

Assessment of Lactic Acid Bacteria Isolated from the Whiteleg Shrimp (*Penaeus vannamei*) as Potential Biocontrol against Pathogenic Vibrio

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ARTICLE INFO

Article History:

Received: Jan. 11, 2024

Accepted: June 3, 2024

Online: Feb. 9, 2025

Keywords:

Aquaculture,
Biocontrol agents,
Lactic acid bacteria,
Pathogenic vibrio,
Probiotics,
Whiteleg shrimp

ABSTRACT

In aquaculture, the use of probiotics holds promise for disease prevention and nutritional enhancement. This study aimed to assess the inhibitory ability of lactic acid bacteria isolated from the hepatopancreas and intestine of healthy whiteleg shrimp (*Penaeus vannamei*) against prevalent shrimp pathogens. Four lactic acid bacteria were isolated from the hepatopancreas, while six isolates were obtained from the intestine. Molecular analysis, based on partial 16S rDNA gene sequencing and subsequent phylogenetic analyses, confirmed that nine isolates belonged to *Pediococcus pentosaceus*, while one isolate showed close relation to *Weissella paramesenteroides*. The cell-free supernatants from these isolates exhibited inhibitory effect against at least two of the three pathogenic *Vibrio* strains—*Vibrio alginolyticus*, *Vibrio harveyi*, and *Vibrio parahaemolyticus*. Among them, supernatants from three isolates displayed strong inhibitory responses against *V. parahaemolyticus*, while eight were strong against *V. harveyi*. Conversely, supernatants from seven isolates demonstrated medium inhibitory activity against *V. alginolyticus*. This investigation highlights the potential of these lactic acid bacteria isolates as promising biocontrol agents against pathogenic *Vibrio* in shrimp.

INTRODUCTION

The excessive use of antibiotics in aquaculture, including the cultivation of whiteleg shrimp (*Penaeus vannamei*), has raised concerns due to the emergence of antibiotic-resistant bacterial strains. Antibiotics are commonly used to prevent and treat bacterial infections in shrimp, ensuring their health and productivity. However, overuse can lead to antibiotic resistance, making treatments less effective and posing a threat to both aquatic life and human health. Furthermore, environmental contamination from antibiotic leaching can affect surrounding water bodies and ecosystems. This trend not only jeopardizes shrimp output and profitability but also disrupts the delicate microbial

balance within shrimp cultivation, leading to disease outbreaks and other production-related challenges (Thornber *et al.*, 2020; Paul, 2022). To ensure sustainable shrimp farming, it is imperative to shift towards responsible antibiotic use and explore alternative disease prevention and management strategies. Among these alternatives, probiotics have emerged as a promising solution for controlling microbial diseases in whiteleg shrimp farms.

The primary microbial species responsible for diseases in whiteleg shrimp farms belong to the *Vibrio* genus, comprising several pathogenic species. *Vibrio harveyi*, *Vibrio parahaemolyticus*, and *Vibrio alginolyticus* are commonly identified as the predominant pathogenic *Vibrio* species associated with acute hepatopancreatic necrosis disease (AHPND) outbreak on whiteleg shrimp farms (Paria *et al.*, 2021; Wan *et al.*, 2021; Yin *et al.*, 2022). Managing *Vibrio* infections pose a significant challenge, particularly within intensive shrimp farming systems characterized by high stocking densities and suboptimal water quality conditions that favor bacterial proliferation (Flegel, 2019). Effective disease prevention and control in shrimp farms necessitate a multifaceted approach, encompassing water quality optimization, stocking density reduction, probiotic administration, and the utilization of other alternative treatments.

Probiotics, live microorganisms administered to shrimp, play a crucial role in promoting a healthy gut microbiome and enhancing the shrimp's immune system. This, in turn, aids in controlling the proliferation of pathogenic bacteria, notably *Vibrio* species. Numerous studies have demonstrated the inhibitory effects of select probiotic strains on *Vibrio* growth and their ability to improve the survival rates of infected shrimp (Amin *et al.*, 2023). Notably, several probiotic strains have exhibited promise in controlling *Vibrio* infections in shrimp, including *Lactobacillus casei*, *Limosilactobacillus fermentum*, and *Lactobacillus plantarum* (Pinoargote *et al.*, 2018; Govindaraj *et al.*, 2021; Thompson *et al.*, 2022). The objective of this study was to explore the potential of hepatopancreas and gastrointestinal tract-derived microorganisms from healthy whiteleg shrimp as sources of anti-*Vibrio* agents.

MATERIALS AND METHODS

1. Growth and maintenance of pathogens culture

Three *Vibrio* species were selected based on their established history of pathogenicity against crustaceans, particularly shrimp: *V. harveyi* (NBRC 15634T), *V. parahaemolyticus* (NBRC 12711T), and *V. alginolyticus* (WLSCC 0021). *Vibrio harveyi* and *V. parahaemolyticus* were sourced from the Biological Resource Center, NITE, Tokyo, Japan, while *V. alginolyticus* was obtained from the culture collection of PT Widya Teknologi Hayati, Yogyakarta, Indonesia. The pathogen culture stocks were initially preserved in a glycerol solution at -70°C . During the experiments, all *Vibrio* cultures were maintained on Marine Agar (MA) at 4°C and subjected to subculturing every four weeks.

2. Isolation of gut microbiota of healthy whiteleg shrimp

The microbiota from the hepatopancreas and intestine of healthy juvenile *P. vannamei* were screened to identify Lactic Acid Bacteria (LAB) strains. Six juvenile shrimp, with sizes measuring 0.5 ± 0.1 and 8 ± 0.5 g, respectively, were sampled for this study. These animals were sourced from the Kartika Jaya shrimp farm located in East Java, Indonesia, during an acute hepatopancreatic necrosis disease (AHPND) outbreak. Shrimps were carefully collected using scoop nets, immediately placed in an icebox with dry ice, and subsequently transported to the Research and Development Laboratory at PT Widya Teknologi Hayati.

Aseptic dissection procedures were followed for the juvenile shrimps, with samples collected from the entire mid-hind gut, including feces, as well as the hepatopancreas. These samples were placed in tubes containing 10mL of sterile 3% NaCl solution before undergoing homogenization. Dilutions of the homogenates were prepared, followed by pour plating onto MRS agar. Plates were then incubated under anaerobic conditions at 37°C for 48h. Colonies were randomly selected from the plates, and the isolates were purified through repeated streaking onto MRS agar. The purified LAB strains were subsequently inoculated into MRS broth (pH 6.5) and incubated for 24h at 30°C. All purified strains were preserved in MRS broth containing 20% glycerol at -70°C. Gram staining and detailed colony morphology observations were recorded for each isolate.

3. Molecular identification of lactic acid bacteria isolates

Total DNA was extracted from a colony using the Presto™ Mini gDNA Bacteria Kit (Geneaid, New Taipei, Taiwan), following the manufacturer's instructions with slight modifications. The quality and quantity of each DNA sample were assessed using a Nanodrop™ 1000 spectrophotometer (Thermo Scientific, USA).

The 16S rDNA was amplified via polymerase chain reaction (PCR) in an AC196-Alpha Cycler (PCR max, UK) using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3') (Vergin *et al.*, 1998). PCR conditions were as follows: initial denaturation at 95°C for 2min followed by 30 cycles of 95°C for 45s, 55°C for 30s, and 72°C for 1min, with a final extension at 72°C for 5min.

The amplified 16S rDNA regions were then sequenced, and the similarity of the homologous sequences was analyzed using the Basic Local Alignment Search Tool (BLAST) program within the National Center for Biotechnology Information database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). In addition, to facilitate phylogenetic analysis the sequence was aligned with reference sequences of LAB using ClustalW algorithm in the molecular evolutionary genetic analysis (MEGA) version 11 software (Tamura *et al.*, 2021). Furthermore, the neighbor-joining method was used for phylogenetic analysis (Saitou & Nei, 1987).

4. Evaluation of inhibitory activity

The inhibitory activity test was conducted using the agar well diffusion method, as described by **Balouiri *et al.* (2016)**. Shrimp pathogenic bacteria, namely *V. harveyi*, *V. parahaemolyticus*, and *V. alginolyticus*, were inoculated onto Marine Agar (MA) plates. Following inoculation, the agar was carefully cut into circular wells, each with a diameter of 6mm, with eleven wells on each plate. Subsequently, each well was loaded with 100µL of supernatant from each LAB isolate and incubated at 30°C for an additional 24h. The supernatant was prepared from LAB cultures grown in de Man-Rogosa-Sharpe (MRS) broth, which were incubated at 37°C for 24h. Afterward, the cultures were centrifuged at 10,000rpm for 10min at 4°C to obtain the supernatant from the culture media.

For the evaluation, un-inoculated MRS broth served as the negative control. Each plate containing *Vibrio* and the supernatant from each LAB isolate was assessed after 24h of incubation, and the diameters of the clearance zones were measured using a caliper. Statistical analyses were conducted using SAS software, version 9.4 (SAS Institute, Cary, NC, USA), with the Duncan multiple range test applied at a significance level of $P < 0.05$.

RESULTS

1. Morphological and molecular identification of lactic acid bacteria isolates

A total of 10 bacterial isolates were collected from the hepatopancreas and gastrointestinal tract of healthy whiteleg shrimp. Among them, four isolates originated from the hepatopancreas (UK 3.1, UK 3.2, UK 3.3, UK 3.4) and six from the intestine (UK 3.12, UK 3.14, UK 3.16, UK 3.17, UK 3.19, UK 3.20). Morphological assessment of bacterial cells revealed that nine isolates exhibited a coccus type, while one isolate displayed a rod-shaped cell morphology. Additionally, all isolates demonstrated white, circular, and opaque colonies on MRS agar plates (Table 1).

Table 1. Cell and colony morphology of the LAB isolates

No.	Source	Isolates	Gram staining	Morphology				
				Cell	Colony			
					Color	Form	Elevation	Opacity
1	Hapatopancreas	UK 3.1	+	Spherical	White	Circular	Convex	Opaque
2	Hapatopancreas	UK 3.2	+	Spherical	White	Circular	Convex	Opaque
3	Hapatopancreas	UK 3.3	+	Spherical	White	Circular	Convex	Opaque
4	Hapatopancreas	UK 3.4	+	Spherical	White	Circular	Convex	Opaque
5	Intestines	UK 3.12	+	Spherical	White	Circular	Convex	Opaque
6	Intestines	UK 3.14	+	Short rods	White	Circular	Convex	Opaque
7	Intestines	UK 3.16	+	Spherical	White	Circular	Convex	Opaque
8	Intestines	UK 3.17	+	Spherical	White	Circular	Convex	Opaque
9	Intestines	UK 3.19	+	Spherical	White	Circular	Convex	Opaque
10	Intestines	UK 3.20	+	Spherical	White	Circular	Convex	Opaque

The molecular analysis of partial 16S rDNA gene sequences indicated that nine isolates shared 100% similarity with *Pediococcus pentosaceus*, while one isolate showed 100% similarity with *Weissella paramesenteroides* (Table 2). Further clarification of their phylogenetic relationships was achieved through phylogenetic analyses, confirming that nine isolates (UK 3.1, UK 3.2, UK 3.3, UK 3.4, UK 3.12, UK 3.16, UK 3.17, UK 3.19, UK 3.20) formed a clade with *Pediococcus pentosaceus*, while isolate UK 3.14 was closely related to *Weissella paramesenteroides* (Fig. 1).

Table 2. Similarity to the closest relatives in GenBank of 16S rDNA sequences of the lactic acid bacteria isolates

Isolates	The most similar sequences in GenBank database		
	Species	Accession number	Sequence similarity
UK 3.1	<i>Pediococcus pentosaceus</i>	OR144304.1	968/968(100%)
UK 3.2	<i>Pediococcus pentosaceus</i>	OR144304.1	755/755(100%)
UK 3.3	<i>Pediococcus pentosaceus</i>	OR144304.1	583/583(100%)
UK 3.4	<i>Pediococcus pentosaceus</i>	OR144304.1	711/711(100%)
UK 3.12	<i>Pediococcus pentosaceus</i>	OR144304.1	968/968(100%)
UK 3.14	<i>Weissella paramesenteroides</i>	MT613524.1	992/992(100%)
UK 3.16	<i>Pediococcus pentosaceus</i>	OR144304.1	920/920(100%)
UK 3.17	<i>Pediococcus pentosaceus</i>	OR144304.1	1013/1013(100%)
UK 3.19	<i>Pediococcus pentosaceus</i>	OR481923.1	912/912(100%)
UK 3.20	<i>Pediococcus pentosaceus</i>	OR144304.1	875/875(100%)

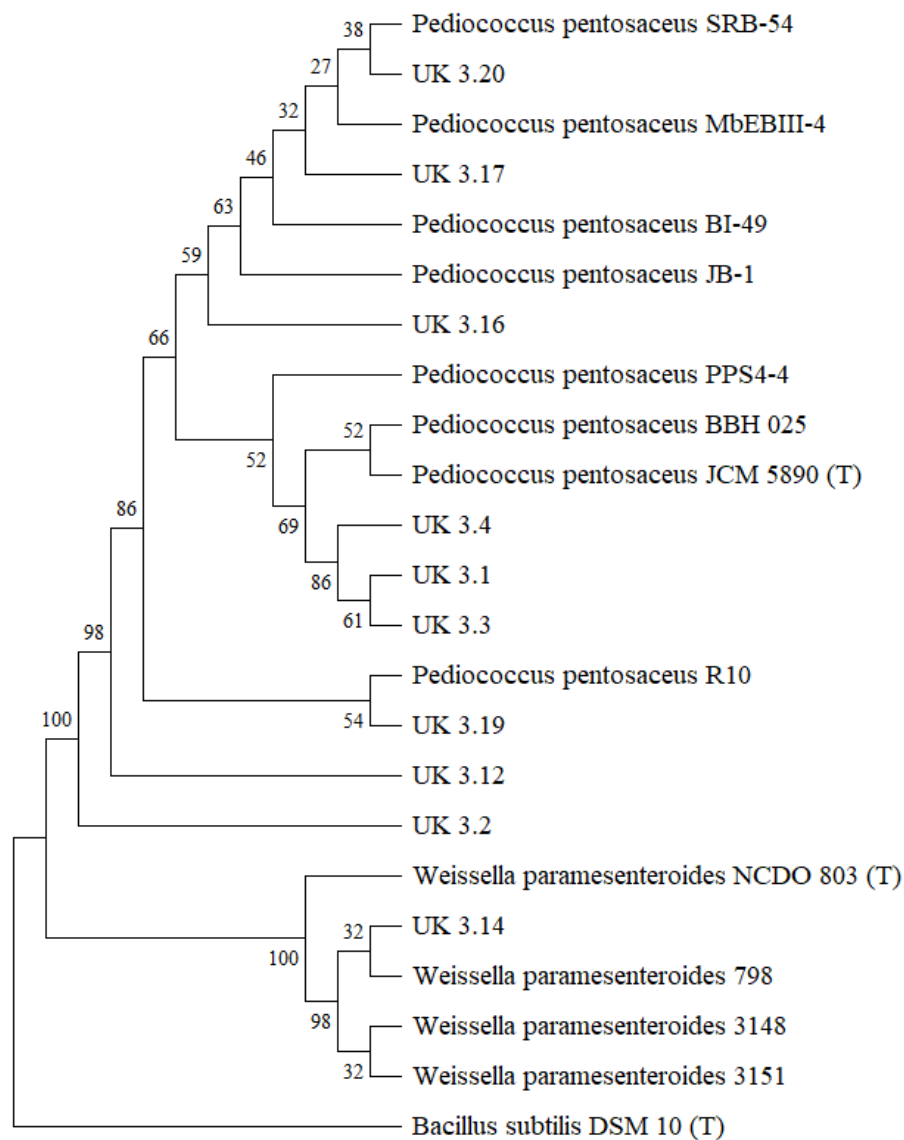


Fig. 1. Neighbor-joining consensus phylogram for partial 16S rDNA sequence of the lactic acid bacteria isolates. Bootstrapping, 1000 replicates. The tree topology is rooted by using *Bacillus subtilis* as the outgroup

2. Inhibitory activity of cell-free supernatants from lactic acid bacteria isolates against pathogenic *Vibrio* strains

Antagonistic potential of lactic acid bacteria (LAB) isolates was evaluated *in vitro* using cell-free supernatant (CFS) agar well diffusion method. Three pathogenic *Vibrio* strains commonly found in shrimp, namely *V. parahaemolyticus*, *V. harveyi*, and *V. alginolyticus*, were employed. The effectiveness of CFS from LAB isolates against these *Vibrio* bacterial strains was determined by measuring inhibition zones. As per the classification criteria proposed by **David and Stout (1971)**, inhibition zones (ZOI) were categorized based on their diameter: >20mm classified as very strong, 10-20mm as strong, 5-10mm as medium, and <5mm as no response.

Among the ten isolates examined, CFS of three isolates (UK3.1, UK3.16, and UK3.20) exhibited a strong inhibitory response against *V. parahaemolyticus*, while eight isolates (UK 3.1, UK 3.2, UK 3.3, UK 3.12, UK 3.16, UK 3.17, UK 3.19, and UK 3.20) were strong against *V. harveyi*. However, the response varied significantly when CFS of the isolates were tested against *V. alginolyticus*, with only seven isolates (UK 3.1, UK 3.2, UK 3.3, UK 3.4, UK 3.17, UK 3.19, and UK 3.20) displaying a medium inhibitory response (Table 3). Notably, UK 3.1 demonstrated the highest overall inhibitory response across all pathogenic *Vibrio* strains tested.

Table 3. Inhibitory activity of cell-free supernatants from lactic acid bacteria isolates

Isolates	Zone of inhibition (mm) ^{1,2}		
	<i>V. parahaemolyticus</i>	<i>V. harveyi</i>	<i>V. alginolyticus</i>
UK 3.1	13.02±3.41 ^a	12.91±0.36 ^a	8.77±0.74 ^a
UK 3.2	9.71±1.10 ^{ab}	10.88±1.69 ^a	7.58±1.32 ^{ab}
UK 3.3	7.67±1.70 ^{ab}	10.38±0.92 ^a	7.01±1.32 ^{ab}
UK 3.4	8.40±1.93 ^{ab}	9.16±2.44 ^a	6.39±1.44 ^{ab}
UK 3.12	8.04±3.12 ^{ab}	10.96±1.23 ^a	4.80±1.44 ^b
UK 3.14	9.62±1.56 ^{ab}	9.32±4.41 ^a	0.00±0.00 ^c
UK 3.16	10.51±1.41 ^{ab}	12.38±1.21 ^a	4.96±0.74 ^b
UK 3.17	7.02±1.69 ^b	10.69±1.26 ^a	5.73±0.84 ^b
UK 3.19	6.74±1.62 ^b	10.59±0.38 ^a	6.94±0.69 ^{ab}
UK 3.20	11.07±0.75 ^{ab}	12.13±0.82 ^a	7.10±0.46 ^{ab}

¹Number with the same letter are not significantly different ($P < 0,05$)

²Data are expressed as mean ± standard deviation (n = 3)

DISCUSSION

Similar to other endothermic and exothermic creatures, shrimps harbor a multitude of beneficial bacteria in their digestive systems. Bacteria capable of producing

various extracellular enzymes vital for digestion have been identified in the stomach, hepatopancreas, and intestine of shrimps. Recent studies have highlighted the presence of beneficial lactic acid bacteria (LAB) in the shrimp's digestive tract. **Maeda et al. (2014)** reported the most prevalent species found in shrimp's whole gut samples as *Lactobacillus plantarum* (31.38% abundance), followed by *Lactococcus lactis* (23.54%), *Vagococcus fluvialis* (11.76%), and *Lactococcus garvieae* (7.84%). Additionally, *Lactobacillus plantarum*, *Lactobacillus fermentum*, and *Pediococcus pentosaceus* have been identified in the shrimp's intestinal tract (**Truc et al., 2019; Haliman et al., 2023**). These studies indicate that the gastrointestinal tract of shrimps serves as a reservoir for beneficial bacteria isolates, with LAB being the predominant species.

In this investigation, lactic acid bacteria were isolated from the hepatopancreas and intestine of healthy whiteleg shrimp. Ten isolates were able to grow on MRS medium selective for *lactobacilli*, indicating that the isolated bacteria belong to the family of lactic acid bacteria. The observed cellular and colony morphology aligned with the molecular identification. Among them, nine isolates belonged to the *Pediococcus*, and one isolate closely related to *Weissella*. *Pediococcus* cells are spherical in shape, as described by **Axelsson (1998)**, while *Weissella* cells are either ovoid or short rods that occur in pairs or short chains, as noted by **Collins et al. (1993)**. *Pediococcus* and *Weissella* are Gram-positive bacteria used as probiotics in aquaculture due to their capability to inhibit various fish and shrimp pathogenic bacteria.

Several studies have reported the probiotic potential of *Pediococcus* and *Weissella*. **Huang et al. (2014)** highlighted the protective effects of *Pediococcus pentosaceus* strain 4012 (LAB4012), isolated from the intestine of cobia fish, against photobacteriosis in cobia fish following a 2-week feeding regimen. The culture supernatant of LAB4012 inhibited the growth of *Vibrio anguillarum* under low pH conditions *in vitro*. Additionally, the incorporation of *Pediococcus pentosaceus* into the diet significantly improved the growth and immune system of *L. vannamei* juveniles, leading to reduced mortality when exposed to *V. parahaemolyticus* (**Hong et al., 2022**). In another study, *Weissella cibaria* KY10 effectively suppressed the development of *V. parahaemolyticus* T.11 within 12 hours under aerobic conditions (**Kanjan et al., 2022**).

Pathogenic *Vibrio* species are commonly active in the hepatopancreas and intestine of shrimps (**Joshi et al., 2014; Garibay-Valdez et al., 2020**). In this study, lactic acid bacteria were isolated from the hepatopancreas and intestine of healthy whiteleg shrimp that survived acute hepatopancreatic necrosis disease (AHPND) outbreak. The CFS from all 10 LAB isolates exhibited antagonistic activity against at least two of the three *Vibrio* strains tested. However, each isolate showed a different category of inhibitory response against each *Vibrio* strain, ranging from medium to strong reactions. Among them, the CFS from UK 3.1 isolate displayed the strongest overall inhibitory response against all three *Vibrio* strains, suggesting its potential as a candidate probiotic.

The differences observed in the inhibitory zone were likely dependent on the secondary metabolites produced by LAB isolates. Previous studies have established that both *Pediococcus pentosaceus* and *Weissella paramesenteroides* possess the capacity to produce metabolites inhibiting pathogen growth. *Pediococcus pentosaceus* SK25 synthesizes a significant quantity of 3-phenyllactic acid, imparting a broad-spectrum antibacterial property (Yu *et al.*, 2015). Furthermore, various strains of *P. pentosaceus* can produce different types of Bacteriocins (Jiang *et al.*, 2021). Conversely, *W. paramesenteroides* DX is known to produce weissellin A, a class IIa bacteriocin with broad-spectrum antimicrobial activity (Papagianni & Papamichael, 2011). These findings provide support for our observations regarding the inhibitory properties of CFS from *Pediococcus pentosaceus* and *Weissella paramesenteroides*.

CONCLUSION

The whiteleg shrimp that is able to survive AHPMD disease is a valuable source for obtaining beneficial bacteria isolates that exhibit antagonistic activity against pathogenic *Vibrio* species. Molecular identification of the isolated lactic acid bacteria revealed nine isolates as *Pediococcus pentosaceus*, while one isolate belonged to *Weissella paramesenteroides*. Among these, *Pediococcus pentosaceus* UK 3.1, derived from the shrimp hepatopancreas, has the most potent inhibitory ability against all tested *Vibrio* strains. Further investigation is necessary to evaluate these isolates as biocontrol agents, particularly in mitigating *Vibrio* infections under field conditions.

ACKNOWLEDGEMENT

This research is funded and facilitated by PT Widya Teknologi Hayati, Yogyakarta, Indonesia.

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