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Isolation of *Weissella cibaria* from Pacific White Shrimp (*Litopenaeus vannamei*) Gastrointestinal Tract and Evaluation of Its Pathogenic Bacterial Inhibition

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Abstract

Objective: The present study reported the isolation of lactic acid bacteria from the shrimp gastrointestinal tract and evaluation of its antagonistic activity against pathogenic bacteria. **Methods/ statistical analysis:** Lactic bacteria from the gastrointestinal tract of alive healthy shrimp were screened on sterilized MRS medium. *Vibrio* sp. inhibition by isolates was conducted by the agar-well diffusion assay. The isolates were identified by 16S rRNA sequencing. Antibacterial activity was investigated against 25 *Vibrio* sp. isolates, *Staphylococcus aureus* ATCC 25023, and *Escherichia coli* ATCC 85922. Hemolytic activity, salt, and antibiotic resistance were also evaluated. **Findings:** Total of 22 isolates exhibited *Vibrio parahaemolyticus* inhibition with antagonistic activities ranged from 220 AU/ml to 460 AU/ml. Sequence analysis of 16S rRNA indicated the 5 isolates belong to *Weissella cibaria* species, namely HN02, HN03, HN04, and HN06 AND HN07. Among these, *W. cibaria* HN05 had highest antagonistic activity and exhibited the broad inhibition spectrum against *Vibrio* sp. as well as *E. coli* ATCC 85922 but not *S. aureus* ATCC 25023. β-hemolytic and salt resistance assays showed that *W. cibaria* HN05 had no β-hemolytic activity and grew well in medium containing 5% of salt. Antibiotic resistance analysis indicated *W. cibaria* HN05 was suppressed by ampicillin, chloramphenicol, oxytetracycline hydrochloride, and chlortetracycline hydrochloride. **Application/improvements:** This is the first study on isolation and

evaluation pathogenic bacteria including *Vibrio* sp. inhibition by *W. cibaria*, which is isolated from shrimp gastrointestinal tract.

Keywords: Antagonistic Activity, Gastrointestinal Tract, Shirmp, *Weissella cibaria*, *Vibrio* sp.

1. Introduction

Pacific white shrimp, *Litopenaeus vannamei*, is the most popular shrimp species due to their survival advantages such as fast growth rate and strong adaptability to the environment [1]. The shrimp is widely cultivated in Southeast Asia, China, India, the United States of America, Mexico, and Latin America, yielding more than 4 million tons in 2016 [2]. However, high frequency of diseases has severely influenced the shrimp farming [1].

In 2009, acute hepatopancreatic necrosis disease (AHPND) was first reported in China then disease outbreaks occurred in various countries in Southeast Asia and America, resulting in up to 100% mortality. *Vibrio* species such as *V. parahaemolyticus* and *V. harveyi* were demonstrated as causative agent of AHPND [3–4]. Meanwhile, *Vibrio* sp. also response for disease on shrimp including luminous vibriosis, tail necrosis, shell disease, red disease, loose shell syndrome, white gut disease, white spot, etc [5–7]. It is estimated shrimp farming industry lost more than 1$ billion US dollar per year [8]. Thus, developing an appropriate strategy to control *Vibrio* species in aquaculture not only increases the productivity but also enhance the product safety.

The infectious disease prevention and control in aquaculture using commercial antibiotics is still the chosen of many farmers due to high inhibiting ability to pathogens. However, overuse of antibiotics can lead to the bloom of drug-resistant bacteria as well as affect aquatic environment and further to human [9–10]. Naturally, *Vibrio* pathogen can be inhibited by other bacteria which are able to secrete anti-bacterial substances during their growth. The bacteria show beneficial effect on the host is called as probiotics and mainly belong to the lactic acid bacteria such as *Bacillus*, *Lactobacillus*, *Bifidobacterium, Streptococcus*, etc [11–12]. Probiotic bacteria strengthen the epithelial barrier, increase adhesion to the intestinal mucosa, inhibit simultaneously pathogens, eliminate competition for pathogenic microorganisms and produce antimicrobial substances as well as modulate of the immune system [13]. Many probiotic bacteria are currently being investigated and applied to aquaculture, especially shrimp farming [14–17]. Thus, the use of probiotics for disease prevention and nutrition improvement in shrimp aquaculture is becoming increasingly popular due to an increasing demand for environment-friendly aquaculture [9].

Weissella cibaria is a Gram-positive and obligate heterofermentative lactic acid bacteria [18]. It is known to produce native substances, including antimicrobial and antifungal agents against Gram-positive bacteria [19], periodontal disease-causing bacteria [20]. Up to date, *W. cibaria* has been used as a probiotic bacterium in different fermented products such as Kimchi [21], cottage cheese [22] Chinese jiang-shui [23], and shrimp paste [24]. *W. cibaria* M3 strain can reduce the fermentation time along with enhancing flavor and

product quality of *Chouguiyu*, a Chinese traditional fermented fish [25]. Thus, this strain has been potential attracted to use as probiotic agent [26].

2. Material and Methods

2.1. Sampling and Lactic Acid Bacteria Screening

The alive healthy shrimp were collected from local shrimp farms at Thua Thien Hue province, Vietnam and delivered to the laboratory. Digestive tracts were collected and dissolved in 500 µl of 0.9% NaCl solution. Two hundred microliters were inoculated in 5 ml of sterilized Man Rogosa Sharpe (MRS) and incubated at 30 °C for 24 h without shaking. One hundred microliters were spread onto MRS supplemented with 0.5% $CaCO_3$ agar medium and cultured at 30 °C for 24 h. The single colony showing halo zone was picked up and subcultured into new MRS agar plate. The culture was repeated five times to select pure colonies.

2.2. Screening for *Vibrio parahaemolyticus* Inhibition Ability

The potential *Vibrio* sp. inhibition by isolates was conducted by the agar well diffusion assay using pure isolate collection against *V. parahaemolyticus* on LB agar (1% tryptone, 0.5% yeast extract, 1% NaCl, and 0.8% agar) medium [27]. A *V. parahaemolyticus* strain was chosen from *Vibrio* collection storing at Institute of Biotechnology, Hue University, Vietnam and was cultured in 5 ml of LB medium overnight. Meanwhile, each potential *Vibrio* sp. inhibition isolate was cultured in 5 ml MRS medium with 180 rmp of shaking, 30 °C for overnight. Then, the cell density of both overnight cultures was measured spectrophotometrically at 600 nm wavelength and diluted to final concentration of 10^8 CFU/ml. Fifty microliters of diluted *V. parahaemolyticus* culture was spread on LB agar medium, while fifty microliters free-cell diluted supernatant culture of isolate was dropped into 6 mm hole made on plate. The plate was incubated at 4 °C for 15 min then transferred into 30 °C incubator for 24 h. The diameter of the zone of clearance (DZC) was measured. The antagonistic activity (AU/ml) was calculated by the following formula [28]:

$$\text{AU / ml} = \frac{\text{Diameter of the zone of clearance}\,(\text{mm}) \times 1000}{\text{Volume taken in the well (µl)}}$$

e *Vibrio* inhibiting capacity was defined based on the antagonistic activity value whereas no inhibition is equal with AU = 0, weak inhibition is equal with $0 < AU \leq 200$, moderate inhibition is equal with $200 < AU \leq 280$, and strong inhibition is equal with AU >280.

2.3. Biochemical and Molecular Identification

The isolate exhibited *V. parahaemolyticus* antagonistic activity was stained by Gram's method [29], was conducted catalase assay [29], and was performed microscopic observation at magnification of 100× (Nikon eclipse 55i, Japan). Molecular identification

of isolate was carried out by sequencing the 16S rRNA fragment. Briefly, the bacterium was cultured in 5 ml of MRS medium at 30 ºC with a shaking speed of 180 rpm overnight. The cell biomass was harvested by centrifugation at 8000 rmp for 5 min. Total genomic DNA was isolated using CTAB buffer as described by Sambrook [30]. The DNA was qualified on 0.8% agarose gel and used as temple to amplify the 16S rRNA sequence with pair primer set of 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′ GGTTACCTTGTTACGACTT-3′). The PCR product was sequenced (Firstbase, Malaysia) and aligned against GenBank database. The strain identity was defined based on the highest nucleotide sequence similarity. The phylogenetic tree was analyzed using Mega X software based on Maximum likelihood with Booststrap of 500 replicates [31].

2.4. Evaluation *Vibrio sp.* Inhibition

To evaluate the *Vibrio* sp. inhibition capacity, the selected isolates exhibited *V. parahaemolyticus* inhibition were examined on *Vibrio* sp. collection storing at Institute of Biotechnology, Hue University, Vietnam. The *Vibrio* sp. collection was isolated from shrimp being infected by acute hepatopancreatic necrosis disease. The evaluation was carried out using agar well diffusion assay as mentioned above. The antagonistic activity was recorded and evaluated.

2.5. Evaluation Antibacterial Against Positive and Negative Bacteria

Staphylococcus aureus ATCC 25023 and *Escherichia coli* ATCC 85922 (Microbiologics, USA) were used as standard for positive and negative bacteria model, respectively. The evaluation was performed by agar well diffusion assay as mentioned above. The DZC around the hole was recorded and converted into antagonistic activity unit.

2.6. Salt Resistance Assay

The single colony of each isolate was cultured in 5 ml MRS medium for 24 h at 37 ºC. Cells biomass was harvested by centrifugation at 8000 rpm for 5 min at 4 ºC. The cells were resuspended in NaCl solution (range from 0% to 25%) to final OD_{600} of 1.0. One hundred microliters of suspension were transferred into nine hundred microliters MRS medium containing the corresponding NaCl concentration. OD_{600} was observed before and after 48 h incubation at 37 ºC.

2.7. Hemolytic Activity

The selected isolates were subjected on agar plate containing 5% sheep blood. The overnight supernatant culture in MRS medium was harvested by centrifugation at 8000 rpm for 14 min at 4 ºC. Fifty microliters were added in the 6 mm diameter hole on blood agar plate. The plate was incubated at 37 ºC for 24 h. Hemolytic activity was accessed as clearance zone [32].

2.8. Antibiotic Resistance Assay

The antibiotic resistance ability of selected isolates was conducted by inoculation the single colony of each isolate on 5 ml MRS medium supplemented with antibiotics (sulfapiridine, sulfathiazole, sulfamethoxazole, oxytetracyclin hydrochloride, chlotetracyclin hydrochloride, ampicillin, and chloramphenicol (Sigma, USA) and kanamycin (Biobasic, Canada)) at 37 °C for 24 h. The antibiotic concentration was ranged from 25 µg/ml to 500 µg/ml. The cell density was taken by measuring at OD_{600}.

3. Results and Discussion

3.1. Isolation and Identification of *Vibrio parahaemolyticus* Inhibiting Bacteria

A total of 23 potential lactic acid bacteria colonies were picked up and purified with serial subculture in which 22 of isolates showed *V. parahaemolyticus* inhibition at different levels (Figure 1). The antagonistic activities ranged from 220 AU/ml to 460 AU/ml. Among them, 16 isolates exhibited strong antagonistic effect with activity over 280 units (Table 1). The isolates were then assigned as HN01 to HN22.

The analyses showed 18 isolates were Gram-positive bacteria and only 2 of them exhibited catalase activity (Table 1). From above results, isolates displayed as Gram-positive bacteria and had the *V. parahaemolyticus* strong inhibition were identified by the 16S RNA fragment sequencing method. DNA sequence alignment confirmed 5 isolates (HN02, HN03, HN04, HN05, and HN07) have 100% similarity to *Weissella cibaria* species and they were deposited on Genbank database as accession number of MK989999, MK990000, MK990001, MK990002, and MK990004, respectively. The phylogenetic tree of strains belongs to *W. cibaria* including HN02, HN03, HN04, HN05, and HN07 is shown in Figure 2.

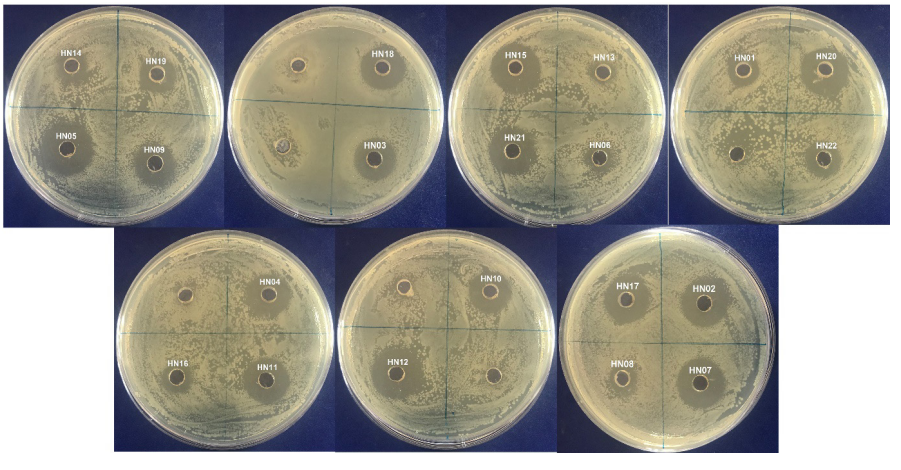

FIGURE 1. Antagonistic activity of isolates against V. parahaemolyticus.

TABLE 1. Isolate collection exhibited V. parahaemolyticus inhibition

No.	Strain name	Antagonistic activity (AU/ml)	Gram profile	Catalase profile
1	HN01	240	+	−
2	HN02	420	+	−
3	HN03	340	+	−
4	HN04	400	+	−
5	HN05	440	+	−
6	HN06	220	+	−
7	HN07	420	+	−
8	HN08	220	+	−
9	HN09	320	+	−
10	HN10	360	+	+
11	HN11	380	+	−
12	HN12	460	−	−
13	HN13	220	−	−
14	HN14	300	+	−
15	HN15	420	−	−
16	HN16	260	−	−
17	HN17	440	+	−
18	HN18	400	−	−
19	HN19	280	+	−
20	HN20	420	+	+
21	HN21	400	+	−
22	HN22	300	+	−

The antagonistic activity is presented as average data of three replicates.
Gram profile: positive gram (+), negative gram (−).
Catalase profile: catalase activity (+), none catalase activity (−).

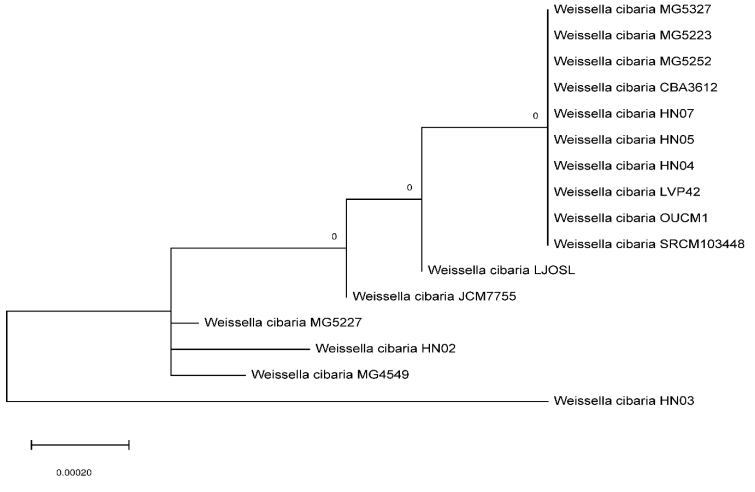

FIGURE 2. Phylogenetic tree among of W. cibaria HN02, W. cibaria HN03, W. cibaria HN04, W. cibaria HN05, W. cibaria HN07, and other W. cibaria.

Probiotic bacteria are considered as potential approach for sustainable aquaculture. Numerous reports have shown supplementation of probiotic bacteria reduce the number of opportunistic pathogenic bacteria, enhance the resistance against pathogenic bacteria as well as increase the immune response, weight gain rate, survival rate [16–17, 33]. The source probiotic bacteria are various such as fermented food [16], animal milk [28], digestive tract, or gut [17, 34]. The present study aims to isolate gut bacteria with potential probiotic against pathogenic *Vibrio*. A collection of 22 isolates from shrimp gastrointestinal tract inhibited *V. parahaemolyticus,* in which 16 isolates exhibited strong antagonistic activity. The results confirm gastrointestinal tract is a good source to isolate potential probiotic lactic acid bacteria.

The isolate collection consists of both gram-positive and gram-negative bacteria. This finding is in accordance with previous reports showed *Vibrio* sp. can be inhibited by gram-positive and gram-negative bacteria [35, 36]. However, due to most of probiotic bacteria applying in aquaculture is gram-positive such as bacillus, lactobacillus,… [36], thus, the gram-positive bacteria are being selected for further study.

The molecular identification showed that isolates are divided into two group *Lactococcus* and *Weissella* genus (data not shown). Dalmacio et al (2011) identified the lactic acid bacteria from fermented shrimp paste by PCR-denaturing gradient gel electrophoresis approach. The author found *W. cibaria* is among of dominant species [24]. Moreover, Leuconostocaceae family where *W. cibaria* belonging, is particular presented in bacteria community associated with shrimp culture system [37]. However, there is no report on identification *W. cibaria* in shirmp gut microbiota up to date.

3.2. Investigation the Board Antibacterial of Selected Isolate

As shown in Table 2, *W. cibaria* HN05 exhibited the strongest antagonistic activity against *V. parahaemolyticus*, thus the strain was selected for further investigations. To investigate the ability for a board *Vibrio* sp. inhibition, *W. cibaria* H05 was tested against 25 different strains of *Vibrio* species. *W. cibaria* H05 inhibited all tested *Vibrio* sp. with antagonistic activity ranged from 220 AU/ml to 500 AU/ml. Interestingly, *W. cibaria* H05 strongly inhibited 22 *Vibrio* species with antagonistic activity >280 AU/ml.

The results on testing against *E. coli* ATCC 85922 and *S. aureus* ATCC 25023 indicated that *W. cibaria* HN05 inhibited *E. coli* growth with antagonistic activity of 240 AU/ml but no *S. aureus* ATCC 25023 inhibition activity was observed.

Our study is the first time described vibrios inhibition activity by the *W. cibaria* which is isolated from shrimp sources. Interestingly, *W. cibaria* isolates exhibited strong antagonistic activity against *Vibrio* sp. up to 500 AU/ml as well as *E. coli* but limited to *S. aureus*. It is well known that *W. cibaria* belongs to probiotic group. *W. cibaria* secretes antibacteria substances such as bacteriocin [18], or modules immune response [38] or inhibits the colonization of pathogenic bacteria [39]. Thus, isolate *W. cibaria* HN05 could be good probiotic candidate for shrimp feed.

TABLE 2. Antagonistic activity of W. cibaria HN05 against Vibrio sp.

No.	Name of *Vibrio* sp.	Antagonistic activity (AU/ml)
1	VTVV4(3)	400
2	VTVV2(8)	200
3	VTVV3(3)	420
4	VTVX3a(13)	300
5	VTVX2a(11)	260
6	VTVV4(1)	240
7	VTVV1(6)	320
8	VTVX1a(9)	200
9	VTVX4a	220
10	VC1	160
11	VTVV4(4)	400
12	VC13	240
13	VTVV2(7)	440
14	VTVX4b	360
15	VC7	340
16	VTVV1(5)	300
17	VC1	400
18	VTVX1b(10)	360
19	VC13	320
20	VTVX2b(12)	360
21	VC14	300
22	VTVX3b(14)	300
23	VC9	320
24	VC12	340
25	VC5	300

The antagonistic activity is presented as average data of three replicates.

3.3. Salt Resistance and Hemolytic Activity

To evaluate salt resistance, *W. cibaria* HN05 was cultured in medium containing NaCl range from 0% to 20%. Overall, increasing NaCl concentration dramatically reduced *W. cibaria* HN05 growth. *W. cibaria* HN05 grew well in the presence of NaCl concentration up to 5 %. However, *W. cibaria* HN05 completely inhibited when increasing NaCl concentration above 10% (Figure 3).

Figure 4 showed the hemolytic activity of *W. cibaria* HN05 on sheep blood agar plate. The results indicated *S. aureus* ATCC 25023 exhibited the β-hemolytic activity whereas no β-hemolytic activity observed in the culture of *W. cibaria* HN05.

The extracellular substances of *W. cibaria* HN05 did not show the β-hemolytic (Figure 4). Our finding is in accordance to report by [32] where is no β-hemolytic observation. Salt resistance is one of importance factors for probiotic applying in shrimp cultivation which salt concentration in water is usually 10‰ or higher. *W. cibaria* HN05 grew well in the medium containing 5% salinity, resulting in advantage when supplement to shrimp pond culture.

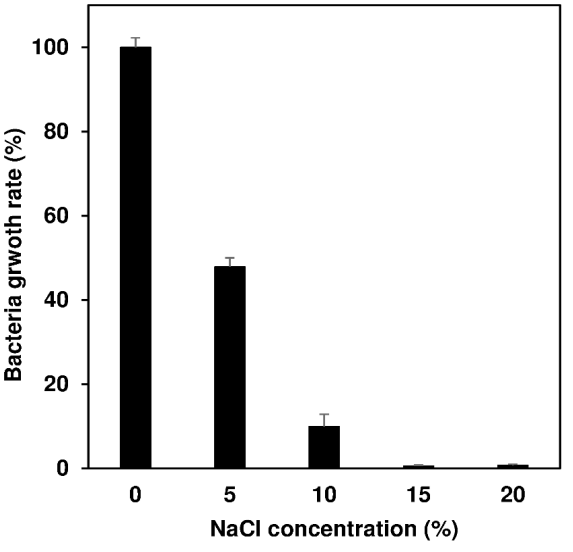

FIGURE 3. NaCl resistance ability of W. cibaria HN05.

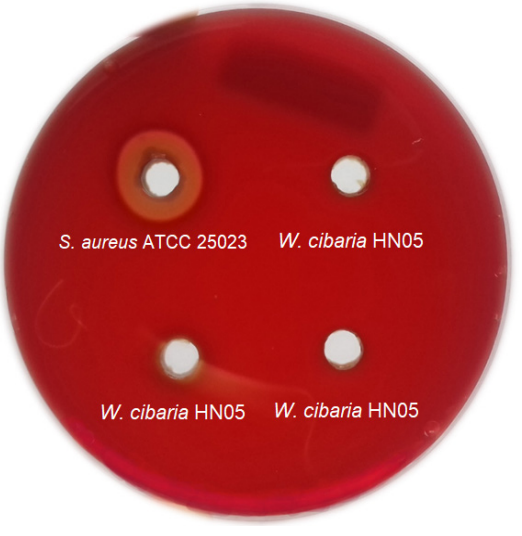

FIGURE 4. Hemolytic activity of W. cibaria HN05 and S. aureus ATCC 25023.

3.4. Antibiotic Resistance

The antibiotic resistance tests showed that *W. cibaria* HN05 grew well in the presence of sulfapyridine, sulfathiazole, sulfamethoxazole, and kanamycin. Oxytetracycline hydrochloride and chlortetracycline hydrochloride inhibited *W. cibaria* HN05 at concentration ≥100 µg/ml. Ampicillin and chloramphenicol completely inhibited *W. cibaria* HN05 at the concentration of 25 µg/ml (Table 3). In the study on safety evaluation of probiotic *W. cibaria,* the strain *W. cibaria* CMU exhibited strong resistant to kanamycin,

vancomycin while was weak against ampicillin, oxytetracycline, tetracycline, etc [32]. In the present study, *W. cibaria* HN05 exhibited resistance to kanamycin and sulfonamides group *in vitro*.

TABLE 3. Antibiotic resistance of W. cibaria HN05

Antibiotic	Concentration (µg/ml)				
	25	50	100	250	500
Sulfapyridine					
Sulfathiazole					
Sulfamethoxazole					
Oxytetracycline hydrochloride			–	–	–
Chlortetracycline hydrochloride			–	–	–
Ampicillin	–	–	–	–	–
Kanamycin					
Chloramphenicol	–	–	–	–	–

–: not growth.

4. Conclusion

W. cibaria was isolated for the first time from shrimp digestive tract. The isolate shows a strong ability to inhibit various pathogens, including *Vibrio* sp. as well as *E. coli*. *W. cibaria* resists to salt at concentration usually higher than that required for shrimp growth and exhibits no β-hemolytic activity. The isolate could be a potential probiotic for shrimp in order to reduce the infection of pathogen bacteria in shrimp intestinal. Further, *in vivo* study is needed to evaluate the probiotic effect of isolate fully.

Conflicts of Interest

The authors declare no conflict of interest.

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