth and improves water quality (Ma et al., 2009; Kolndadacha et al., 2011). Zhang et al. (2011) and Tran et al. (2019) reported that several *Pseudomonas* species could remove nitrogen. In the present study, two bacterial isolates, AM1 and ST1.1 showed the highest efficiency in removing nitrogen. According to 16S rRNA sequencing, these candidates were identified as *P. stutzeri* and *P. oleovorans*. Based on *in vitro* results, the concentration of NH_4^+-N, NO_2^--N, and NO_3^--N decreased significantly after 96 h incubation, suggesting that strains AM1 and ST11 have a strong ability to oxidize and assimilate nitrogen in the water. Similar results were also reported with strains of *P. putida* Y-9 (Xu et al., 2017), *Pseudomonas* sp. (Tran et al., 2019), and *P. fluorescens* NB14 (Duo et al., 2021). Yang et al. (2017) reported that the removal efficiency of total nitrogen was directly related to bacterial growth. In addition, the results of this study indicated that strain AM1 has a strong ability to remove nitrogen compounds than ST11.

In water-supplemented conditions, the effects of *P. stutzeri* and *P. oleovorans* application had no impact on shrimp growth parameters (final weigh, WG, DWG, and SGR), but the survival rate and biomass of shrimp in the treatment supplemented with *P. stutzeri* AM1 (T1) were significantly greater than those in control one. These results suggest that the application of *P. stutzeri* AM1 via water addition improved the survival of whiteleg shrimp. This observation is similar to Balcázar et al. (2007) report, where the application of *P. aestumarina* SLV22 at 10^5 CFU g^{-1} diet for 28 days enhanced the survival rate of whitelegs shrimp. Ngo and Ravi (2009) also reported that *Penaeus latisulcatus* shrimp fed *P. synxantha* and *P. aeruginosa* (2×10^6 CFU kg^{-1} diet) diets for 84 days had a significantly higher survival rate. Some previous studies demonstrated that the increased survival of shrimp supplemented with probiotics (*Lactobacillus plantarum, Bacillus* sp., *Pediococcus acidilactici, P. fluorescens)* might be related to the decreased abundance of *Vibrio* spp. in the digestive tract (Lone *et al*., 1999; Wang et al., 2007**;** Castex et al., 2008; Vanmaele et al., 2015). In addition, Borges et al. (2008) reported that probiotics promoted the health of animals through improved water quality. In our study, water quality parameters, including pH, temperature, DO and alkalinity were within suitable ranges for the culture of whiteleg shrimp (Law 1988; Wybanet al., 1995; Nonwachaiet al., 2011; Boyd, 2007) and were not significantly different between the treatments. However, the TAN, NO_2^--N, NO_3^--N, and total S^{2-} concentrations were lower when the probiotic (*P. stutzeri* or *P. oleovorans*) was applied compared with no probiotic application. This could be suggested that *P. stutzeri* and *P. oleovorans* played an important role in improving water quality.

In conclusion, based on the *in vivo* and *in vitro* experiments, the two candidate probiotics *P. stutzeri* AM1 and *P. oleovorans* ST11 isolated from the shrimp pond sediment, had the clear ability to remove nitrogen compounds in wastewater from shrimp culture. Additionally, these isolates also effectively enhanced the survival rate of shrimp and water quality, which is very important for application in shrimp farms. Therefore, these strains can be used as a potential probiotic in aquaculture.

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