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Original Research Articles

Isolation of *Lactobacillus plantarum* CMT1 from shrimp intestine and its effects on growth and survival of the whiteleg shrimp, *Litopeneaus vannamei*

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Probiotics are widely applied in aquaculture, which can improve the growth, survival and health of aquatic animals. The aim of this study was to isolate and evaluate a *Lactobacillus* strain from the intestine of healthy whiteleg shrimp, *Litopenaeus vannamei*. A total of 20 *Lactobacillus* isolates showed rod-shaped morphology, gram-positive, non-motile, non-spore forming, and catalase and oxidase negative. Among the isolates, strain CMT1 exhibited the highest antibacterial activity against *Vibrio parahaemolyticus* and extracellular enzyme activity, which was selected and identified as *Lactobacillus plantarum* CMT1 based on 16S rDNA gene sequencing. This strain was also chosen to evaluate its effects on growth and survival of the whiteleg shrimp as a feed supplement. Four different diets were prepared, including the control (a commercial diet without *L. plantarum* CMT1) and three different concentrations of *L. plantarum* CMT1, viz. T1 (10^6 CFU kg diet^{-1}), T2 (10^7 CFU kg diet^{-1}), and T3 (10^8 CFU kg diet^{-1}). After a 56-day feeding trial, the growth parameters in the T3 group had the highest values and showed a significant difference compared to the other groups. The survival rates of shrimp fed T2 and T3 diets were significantly higher than those in the control diet ($p < 0.05$). The lowest FCR value was recorded in the T3 group and showed a significant difference compared to the control group ($p < 0.05$). In addition, *Lactobacillus* counts in T2 and T3 groups increased significantly from day 28, which indicated that *L. plantarum* CMT1 could sustain its population in the intestine of shrimp. However, the total *Vibrio* sp. count in the T2 and T3 groups was significantly lower than that in the control group ($p < 0.05$). In conclusion, *L. plantarum* CMT1 could be used as a potential probiotic for shrimp aquaculture, and the recommended level of the probiotic strain CMT1 is 10^8 CFU kg diet^{-1}.

INTRODUCTION

Shrimp aquaculture in Vietnam has continuously developed during the last few years, which is an economically important part of the aquaculture industry. According to the Directorate of Fisheries,[1] the production of shrimp was reported to increase from 1.06 million metric tons in 2022 to 1.12 million metric tons in 2023. However, with intensive and super-intensive cultivation technology, shrimp farming is facing several challenges with regard to disease outbreaks and water quality degradation. In the past decades, the use of antibiotics and other chemicals has been commonly applied to control bacterial diseases and enhance the growth of cultured species.[2] Reverter et al.[3] reported that antibiotic overuse may result in other problems such as environmental effects, food safety, and bacterial multidrug resistance. As a result, many alternative findings have been developed to reduce the use of antibiotics in shrimp culture. In the recent past, probiotics have been considered one of the most promising ways to control disease and improve growth, survival, and water quality.[4]

Lactobacillus are lactic acid producing bacteria that have been commonly used as probiotic candidates in the aquaculture industry.[5] They could be found in fermented foods and in the gastrointestinal tracts of fish, prawns, and shrimp.[6] The species of the genus *Lactobacillus* are gram-positive, non-motile, and non-sporulation bacteria that can produce lactic acid. Normally, *Lactobacillus* species could be administered to aquatic animals as a feed additive. They

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are capable of inhibiting pathogenic bacteria, improving the intestinal microbial balance, producing digestive enzymes, and promoting growth performance and disease resistance.[5] Several studies have reported that dietary supplementation of *L. plantarum* could improve growth performance, immune parameters, and disease resistance in fish species such as rainbow trout, common carp, and Nile tilapia.[7-9] In a recent study, Zheng et al.[10] demonstrated that dietary administration of *L. plantarum* improved the composition and diversity of beneficial bacteria in the gut of whiteleg shrimp. Moreover, the whiteleg shrimp fed *L. plantarum* were isolated from kefir, which could enhance their survival, growth, and immune response.[11] Although *L. plantarum* has been widely used as a probiotic in whiteleg shrimp culture, most of them are non-shrimp sourced, which could not show the most effective results. This is because the selected strain of probiotic is one of the crucial factors for evaluating probiotic effectiveness.[12]

The objective of this study is to screen and isolate potential probiotic *L. plantarum* from the intestine of healthy whiteleg shrimp through *in vitro* assays. The isolated strain was screened based on morphology, biochemical characteristics, antimicrobial activity, and extracellular enzyme production. In addition, an *in vivo* experiment was also conducted to assess the effects of *L. plantarum* CMT1 on the growth performance and survival of whiteleg shrimp when administered in feed.

MATERIALS AND METHODS

ISOLATION AND SCREENING OF *LACTOBACILLUS* SPP.

A total of 120 whiteleg shrimp (8-10 g) were collected from six different extensive and semi-intensive ponds in Dam Doi district, Ca Mau province, Vietnam. Shrimp samples were rinsed with 70% ethanol. Each whole gut of shrimp was dissected out and homogenized in an Eppendorf tube with 1.5 ml of sterile saline solution (0.85% NaCl). Then, 100 µL of homogenate was aseptically spread onto De Man–Rogosa–Sharpe (MRS) agar plates, and incubated at 37°C for 24–72 hours. After incubation, each colony was purified on new MRS plates by the streaking method. All isolates were determined Gram-stain, catalase, oxidase, motility, and spore-forming tests based on the methods of Mohamad et al.[12] The isolated strains were stored at -80°C in Eppendorf tubes containing MRS broth with 20% glycerol for further study.

The genomic DNA of *Lactobacillus* isolates was extracted using a genomic DNA purification kit following the manufacturer's instructions. The 16s rDNA gene was amplified using 27F (5'-AGAGTTTGATCCTGG CTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') primers. Sequences identified were compared directly with sequences obtained from Gen Bank using BLAST at the NCBI.

ANTIMICROBIAL ACTIVITY OF THE ISOLATED STRAINS

The antimicrobial activity of *Lactobacillus* isolates against pathogenic bacteria, *Vibrio parahaemolyticus* was performed using the agar disk diffusion method described by Noordiana et al.[13] *Vibrio parahaemolyticus* was stored in the lab of aquatic probiotic, College of Aquaculture and Fisheries, Can Tho University. In brief, the fresh cultures of isolated *Lactobacillus* and *V. parahaemolyticus* were cultured in MRS broth and TSB broth, respectively. After incubation at 37°C for 24 h, the bacteria were collected by centrifugation at 10000 rpm for 4 min and then diluted with sterile saline solution to obtain an absorbance of 0.8 at 600 nm using a spectrophotometer (Shimadzu UV-1900i, Malaysia), which was equivalent to a density of 10^7 CFU mL^{-1} based on the standards bacterial growth curves (data not shown). Then, 100 µL of pathogen solution was spread over MHA plates. Four wells of 6.0 mm in diameter were made and filled with 100 µl of *Lactobacillus* isolates. The plates were incubated at 37°C for 24 h, and the inhibition zone diameter was measured in millimeters by caliper. The antimicrobial activity test was conducted in two replicates.

SCREENING OF EXTRACELLULAR ENZYME ACTIVITY OF THE ISOLATED STRAINS

Three isolated strains with high antibacterial activity were selected and cultured in MRS broth at 37°C for 24 h. After incubation, cell-free supernatant (CFS) was harvested to measure extracellular α-amylase, protease, and leu-aminopeptidase activity. Amylase activity was determined based on the method of Bernfeld et al. (1955) with 2% starch solution as the substrate. The activity of protease was measured according to the method of Lowry et al.,[14] using the casein hydrolysis method. All enzyme activities were expressed as U mg $protein^{-1}$. The concentration of soluble protein was determined following the method of Lowry et al.,[14] using bovine serum albumin as a standard.

EFFECT OF *LACTOBACILLUS PLANTARUM* CMT1 ON GROWTH AND SURVIVAL OF WHITELEG SHRIMP

Juveniles whiteleg shrimp (0.90 ± 0.05g) were obtained from a local hatchery in Can Tho city, Vietnam, and acclimatized to a 4000-L composite tank for 5 days prior to the experiment. After acclimatizing, shrimp were randomly distributed to 12 composite tanks (500 L capacity) in recirculating aquaculture systems. Each tank contained 100 shrimp in 400 L of water at 15 ppt salinity. Shrimp were fed with different experimental diets four times a day (6:00, 12:00, 17:00, and 20:00). Dietary experiments included a commercial diet (Grobest Landfound Co. Ltd., Vietnam) without *L. plantarum* CMT1 (control), T1, T2, and T3 containing 10^6, 10^7 and 10^8 CFU kg^{-1} *L. plantarum* CMT1, respectively. The experimental diets were prepared as described by Zheng et al.[10] The bacteria solution was sprayed and mixed homogeneously to a commercial feed (Grobest Landfound Co. Ltd., Vietnam) to reach the desired concentration. The bac-

terial density in the diets were then checked using MRS plate count method. The amount of uneaten feed was collected after 1 h of feeding and dried in an oven at 80℃. During a 56-day feeding experiment, water quality, including temperature and pH, was checked daily using a Hana HI98128 pH/temperature tester. Dissolved oxygen (DO), total ammonia nitrogen (TAN), and nitrite (NO_2^--N) were weekly determined following APHA.[15] At the end of the experiment, growth parameters such as initial weight, final weight, weight gain (WG), daily weight gain (DWG), specific growth rate (SGR), feed conversion ratio (FCR), and survival rate (%) were calculated based on a method previously described by Tu et al.[16] In this experiment, three shrimp from each tank were randomly sampled every week for the determination of intestinal bacterial counts. Briefly, the intestine of shrimp was weighed and homogenized in sterile normal saline (0.85%). An aliquot of 100 µL from appropriated dilutions was spread on MRS agar and TCBS agar plates to determine total *Vibrio* sp. and *Lactobacillus* sp., respectively.

STATISTICAL ANALYSIS

All experimental data were analyzed by one-way analysis of variance (ANOVA) and a Tukey multiple range test using SPSS 22.0 software. Statistically significant level was set at $p < 0.05$. Values were presented as mean ± standard deviation (SD).

RESULTS

ISOLATION AND CHARACTERIZATION OF *LACTOBACILLUS* CANDIDATES

A total of 20 *Lactobacillus* isolates (CMT1-CMT20) were obtained from the intestine of healthy whiteleg shrimp. All isolates were gram-positive microorganisms with a rod morphology. The isolated strains showed negative results for catalase, oxidase, motility, and spore-forming tests (**Table 1**). During the preliminary screening, ten of 20 isolates exhibited antimicrobial activity against *V. parahaemolyticus*. Among these 10 isolates, only 3 isolates, CMT1, CMT6, and CMT20 were selected for further study due to their strong antimicrobial performance. The isolated bacterial strain was first identified as *Lactobacillus* sp. based on its morphology, biochemical characteristics, antibacterial activity, and extracellular enzyme activity. PCR analysis using 16S rDNA gene sequences showed 99% identity with *Lactobacillus plantarum* for CMT1.

EXTRACELLULAR ENZYME ACTIVITIES

The highest amylase activity (U mg protein^{-1}) was recorded in strain CMT1, and there was a significant difference as compared with the strains CMT6 and CMT20 (**Figure 1A**). Similar results were observed in protease activity. Specifically, the value in strain CMT1 was significantly higher than that in strains CMT6 and CMT20 (**Figure 1B**).

EFFECTS OF *LACTOBACILLUS PLANTARUM* CMT1 ON GROWTH PERFORMANCE AND SURVIVAL OF WHITELEG SHRIMP

The effect of *L. plantarum* CMT1 on water quality in shrimp tanks is presented in **Table 2**. Water temperature, pH, DO, and alkalinity in each tank were within a suitable range for shrimp growth and survival, and there was no significant difference among all groups. Although TAN and NO_2^--N values in the control group were slightly higher than those of the other groups, no significant difference was found.

During the experimental period, the total *Lactobacillus* count in the intestine of shrimp significantly increased from day 28 of the experimental period (**Figure 2A**). Significant differences in *Lactobacillus* count were found among T2, T3, and the control groups at days 28, 42, and 56 ($p < 0.05$). As for *Vibrio* counts, the levels were significantly decreased ($p < 0.05$) by increasing the dose of *L. plantarum* CMT1 supplementation. To be specific, the number of *Vibrio* in the T2 and T3 groups was lower than that in the control group (**Figure 2B**).

After a 56-day feeding period, the final weight, WG, DWG, and SGR were significantly enhanced in the T3 group (10^8 CFU kg diet^{-1}) compared to the control, T1, and T2 groups (**Table 3**). However, there were no significant differences in final weight, WG, and DWG between the control and T1 groups or between T1 and T2 groups ($p > 0.05$). Additionally, the survival rates of shrimp fed T2 and T3 diets were significantly higher than those of the control group. Conversely, the lowest FCR value (1.24±0.72) was observed in the T3 group, and there was a significant difference as compared with the control group ($p > 0.05$).

DISCUSSION

Probiotics are one of the potential solutions to support the development of sustainable shrimp farming practices. Therefore, the use of probiotics in shrimp culture has been increasing from day to day. In fact, the application of probiotics in sufficient quantities helps to enhance the growth performance, feed conversion efficiency, immune responses, disease resistance, and intestinal health of aquatic organisms, including shrimp species.[17,18] According to Mohamad et al.,[12] most *Lactobacillus* species were commonly detected in the intestinal tracts of shellfish, crabs, and shrimp. Therefore, the digestive gut of a healthy shrimp could be the best source for the isolation of potential probiotic strains. In the present study, 20 *Lactobacillus* isolates were identified based on morphological and biochemical tests. Due to the strong antimicrobial activity against *V. parahamolyticus* and extracellular enzyme activity, the isolated strain CMT1 was selected and confirmed as *L. plantarum* using 16S rDNA gene sequencing, which was further used for evaluation on a laboratory scale in feed application.

The present result showed that the application of feed supplemented with *L. plantarum* CMT1 significantly enhanced the growth performance of the whiteleg shrimp, wherein growth parameters in the T3 group (10^8 CFU kg^{-1})

Table 1. Morphology and antimicrobial activity against *Vibrio parahaemolyticus* of 20 *Lactobacillus* spp.

No.	Isolated strains	Cell shape	Gram staining	Catalase test	Oxidase test	Motility test	Spore formation ability	Antibacterial activity (mm)
1	CMT1	short-rod	+	-	-	-	-	12.1 ± 0.3
2	CMT2	long-rod	+	-	-	-	-	8.0 ± 0.3
3	CMT3	long-rod	+	-	-	-	-	ND
4	CMT4	short-rod	+	-	-	-	-	ND
5	CMT5	short-rod	+	-	-	-	-	7.0 ± 0.2
6	CMT6	short-rod	+	-	-	-	-	9.3 ± 0.1
7	CMT7	short-rod	+	-	-	-	-	8.6 ± 0.2
8	CMT8	long-rod	+	-	-	-	-	ND
9	CMT9	long-rod	+	-	-	-	-	ND
10	CMT10	long-rod	+	-	-	-	-	7.1 ± 0.4
11	CMT11	long-rod	+	-	-	-	-	ND
12	CMT12	long-rod	+	-	-	-	-	ND
13	CMT13	short-rod	+	-	-	-	-	8.6 ± 0.3
14	CMT14	short-rod	+	-	-	-	-	7.3 ± 0.1
15	CMT15	short-rod	+	-	-	-	-	ND
16	CMT16	short-rod	+	-	-	-	-	ND
17	CMT17	short-rod	+	-	-	-	-	ND
18	CMT18	long-rod	+	-	-	-	-	7.6 ± 0.1
19	CMT19	short-rod	+	-	-	-	-	ND
20	CMT20	short-rod	+	-	-	-	-	9.1 ± 0.1

(ND: non-detected)

were significantly higher than those in the other groups. Similar results were reported in other species, including rainbow trout (*Oncorhynchus mykiss*) fed *L. plantarum* at 2 × 10^7 CFU g^{-1} feed for 72 days,[7] Nile tilapia (*Oreochromis niloticus*) fed *L. plantarum* at 10^8 CFU g $diet^{-1}$ for 28 days,[9] and giant prawn (*Macrobrachium rosenbergii*) fed *L. plantarum* at 10^9 CFU g $diet^{-1}$ for 90 days.[19] As reported by Xie et al.,[20] increased activity of digestive enzymes could improve nutrient absorption ability, hence promoting growth performance. Based on the results of this study, *L. plantarum* strain CMT1 isolated from shrimp could produce digestive enzymes such as protease and amylase. In addition, probiotics have been demonstrated to increase epithelial cell microvilli in the intestine, which provide a great surface area for the absorption of available nutrients.[21] The dietary supplementation of *L. plantarum* could significantly improve the survival rate of whiteleg shrimp, and similar results were observed in blue swimming crab (*Portunus pelagicus*),[22] bighead catfish (*Clarias macrocephalus*),[23] and olive flounder (*Paralichthys olivaceus*).[24] Moreover, shrimp fed diet supplemented with *L. plantarum* CMT1 at a dose of 10^8 CFU kg^{-1} significantly decreased FCR compared to the control diet. This result is similar to the finding of Dash et al.,[19] who reported lower FCR in freshwater prawn *M. rosenbergii* fed with *L. plantarum*. Total *Lactobacillus* counts in the intestines of shrimp fed T2 and T3 diets were sig-

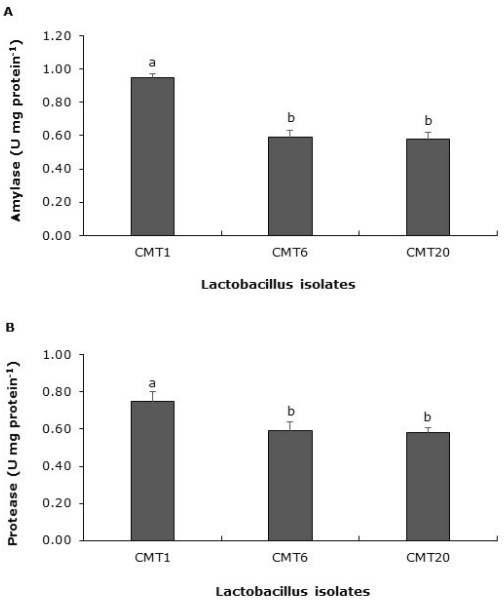

Figure 1. Extracellular enzyme activities (A) Amylase and (B) Protease of three *Lactobacillus* isolates.

Table 2. Water quality parameters recorded during the experiment period

Parameters	Treatments			
	Control	T1	T2	T3
Temperature	29.0 ± 0.2	29.0 ± 0.2	29.0 ± 0.2	29.1 ± 0.2
pH	7.75 ± 0.03	7.73± 0.02	7.70 ± 0.04	7.76 ± 0.04
DO (mg L^{-1})	4.7 ± 0.1	4.8 ± 0.1	4.8 ± 0.1	4.7 ± 0.1
Alkalinity (mg$CaCO_3$ L^{-1})	122.1 ± 8.7	121.7 ± 8.4	122.9 ± 6.9	122.4 ± 7.8
NO_2^--N (mg L^{-1})	0.815 ± 0.149	0.599 ± 0.120	0.594 ± 0.100	0.582 ± 0.138
TAN (mg L^{-1})	1.376 ± 0.061	1.207 ± 0.034	1.221 ± 0.044	1.051 ± 0.028

nificantly higher than those in the other diets from day 14 to the end of the experiment, but no significant difference was observed in the T2 and T3 groups. In contrast with the number of *Vibrio* counts, the levels of the T2 and T3 groups significantly decreased compared to the control and T1 groups. These findings suggested that *L. plantarum* CMT1 could inhibit the growth of pathogenic bacteria in the gut of shrimp. Similarly, Kongnum and Hongpattarakere[25] reported that the administration of *L. plantarum* MRO3.12 in feed effectively inhibited non-fermenting *Vibrios* in the guts of shrimp. This could be explained by the fact that lactic acid bacteria are capable of producing antimicrobial substances and competing for nutrients and space from harmful bacteria.[26] Furthermore, the high density of beneficial bacteria in the gastrointestinal tracts of shrimp could reduce and eliminate the colonization of pathogenic bacteria.[27]

In conclusion, the present study isolated *L. plantarum* CMT1 from the intestines of shrimp collected from extensive shrimp farming, which could be used as a potential probiotic candidate. In an *in vivo* experiment, the diet supplementation of *L. plantarum* CMT1 had a positive effect on the survival and growth performance of shrimp, and the suggested dose of the probiotic strain CMT1 is 10^8 CFU kg $diet^{-1}$.

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AUTHORS' CONTRIBUTION

Conceptualization: Thi Cam Tu Phan (Equal), Thi Thanh Hien Tran (Equal). Methodology: Thi Cam Tu Phan (Equal), Truong Giang Huynh (Equal), Thi Thanh Hien Tran (Equal). Formal Analysis: Thi Cam Tu Phan (Equal), Thi Thu Nguyen

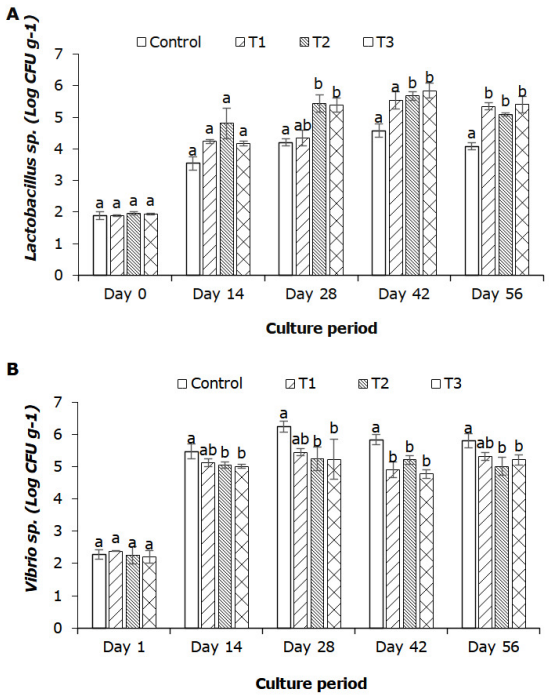

Figure 2. Total density of *Lactobacillus* sp. (A) and *Vibrio* sp. (B) in the intestine of whiteleg shrimp. The different lowercase letters in each bar show significant difference among the test groups at the same sampling period ($p < 0.05$).

Table 3. Growth performance of the whiteleg shrimp after 56 days of feeding

Parameters	Treatments			
	Control	T1	T2	T3
Initial weight (g)	1.04±0.00^{a}	1.05±0.00^{a}	1.05±0.00^{a}	1.05±0.00^{a}
Final weight (g)	13.21±0.03^{a}	13.27±0.03^{ab}	13.33±0.04^{b}	13.58±0.02^{c}
WG (g)	12.16±0.03^{a}	12.28±0.01^{ab}	12.28±0.01^{b}	12.53±0.01^{c}
DWG (g day^{-1})	0.2027±0.0005^{a}	0.2036±0.0002^{ab}	0.2047±0.0001^{b}	0.2088±0.0001^{c}
SGR (% day^{-1})	4.231±0.001^{a}	4.223±0.003^{a}	4.236±0.001^{a}	4.238±0.005^{b}
Survival rate (%)	71.7±1.67^{a}	78.3±1.67^{ab}	80.7±0.67^{b}	83.3±1.67^{b}
FCR	1.54±0.89^{a}	1.4±0.81^{ab}	1.31±0.76^{ab}	1.24±0.72^{b}

Values (mean ± SD, n = 3) with different letters in the same row indicate significant differences among the test groups ($p < 0.05$).

(Equal). Writing – original draft: Thi Cam Tu Phan (Lead). Writing – review & editing: Thi Cam Tu Phan (Equal), Thi Kim Lien Nguyen (Equal), Truong Giang Huynh (Equal), Thi Thanh Hien Tran (Equal). Funding acquisition: Thi Cam Tu Phan (Lead).

ETHICAL CONDUCT APPROVAL – IACUC

The experiment complied with the regulations of Can Tho University and Vietnam's policy for animals used in research.

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