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Assessment of Antibiotic Susceptibility and Biofilm Formation in Bacteria Isolated from White-Leg Shrimp in Traditional Markets, Can Tho City, Vietnam

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ABSTRACT

In shrimp aquaculture, antibiotics are used to control bacteria, particularly species capable of growing on thiosulfate citrate bile salts sucrose (TCBS) agar. However, the overuse of antibiotics may lead to the emergence of antibiotic-resistant bacteria and the presence of antibiotic residues in food products. The present study aimed to evaluate the antibiotic susceptibility of bacterial isolates recovered from market shrimp against nine antibiotics, as well as their biofilm-forming capacity. The investigation focused on isolating bacteria capable of growing on TCBS agar, assessing antibiotic susceptibility using the disk diffusion method, and determining biofilm formation by the 1% crystal violet staining assay. From four shrimp samples collected at local markets, a total of 18 bacterial isolates were obtained, exhibiting yellow, green, and black colonies on TCBS agar. All isolates were resistant to at least one antibiotic and were capable of forming biofilms. Specifically, all 18/18 isolates were resistant to cefotaxime (100%), while resistance rates to azithromycin, ampicillin, and erythromycin ranged from 77.8% to 83.3%. Lower resistance frequencies were observed for doxycycline, tetracycline, and trimethoprim-sulfamethoxazole (16.7-22.2%), and only 5.6% of isolates were resistant to ciprofloxacin. Notably, all isolates remained susceptible to levofloxacin (100%). The multiple antibiotic resistance (MAR) index indicated that different isolates exhibited high levels of multidrug resistance, particularly Mar2.2, Mar3.4, and Mar3.5 (MAR = 0.67). Moreover, all 18 isolates demonstrated biofilm-forming ability, with Mar1.2, Mar2.4, and Mar4.4 producing significantly greater biofilm biomass compared to the remaining isolates. Phylogenetic analysis based on 16S rRNA gene sequencing revealed that Mar1.2 and Mar2.2 belonged to the genus *Vibrio*, Mar2.4 and Mar4.4 were assigned to the genus *Shewanella*, whereas Mar3.4 and Mar3.5 were classified within the genus *Providencia*.

Keywords: Antibiotic, Biofilm, Marketable whiteleg shrimp, *Shewanella*, *Vibrio*, *Providencia*

INTRODUCTION

Antibiotics are applied in aquaculture to control pathogenic bacteria. However, their use must be strictly managed to ensure food safety (Okocha et al., 2018). In whiteleg shrimp, acute hepatopancreatic necrosis disease can cause mortality rates of up to 80-100% (FAO, 2020), while white feces disease, associated with microbial imbalance and the dominance of *Vibrio* spp. (Boopathi et al., 2023), reduces growth performance. Although antibiotics have been applied as an effective control measure, their extensive use has led to alterations in biodiversity and the composition of natural microbial communities in aquatic environments, as well as adverse effects on human health (Chen et al., 2025). Seafood products containing antibiotic residues may disrupt the human gut microbiota, promote the proliferation of antibiotic-resistant bacteria (Sadighara et al., 2023), and increase the risk of opportunistic infections (Pelić et al., 2024).

The different mechanisms of antibiotic resistance have been documented in bacteria, including active efflux of antibiotics, reduced cell membrane permeability, modification or replacement of metabolic pathways, acquisition and accumulation of antibiotic resistance genes, and biofilm formation (Elshobary et al., 2025). Among these mechanisms, biofilm represents a highly adaptive lifestyle characterized by a three-dimensional spatial structure embedded within an extracellular polymeric substance (EPS) matrix, forming complex multispecies microbial communities with enhanced tolerance to environmental stress and antimicrobial agents (Flemming et al., 2016).

Biofilm development is a dynamic and multistep process. Initially, planktonic bacterial cells reversibly attach to surfaces (Liu et al., 2024), followed by irreversible adhesion mediated by EPS secretion. Subsequently, cells proliferate and aggregate to form microcolonies, which gradually develop into mature biofilms with complex three-dimensional architectures and internal nutrient channel networks (Liu et al., 2024). At the mature stage, biofilms generate heterogeneous microenvironments that enhance bacterial adaptability and survival under unfavorable conditions (Liu et al., 2024). Finally, the dispersion stage enables cells to detach from the biofilm and colonize new surfaces, thereby facilitating bacterial dissemination and long-term persistence across diverse environments (Flemming et al., 2016).

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Multiple biological mechanisms operating within biofilms contribute to increased tolerance against antimicrobial agents (Chambless et al., 2006). The EPS matrix can reduce biofilm permeability, thereby limiting antibiotic penetration and diffusion into deeper biofilm layers (Azeem et al., 2025). In addition, biofilms provide favorable conditions for horizontal gene transfer (HGT), enabling the exchange of genetic material, particularly antibiotic resistance genes (ARGs), via plasmid-mediated conjugation. The dissemination rate of ARGs has been reported to be significantly higher in biofilm-associated bacteria than in planktonic cells (Michaelis et al., 2023). Furthermore, bacteria within biofilms communicate through quorum-sensing (QS) systems, which coordinate gene expression associated with antimicrobial resistance. In Pseudomonas aeruginosa, QS has been demonstrated to regulate the expression of antioxidant enzymes such as superoxide dismutase and catalase, thereby enhancing bacterial tolerance to oxidative stress induced by hydrogen peroxide (Khan et al., 2021).

The present study aimed to evaluate antibiotic susceptibility and biofilm-forming ability of bacterial isolates recovered from TCBS agar from whiteleg shrimp (*Litopenaeus vannamei*) purchased at selected markets in Can Tho City, Vietnam.

MATERIALS AND METHODS

Sample collection

Whiteleg shrimp samples were collected once from four local markets in Can Tho City between January 2025 and April 2025, including Tan An Market (Mar1; 10°01′41.0″N, 105°47′12.8″E), Cai Rang Market (Mar2; 10°00′22.2″N, 105°45′02.8″E), Xuan Khanh Market (Mar3; 10°01′35.2″N, 105°46′37.9″E), and An Hoa Market (Mar4; 10°02′55.4″N, 105°46′18.7″E). The selection criteria included commercial shrimp with a body length of 10-12 cm, corresponding to approximately 80-90 individuals per kg. When shrimp reach the mentioned size, they are regarded as market-size and widely consumed. Therefore, this selection ensures practical relevance and accurately reflects the microbiological status of products currently in circulation. At each market, 20 shrimp were collected from a single vendor. After sampling, the samples were stored in insulated containers with gel ice packs (2-8°C) and transported promptly to the laboratory for analysis, ensuring that the time between collection and processing did not exceed 24 h (STAMEQ, 2019).

Isolation

Shrimp samples were surface-sterilized with 70% ethanol to remove external microorganisms and minimize cross-contamination. Sterile scissors and forceps were used to aseptically collect the hepatopancreas and intestines from 10 shrimp individuals obtained from a single vendor at one sampling location (market). The pooled tissues were homogenized in 10 mL of sterile physiological saline using a stomacher (Seward Ltd., UK). The remaining individuals (10 shrimp) were preserved as reserve material at 2-8°C for up to 48 h to allow repeat isolation in case of experimental failure or contamination. The samples were streaked onto thiosulfate citrate bile salts sucrose (TCBS, Himedia, India) agar plates, followed by incubation at 37°C for 24h. The isolation procedure was designed to target bacteria capable of growing on TCBS medium, which is commonly used for the selective screening and presumptive identification of pathogenic *Vibrio* species associated with aquatic animals. On TCBS agar, bacterial colonies typically appear in three characteristic color types, including yellow colonies produced by sucrose-fermenting strains that acidify the medium and cause a color change of the bromothymol blue indicator; blue colonies produced by non-sucrose-fermenting strains; and black colonies produced by strains capable of hydrogen sulfide (H_2S) production in the presence of ferric citrate (Kaysner et al., 2019). After 24h incubation, colonies were grouped based on morphological characteristics (color and shape), and at least one representative colony from each morphological group was randomly selected and streaked onto the surface of TCBS agar plates. The isolates were purified by repeated subculturing until single, morphologically uniform colonies were obtained on the same plate.

Colony, cellular, and biochemical characteristics

Colony morphology was directly observed on TCBS agar after 24h of incubation. Characteristics, including colony color, shape, elevation, margin, and diameter, were recorded under a stereo microscope (Carl Zeiss, Germany) in accordance with standard microbiological guidelines (ASM, 2016). Cell morphology was recorded under a light microscope (Carl Zeiss Microscopy GmbH, Germany) at 1000× magnification. Biochemical properties were evaluated using catalase, oxidase, indole, methyl red, protease, and gelatinase assays according to previously published. Bacterial motility was determined using the deep-stab method in semi-solid agar as described by Hardy Diagnostics (2016). Briefly, isolates were inoculated into TSB supplemented with 1% NaCl and 0.4% (w/v) agar, incubated at 37°C for 24 h, and motility was assessed based on diffuse growth away from the inoculation line.

Gram staining and oxidase tests were performed using commercial diagnostic kits (Nam Khoa, Vietnam) according

to the manufacturer's instructions.

Catalase activity was assessed using the slide drop method described by Reiner (2010). A drop of 3% H_2O_2 (Xilong, China) was added directly onto bacterial cells placed on a clean glass slide, and the immediate formation of oxygen bubbles was recorded as a positive reaction.

The indole test was conducted following the method described by Burhana et al. (2024). Bacterial isolates were inoculated into 5 mL of tryptone/tryptophan broth supplemented with 1% NaCl and incubated at 37˚C for 24h. Subsequently, 1 mL of Kovac's reagent (Merck, Germany) was added, and the appearance of a red ring at the interface indicated indole production.

Gelatinase activity was evaluated according to Medina and Baresi (2007). Bacterial strains were streaked onto gelatin agar plates (8 g/L gelatin, 15 g/L agar, and 10 g/L NaCl) and incubated at 37˚C for 48h. After incubation, the plates were flooded with trichloroacetic acid (TCA). Gelatinase hydrolyzes gelatin into soluble peptides. Upon TCA addition, the unhydrolyzed gelatin precipitates to form an opaque background, whereas the hydrolyzed regions remain clear, resulting in the formation of a halo surrounding the colony.

Protease activity was determined following the method described by Zheng et al. (2024). Isolates were inoculated onto skim milk agar plates (20 g/L skim milk, 10 g/L NaCl, and 15 g/L agar) and incubated at 37˚C for 48h. Proteolytic activity was identified by the presence of clear zones surrounding bacterial colonies.

Amylase activity was assessed according to Hu and Liu (2021). Bacterial isolates were spot-inoculated onto starch agar plates (peptone 5 g/L, NaCl 10 g/L, soluble starch 2 g/L, and agar 15 g/L) and incubated at 37˚C for 48h. Plates were subsequently exposed to iodine vapor for 5 min, and the appearance of clear zones around colonies was interpreted as positive amylase activity.

Antibiotic susceptibility testing

The antibiotic susceptibility of the isolated bacterial strains was determined using the Kirby-Bauer disk diffusion method. The bacteria were cultured in TSB supplemented with 1% NaCl at 37˚C for 18-24 h. The bacterial suspension was then adjusted to an optical density at 600 nm of approximately 0.5 (10^8 CFU/mL; Chen et al., 2019; Guo et al., 2019) and evenly spread onto the surface of TSA supplemented with 1% NaCl using a sterile cotton swab. Antibiotic disks containing ampicillin, cefotaxime, ciprofloxacin, levofloxacin, azithromycin, erythromycin, doxycycline, tetracycline, and trimethoprim-sulfamethoxazole (Nam Khoa, Viet Nam) were placed on the agar surface and incubated at 37˚C for 24h. After incubation, the diameters of the inhibition zones were measured to evaluate and classify the antibiotic susceptibility of the bacterial strains according to the standards and interpretive breakpoints established by the Clinical and Laboratory Standards Institute (CLSI, 2016; Table 1).

The multiple antibiotic resistance (MAR) index for each bacterial isolate was calculated according to the formula described by Ahmat et al. (2024), including MAR = A/B, where A represents the number of antibiotics ineffective on the strain, and B represents the total number of antibiotics tested on the strain.

Table 1. Interpretive criteria for antimicrobial susceptibility testing

Zone diameter (mm) interpretive criteria / Antibiotics	Susceptible	Intermediate	Resistant
Ampicillin (10 µg)	≥17	14-16	≤13
Cefotaxime (30 µg)	≥26	23-25	≤22
Ciprofloxacin (5 µg)	≥21	16-20	≤15
Levofloxacin (5 µg)	≥17	14-16	≤13
Azithromycin (15 µg)	≥18	14-17	≤13
Erythromycin (15 µg)	≥23	14-22	≤13
Doxycycline (30 µg)	≥15	12-14	≤11
Tetracycline (30 µg)	≥15	12-14	≤11
Trimethoprim-sulfamethoxazole (1.25/23.75 µg)	≥16	11-15	≤10

Evaluation of biofilm-forming ability

Bacterial strains were cultured in TSB for 24h and subsequently adjusted to an optical density at 600 nm of 0.5 before experimental assays. After that, the bacterial suspension was added to TSB medium supplemented with 1% NaCl and 1% glucose at a 1:9 (v/v) ratio (100 µL:900 µL) and incubated statically at 37˚C. After 48 h of incubation, the culture medium was discarded, and each tube was stained with 1 mL of 1% (w/v) crystal violet solution for 30 min (Nirmala et al., 2024). The staining solution was then removed, and the tubes were rinsed three times with reverse

osmosis (RO) water to remove excess dye. Subsequently, 1 mL of 95% ethanol was added to each well to solubilize the crystal violet bound to the biofilm. Then, 200 µL of the solution was transferred to a microtiter plate, and the optical density was measured at 600 nm using a spectrophotometer (Multiskan™ SkyHigh spectrophotometer, Thermo Scientific, Singapore; Abdel-Fatah et al., 2024). The negative control consisted of TSB supplemented with glucose without bacterial inoculation. The control tubes were subjected to the same incubation, biofilm staining, washing, and OD measurement procedures as the bacterial treatments.

Biofilm formation was evaluated according to the method described by Stepanović et al. (2007). Based on optical density (OD) values, bacterial isolates were classified into four categories, including non-biofilm producers, weak, moderate, and strong biofilm producers. The classification thresholds were defined as follows, including $OD \leq ODc$, non-biofilm producers; $ODc < OD \leq 2 \times ODc$, weak biofilm producers; $2 \times ODc < OD \leq 4 \times ODc$, moderate biofilm producers; and $OD > 4 \times ODc$, strong biofilm producers. The cutoff OD value (ODc) was calculated as the mean OD of the negative control plus three times its standard deviation (ODc = mean OD of negative control + 3 × SD).

Bacterial identification by molecular methods

DNA was extracted according to Tran et al. (2026). Bacterial cells were lysed using the lysis buffer for 15 minutes, and centrifuged (Eppendorf, Hamburg, Germany) at 12,000 revolutions per minute for 20 minutes. 0.5 mL of the supernatant was transferred to a new tube and mixed with 1 mL of 95% ethanol to precipitate DNA for 30 minutes, then the mixture was centrifuged to collect the pellet. The DNA pellet was washed twice with 70% ethanol and dried at 45°C for 20 minutes. Finally, the DNA was resuspended in 0.1 mL of 0.1X TE buffer and stored at -20°C.

Phylogenetic analysis based on 16S rRNA gene sequences was conducted to confirm the taxonomic position and evaluate the genetic relationships among bacterial isolates exhibiting high MAR indices and strong biofilm-forming capacity. Genomic DNA was used as template for PCR amplification with the universal bacterial primers 27F (forward; 5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (reverse; 5′-TACCTTGTTACGACTT-3′; Weisburg et al. 1991; Park et al. 2021). PCR was performed under the following conditions, initial denaturation at 95°C for 3 minutes; 30 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1.5 minutes; and a final extension at 72°C for 5 minutes. Specific and clear PCR amplicons were submitted to the DNA-Sequencing company (Can Tho city, Vietnam) for Sanger sequencing.

rRNA sequences of closely related taxa were selected based on BLASTn searches and retrieved from the NCBI nucleotide database (GenBank). The sequence dataset was aligned in BioEdit (version 7.2.1) and trimmed to the shared overlapping region to reduce bias due to unequal sequence lengths. The curated alignment was imported into MEGA12 to reconstruct a Maximum Likelihood phylogeny under the Kimura 2-parameter model with gamma-distributed rate heterogeneity (K2+G). The substitution model was selected using MEGA's built-in model selection procedure. Node support was assessed using 1,000 bootstrap replicates, and all analyses were conducted in MEGA12 (Kumar et al., 2024).

RESULTS AND DISCUSSION

A total of 18 bacterial strains were isolated from shrimp samples collected at traditional markets in Can Tho City (Table 2). Among the isolates, six produced green colonies, nine produced yellow colonies, and three produced black colonies on TCBS agar (Table 2; Figure 1D-F). Microscopic examination revealed that all isolates exhibited a short rod-shaped morphology.

Biochemical characterization demonstrated considerable phenotypic diversity among the isolates. Motility was observed in 8 out of 18 strains (44.4%), whereas all isolates were negative for indole production (100%) and positive for the methyl red reaction (100%). Hydrogen sulfide (H_2S) production was detected in 3 isolates (16.7%). Extracellular enzymatic activities were also observed in a subset of isolates, including gelatinase (4/18; 22.2%), protease (5/18; 27.8%), and amylase (4/18; 22.2%).

TCBS agar is widely used for the selective isolation of *Vibrio* spp. due to the presence of inhibitory components that strongly suppress Gram-positive bacteria and partially inhibit Gram-negative bacteria. However, different studies have demonstrated that TCBS agar is not fully selective for *Vibrio* spp. and may also support the growth of non-*Vibrio* bacteria exhibiting *Vibrio*-like colony phenotypes (Aboyadak et al., 2017; Alhusayni et al., 2024). Aboyadak et al. (2017) reported that *Aeromonas hydrophila* is capable of growing on TCBS agar and producing yellow colonies similar to those of *Vibrio cholerae* and *Vibrio alginolyticus* (*V. alginolyticus*). In that study, bacterial species were subsequently confirmed by PCR using species-specific primers targeting the *ompW* gene for *Vibrio cholerae* and the *gyrB* gene for *V. alginolyticus*. Furthermore, *Enterococcus* spp. have been reported to form yellow colonies, whereas Proteus mirabilis can produce H_2S, resulting in black colonies on TCBS agar (Alhusayni et al., 2024).

Table 2. Biological and biochemical characteristics of bacterial isolates collected from shrimps in traditional markets of Can Tho City, Vietnam (January to April 2025)

Bacterial strains	Colony morphology				Cell morphology			Biochemical characteristics							
	Color	Shape	Evalution	Margin	Shape	1	2	3	4	5	6	7	8	9	10
Mar1.1	Yellow	Circular	Raised	Entire	Bacilli	+	-	+	+	-	+	-	-	+	-
Mar1.2	Yellow	Circular	Raised	Undulate	Bacilli	+	-	+	-	-	+	-	-	+	-
Mar1.3	Green	Circular	Convex	Entire	Bacilli	-	-	-	-	-	+	+	-	+	+
Mar2.1	Black	Circular	Raised	Entire	Bacilli	-	-	-	-	-	+	+	+	+	+
Mar2.2	Yellow	Circular	Flat	Undulate	Bacilli	-	-	+	-	-	+	-	-	-	+
Mar2.3	Yellow	Circular	Raised	Entire	Bacilli	-	-	-	-	-	+	+	-	+	-
Mar2.4	Black	Circular	Raised	Entire	Bacilli	+	-	-	+	-	+	+	-	-	-
Mar2.5	Green	Circular	Raised	Entire	Bacilli	+	-	-	-	-	+	-	-	-	-
Mar3.1	Yellow	Circular	Flat	Entire	Bacilli	-	-	-	-	-	+	-	-	-	-
Mar3.2	Yellow	Circular	Flat	Entire	Bacilli	+	-	-	-	-	+	-	-	-	-
Mar3.3	Green	Circular	Flat	Entire	Bacilli	+	-	-	-	-	+	-	-	-	-
Mar3.4	Yellow	Circular	Raised	Entire	Bacilli	-	-	-	-	-	+	-	-	-	-
Mar3.5	Yellow	Circular	Raised	Entire	Bacilli	+	-	-	+	-	+	-	-	-	+
Mar3.6	Green	Circular	Raised	Entire	Bacilli	-	-	-	-	-	+	-	+	-	-
Mar4.1	Green	Circular	Raised	Entire	Bacilli	-	-	-	-	-	+	-	+	-	-
Mar4.2	Yellow	Circular	Raised	Entire	Bacilli	-	-	-	-	-	+	-	-	-	-
Mar4.3	Green	Circular	Raised	Entire	Bacilli	+	-	-	-	-	+	-	-	-	-
Mar4.4	Black	Circular	Raised	Entire	Bacilli	-	-	+	-	-	+	+	+	-	-

Colony characteristics were recorded on TCBS agar after 24 h of incubation. Cell morphology was observed under a light microscope after bacterial cultures were grown in TSB supplemented with 1% NaCl for 24 h. Symbols: Positive (+), negative (−). The analyzed parameters included: (1) Motility, (2) Gram staining, (3) Oxidase, (4) Catalase, (5) Indole, (6) Methyl red, (7) H_2S production, (8) Gelatinase, (9) Protease, and (10) Amylase.

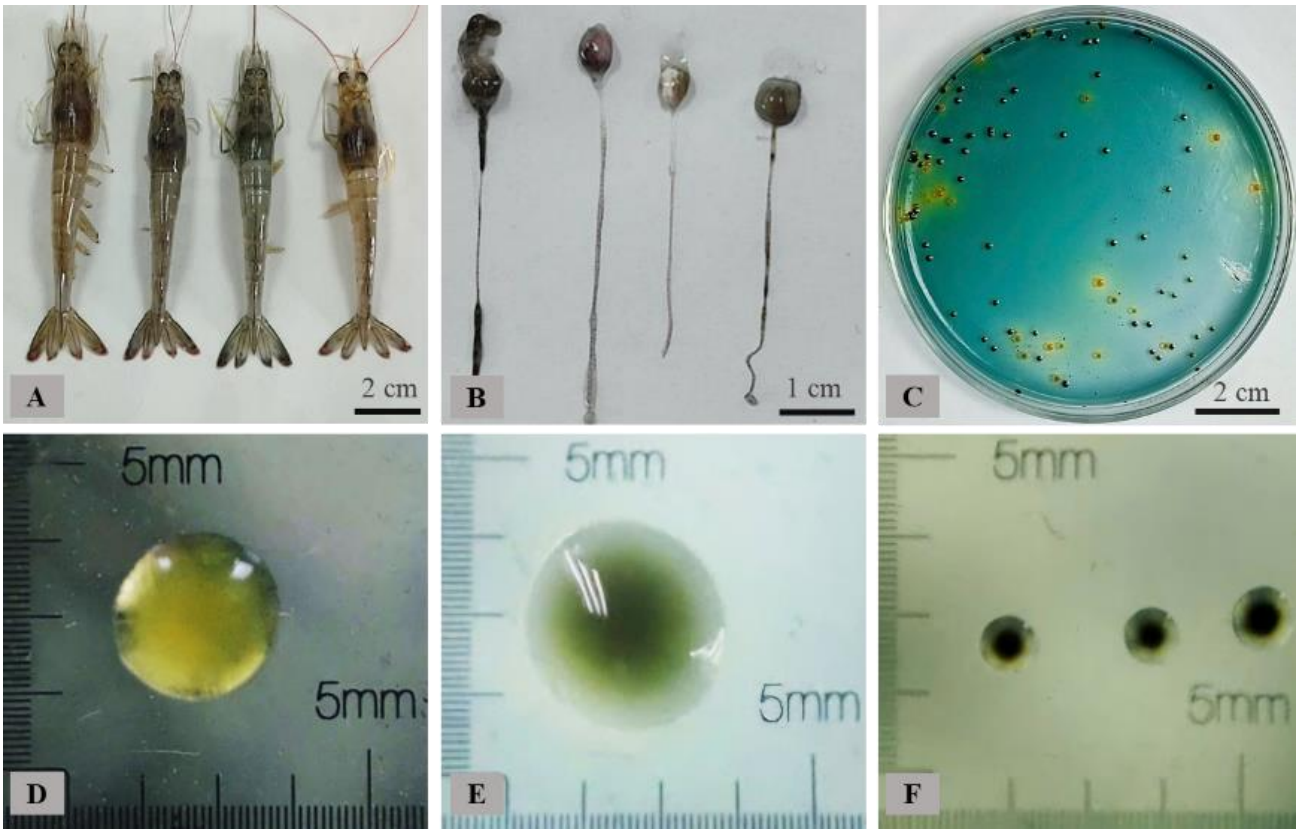

Figure 1. Samples and morphological characteristics of bacterial isolates from shrimps in traditional markets of Can Tho City, Vietnam (January to April 2025). **A**: Marketable shrimp in Cai Rang market (Mar2), **B:** Hepatopancreas and intestine (Mar2), **C**: The Mar2 sample was plated on TCBS agar, **D**: Yellow colonies, **E**: Green colonies, **F**: Black colonies.

Antibiotic susceptibility

The antibiotic susceptibility profiles of the 18 bacterial isolates are shown in Table 3, Figure 2, and Figure 3A. Overall, most isolates exhibited high resistance to the nine tested antibiotics. The bacterial isolates exhibited a high level of resistance to cefotaxime (18/18), of which 17 revealed no inhibition zones. In addition, 15 isolates were resistant to ampicillin. In contrast, fluoroquinolones (ciprofloxacin and levofloxacin) demonstrated better inhibitory activity against different isolates, particularly Mar2.5, Mar3.2, and Mar4.2, although considerable variability in susceptibility was observed. Tetracyclines (doxycycline and tetracycline) exhibited relatively effective antibacterial activity in multiple isolates, with inhibition zone diameters exceeding 20 mm in different strains. Trimethoprim-sulfamethoxazole remained effective against five isolates, with inhibition zones ranging from 21.3 to 24.0 mm. The overall resistance rates are summarized in Figure 3B. Cefotaxime and ampicillin indicated the highest resistance rates (100% and 83.3%, respectively), followed by macrolides (erythromycin, 83.3%; azithromycin, 77.8%) and trimethoprim-sulfamethoxazole (22.2%). Fluoroquinolones exhibited moderate resistance levels (ciprofloxacin, 5.6%; levofloxacin, 0%), whereas tetracyclines revealed the lowest resistance rates (doxycycline,16.7%; tetracycline, 22.2%).

MAR index values ranged from 0.22 to 0.67 (Table 3), indicating considerable variability in resistance levels among the isolates. Notably, Mar2.2, Mar3.4, and Mar3.5 exhibited the highest MAR values (0.67). Most isolates indicated MAR values between 0.40 and 0.60, including Mar1.1, Mar1.2, Mar1.3, Mar2.1, Mar2.5, Mar3.1, Mar3.3, Mar3.6, Mar4.1, Mar4.2, Mar4.3, and Mar4.4. In contrast, Mar2.4, Mar3.2, and Mar4.1 exhibited lower MAR values (< 0.40). MAR values exceeding 0.2 in aquatic food products are considered indicators of high-risk sources of antibiotic contamination (Hu et al., 2020). In the present study, the high proportion of isolates with MAR values above the mentioned threshold suggests that multidrug resistance is prevalent among bacterial populations isolated from the intestines of whiteleg shrimp, reflecting potential antibiotic selective pressure in aquaculture environments. The findings are consistent with previous report in Vietnam on antibiotic resistance in *Vibrio* spp. Ho et al. (2019) reported MAR values ranging from 0.487 to 0.519 among 240 *Vibrio* spp. isolates tested against 24 antibiotics. Meanwhile, Truyen and Nguyen (2022) reported an average MAR value of 0.259 for 150 *Vibrio* spp. isolates tested with 10 antibiotics. Ngo et al. (2022) also observed a wide MAR range from 0.15 to 1.00 when evaluating 86 *Vibrio* spp. isolates against 13 antibiotics. Variations in MAR values among studies may be attributed to differences in the number of isolates tested, antibiotic panels used, and sampling sources, as well as environmental conditions. In the present study, the use of 18 isolates and nine antibiotics may have contributed to the relatively higher MAR values observed compared to some previous studies. The MAR index in the present study was calculated based on disk diffusion antibiotic susceptibility testing. Therefore, the obtained values primarily reflect phenotypic resistance under the experimental conditions and depend on the selected antibiotic panel, and may not fully represent the complete antibiotic resistance spectrum of the bacterial isolates. Consequently, future studies integrating minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determinations, together with molecular analysis of antibiotic resistance genes, would provide a more comprehensive understanding of resistance mechanisms and levels in the isolated bacterial strains.

Table 3. Inhibition zone diameters and antibiotic resistance profiles of bacterial strains isolated from shrimps in traditional markets of Can Tho City, Vietnam (January to April 2025)

Bacterial strains	Diameter of halo (mm)									MAR index
	AMP	CTX	CIP	LEV	AZM	ERY	DOX	TET	SXT	
Mar1.1	0.0±0.0^{R}	0.0±0.0^{R}	19.7±0.6^{I}	19.3±0.6^{S}	10.7±1.2^{R}	16.3±1.5^{S}	20.0±0.0^{S}	16.0±0.0^{S}	10.0±0.0^{R}	0.44
Mar1.2	0.0±0.0^{R}	0.0±0.0^{R}	17.3±1.2^{I}	17.3±1.2^{S}	0.0±0.0^{R}	9.3±1.2^{R}	18.7±1.2^{S}	11.3±1.2^{I}	9.0±1.0^{R}	0.56
Mar1.3	0.0±0.0^{R}	0.0±0.00^{R}	8.3±0.6^{R}	13.7±1.5^{I}	21.7±0.6^{S}	9.3±1.2^{R}	18.7±1.2^{S}	22.0±2.0^{S}	16.7±1.2^{S}	0.44
Mar2.1	0.0±0.0^{R}	0.0±0.0^{R}	25.3±0.6^{S}	29.3±1.2^{S}	0.0±0.0^{R}	0.0±0.0^{R}	26.0±1.7^{S}	26.3±1.5^{S}	24.0±2.0^{S}	0.44
Mar2.2	0.0±0.0^{R}	0.0±0.0^{R}	23.3±1.2^{S}	22.0±0.0^{S}	0.0±0.0^{R}	0.0±0.0^{R}	0.0±0.0^{R}	9.3±0.6^{R}	11.7±0.6^{I}	0.67
Mar2.3	0.0±0.0^{R}	0.0±0.0^{R}	31.7±2.9^{S}	30.7±1.2^{S}	18.7±1.2^{S}	0.0±0.0^{R}	23.7±1.5^{S}	23.3±1.2^{S}	22.7±1.2^{S}	0.33
Mar2.4	22.7±1.3^{S}	12.3±0.6^{R}	21.0±1.7^{S}	24.3±0.6^{S}	0.0±0.0^{R}	0.0±0.0^{R}	32.7±1.2^{S}	30.7±1.2^{S}	21.3±1.2^{S}	0.33
Mar2.5	0.0±0.0^{R}	0.0±0.0^{R}	36.3±1.5^{S}	31.3±1.2^{S}	0.0±0.0^{R}	0.0±0.0^{R}	27.3±1.2^{S}	27.3±1.2^{S}	20.7±1.2^{S}	0.44
Mar3.1	0.0±0.0^{R}	0.0±0.0^{R}	18.0±0.0^{I}	19.3±1.2^{S}	0.0±0.0^{R}	0.0±0.0^{R}	26.7±1.2^{S}	26.0±1.0^{S}	16.3±1.5^{S}	0.44
Mar3.2	30.0±0.0^{S}	0.0±0.0^{R}	28.0±0.0^{S}	26.3±1.5^{S}	22.7±1.2^{S}	12.3±1.6^{R}	26.0±0.0^{S}	27.3±1.2^{S}	19.7±0.6^{S}	0.22
Mar3.3	0.0±0.0^{R}	0.0±0.0^{R}	20.0±0.0^{I}	20.7±1.2^{S}	10.7±1.2^{R}	12.0±2.0^{R}	20.7±2.3^{S}	20.7±1.2^{S}	0.0±0.0^{R}	0.56
Mar3.4	0.0±0.0^{R}	0.0±0.0^{R}	16.0±0.0^{I}	13.0±1.0^{I}	0.0±0.0^{R}	0.0±0.0^{R}	12.7±1.2^{I}	10.7±1.2^{R}	0.0±0.0^{R}	0.67
Mar3.5	0.0±0.0^{R}	0.0±0.0^{R}	20.0±0.0^{I}	20.0±0.0^{S}	0.0±0.0^{R}	0.0±0.0^{R}	8.0±0.0^{R}	8.7±0.6^{R}	15.7±0.6^{I}	0.67
Mar3.6	0.0±0.0^{R}	0.0±0.0^{R}	16.0±0.0^{I}	17.3±1.2^{S}	0.0±0.0^{R}	11.3±1.2^{R}	20.0±0.0^{S}	22.7±1.2^{S}	17.3±2.3^{S}	0.44
Mar4.1	17.7±2.5^{S}	0.0±0.0^{R}	26.0±0.0^{S}	22.0±0.0^{S}	0.0±0.0^{R}	0.0±0.0^{R}	23.3±1.2^{S}	22.0±0.0^{S}	19.3±1.2^{S}	0.33
Mar4.2	0.0±0.0^{R}	0.0±0.0^{R}	28.7±1.2^{S}	26.0±0.0^{S}	0.0±0.0^{R}	0.0±0.0^{R}	26.0±0.0^{S}	25.3±0.6^{S}	21.3±1.2^{S}	0.44
Mar4.3	0.0±0.0^{R}	0.0±0.0^{R}	28.0±0.0^{S}	23.3±2.9^{S}	0.0±0.0^{R}	0.0±0.0^{R}	26.7±1.2^{S}	24.3±0.6^{S}	18.0±2.0^{S}	0.44
Mar4.4	0.0±0.0^{R}	0.0±0.0^{R}	21.3±1.2^{S}	20.7±1.2^{S}	20.7±1.2^{S}	0.0±0.0^{R}	0.0±0.0^{R}	0.0±0.0^{R}	19.3±1.2^{S}	0.44

Values represent the inhibition zone diameters and are expressed as the mean of three replicates ± standard deviation (SD). R: Resistant, I: Intermediate, S: Sensitive, MAR: Multiple antibiotic resistance index, AMP: Ampicillin, CTX: Cefotaxime, CIP: Ciprofloxacin, LEV: Levofloxacin, AZM: Azithromycin, ERY: Erythromycin, DOX: Doxycycline, TET: Tetracycline, and SXT: Trimethoprim-sulfamethoxazole.

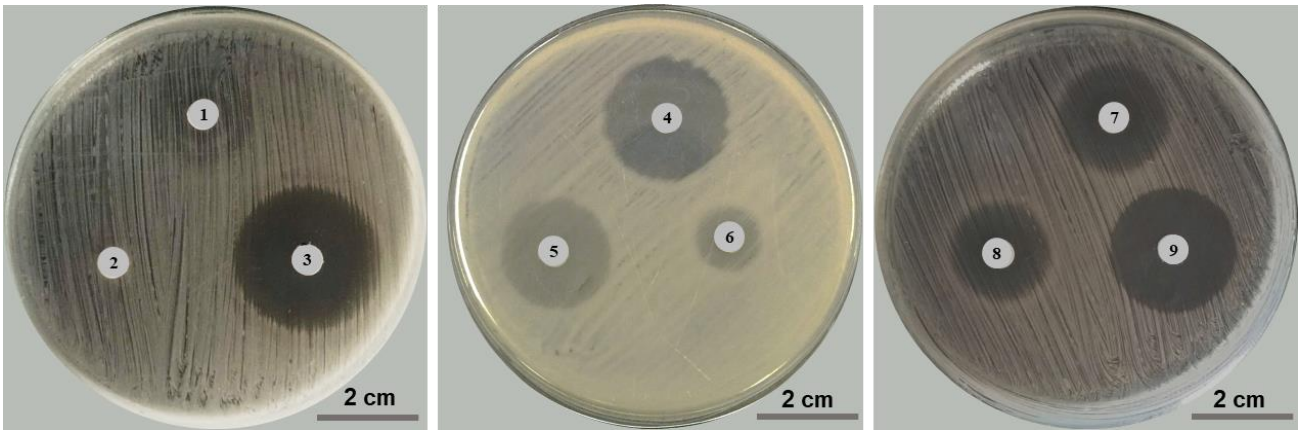

Figure 2. Antibiotic susceptibility rates of the bacterial isolates collected from shrimps in traditional markets of Can Tho City, Vietnam (January to April 2025). 1: Ampicillin, 2: Cefotaxime, 3: Ciprofloxacin, 4: Levofloxacin, 5: Azithromycin, 6: Erythromycin, 7: Doxycycline, 8: Tetracycline, and 9: Trimethoprim–sulfamethoxazole.

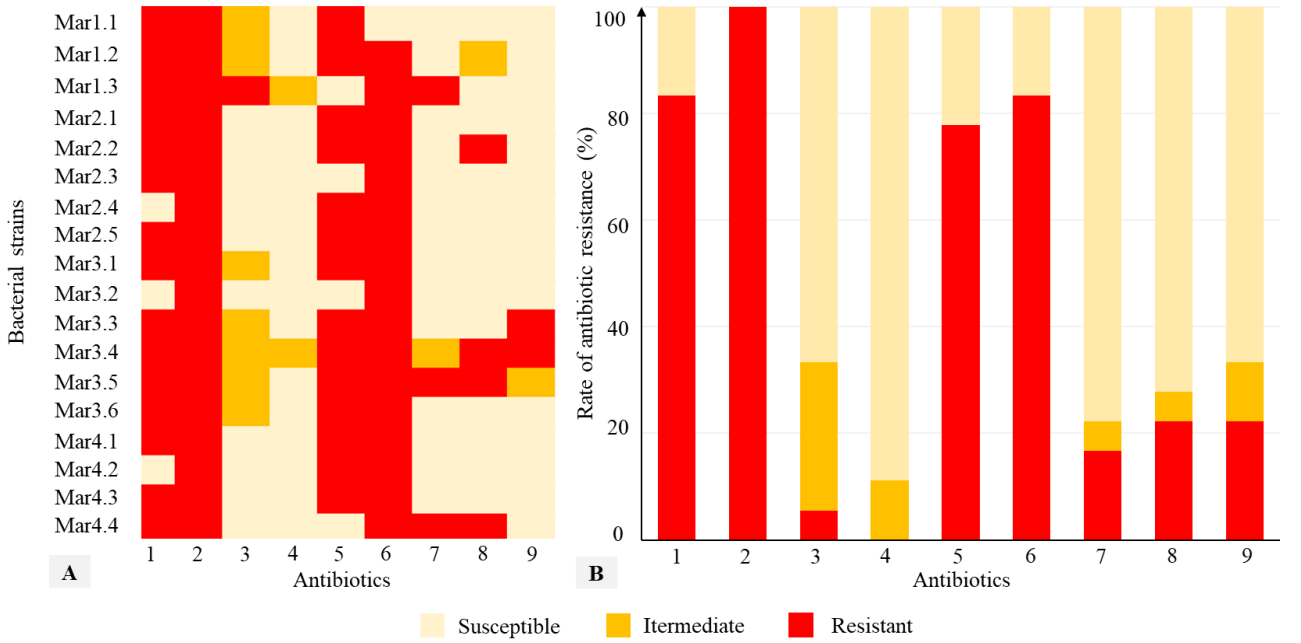

Figure 3. Antibiotic resistance patterns of bacterial isolates recovered from shrimp samples in Can Tho City, Vietnam (January to April 2025). Heatmap illustrating the antibiotic susceptibility profiles of bacterial strains **(A)**. The color gradient represents increasing levels of antibiotic resistance, ranging from green (high susceptibility) to red (high resistance). Percentage of antibiotic resistance among the tested isolates **(B)**. Antibiotics tested included ampicillin (1), cefotaxime (2), ciprofloxacin (3), levofloxacin (4), azithromycin (5), erythromycin (6), doxycycline (7), tetracycline (8), and trimethoprim-sulfamethoxazole (9).

Biofilm-forming ability

The biofilm-forming ability of the 18 bacterial isolates (Mar1.1-Mar4.4) was evaluated based on OD_{600} values, which ranged from 0.36 to 1.69 (Table 4 and Figure 4). The cut-off OD value (ODc) was determined as 0.13, calculated as the mean optical density of the negative control plus three times the standard deviation (mean + 3SD), following the method described by Stepanović et al. (2007). The results indicated that the majority of isolates exhibited moderate to strong biofilm-forming capacity. Specifically, the strong biofilm-forming group (+++) included isolates Mar1.2, Mar1.3, Mar2.2, Mar2.3, Mar2.4, Mar2.5, Mar3.2, Mar3.5, Mar3.6, and Mar4.4. Among these, the highest OD_{600} values were recorded for Mar1.2 (1.69 ± 0.22) and Mar2.4 (1.43 ± 0.12). The moderate biofilm-forming group (++) comprised isolates Mar1.1, Mar2.1, Mar3.1, Mar3.3, Mar3.4, Mar4.1, Mar4.2, and Mar4.3. In contrast, the negative control exhibited a low OD_{600} value (0.09 ± 0.01), confirming the absence of biofilm formation. The observed OD_{600} values among the tested isolates indicate substantial variability in biofilm-forming ability among the tested isolates. Notably, isolates Mar1.2, Mar2.4, and Mar4.4, which exhibited OD_{600} values more than four times higher than ODc, revealed biofilm formation and were significantly different from the remaining isolates ($p < 0.05$). The biofilm-forming ability of these strains may play a critical role in the establishment and stabilization of adherent microbial communities in the intestinal environment of whiteleg shrimp. Biofilms are considered complex multispecies microbial communities with

highly structured architectures (Prabhukhot et al., 2023), in which fast-growing and strongly adherent species often gain a competitive advantage, thereby shaping the structure and functional dynamics of microbial communities in aquatic environments (Valiei et al., 2024). Therefore, the biofilm-forming isolates were selected for subsequent 16S rRNA gene analysis to elucidate the genetic characteristics of antibiotic-resistant bacteria with high biofilm-forming potential.

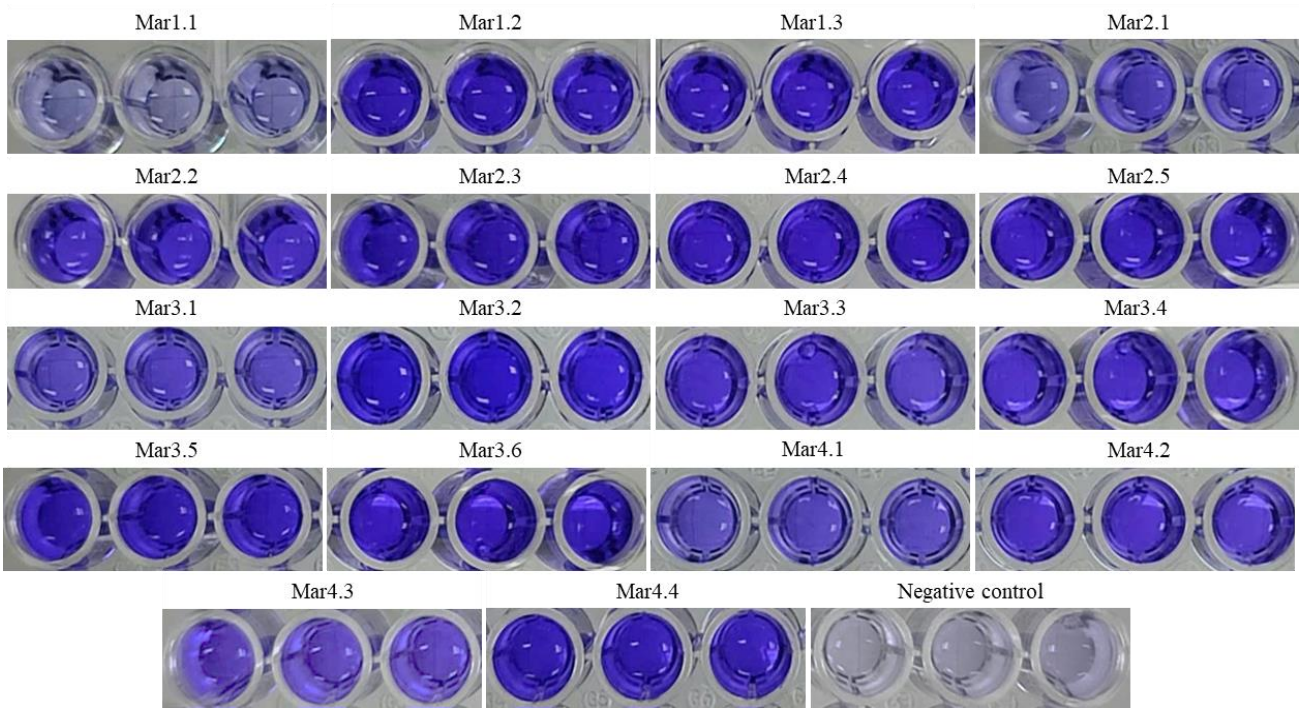

Figure 4. The biofilm was suspended in ethanol, and absorbance was measured at 600 nm in a 96-well plate.

Table 4. The OD value of biofilm formation recovered from shrimp samples in Can Tho City, Vietnam (January to April 2025)

Number	Bacterial strains	OD_{600}	Levels	Number	Bacterial strains	OD_{600}	Levels
1	Mar1.1	0.50 ± 0.09^{cde}	++	11	Mar3.3	0.51 ± 0.12^{cde}	++
2	Mar1.2	1.69 ± 0.22^{a}	+++	12	Mar3.4	0.49 ± 0.12^{cde}	++
3	Mar1.3	0.63 ± 0.07^{cde}	+++	13	Mar3.5	0.69 ± 0.27^{c}	+++
4	Mar2.1	0.39 ± 0.05^{de}	++	14	Mar3.6	0.57 ± 0.17^{cde}	+++
5	Mar2.2	0.60 ± 0.17^{cde}	+++	15	Mar4.1	0.39 ± 0.09^{e}	++
6	Mar2.3	0.68 ± 0.21^{cd}	+++	16	Mar4.2	0.36 ± 0.09^{ef}	++
7	Mar2.4	1.43 ± 0.12^{ab}	+++	17	Mar4.3	0.45 ± 0.14^{cde}	++
8	Mar2.5	0.62 ± 0.23^{cde}	+++	18	Mar4.4	1.25 ± 0.37^{b}	+++
9	Mar3.1	0.50 ± 0.16^{cde}	++	19	Negative control	0.09 ± 0.01^{f}	-
10	Mar3.2	0.56 ± 0.17^{cde}	+++	20	ODc	0.13	

No biofilm producer (-), weak biofilm producer (+), moderate biofilm producer (++), and strong biofilm producer (+++). Mean ± SD values in the table with the same letters are not significantly different at the 5% significance level according to Tukey's test.

Phylogenetic tree

The 16S rRNA-based phylogenetic tree of six isolates recovered from the gut of white leg shrimp revealed three major clusters corresponding to the genera *Vibrio*, *Providencia*, and *Shewanella*, with *Pseudoalteromonas haloplanktis* used as an outgroup to appropriately root the tree (Figure 5). Within the *Vibrio* cluster, the two isolates (Mar1.2 and Mar2.2) grouped closely with reference sequences related to the *V. alginolyticus* and *Vibrio parahaemolyticus* complex, suggesting the presence of marine or opportunistic vibrio commonly detected in shrimp gut communities and potentially associated with microbiome dysbiosis and gastrointestinal disease manifestations in intensive farming systems (Boopathi et al., 2023; Xiong et al., 2024). Notably, the placement of the two *Vibrio* isolates in different subclades indicates intra-genus diversity, implying possible differences in adhesion, biofilm-forming capacity and multidrug-resistant phenotypes that should be validated by biofilm quantification assays and antimicrobial susceptibility testing; recent studies have reported links between multidrug resistance, antibiotic resistance genes (ARGs), and biofilm formation in *Vibrio parahaemolyticus* from aquaculture settings (Nguyen et al., 2024). The two *Providencia* isolates (Mar3.4 and Mar3.5) formed a strongly supported subcluster near *P. rettgeri*, indicating close phylogenetic relatedness and suggesting that

they may share a similar intestinal niche; under antibiotic selection pressure, gut microbial communities can shift toward enrichment of ARG-carrying bacteria and conditions favorable for horizontal gene transfer, thereby increasing persistence and biofilm-associated risks (Zhao et al., 2021; Xiong et al., 2024). Finally, the two *Shewanella* isolates (Mar2.4 and Mar4.4) were nested within the *Shewanella* clade; this genus is considered a potential reservoir of resistance determinants and may contribute to the resistance network across aquatic environments and the seafood production chain, underscoring the importance of screening for ARGs and assessing biofilm formation in parallel with phylogenetic identification (Sher et al., 2025).

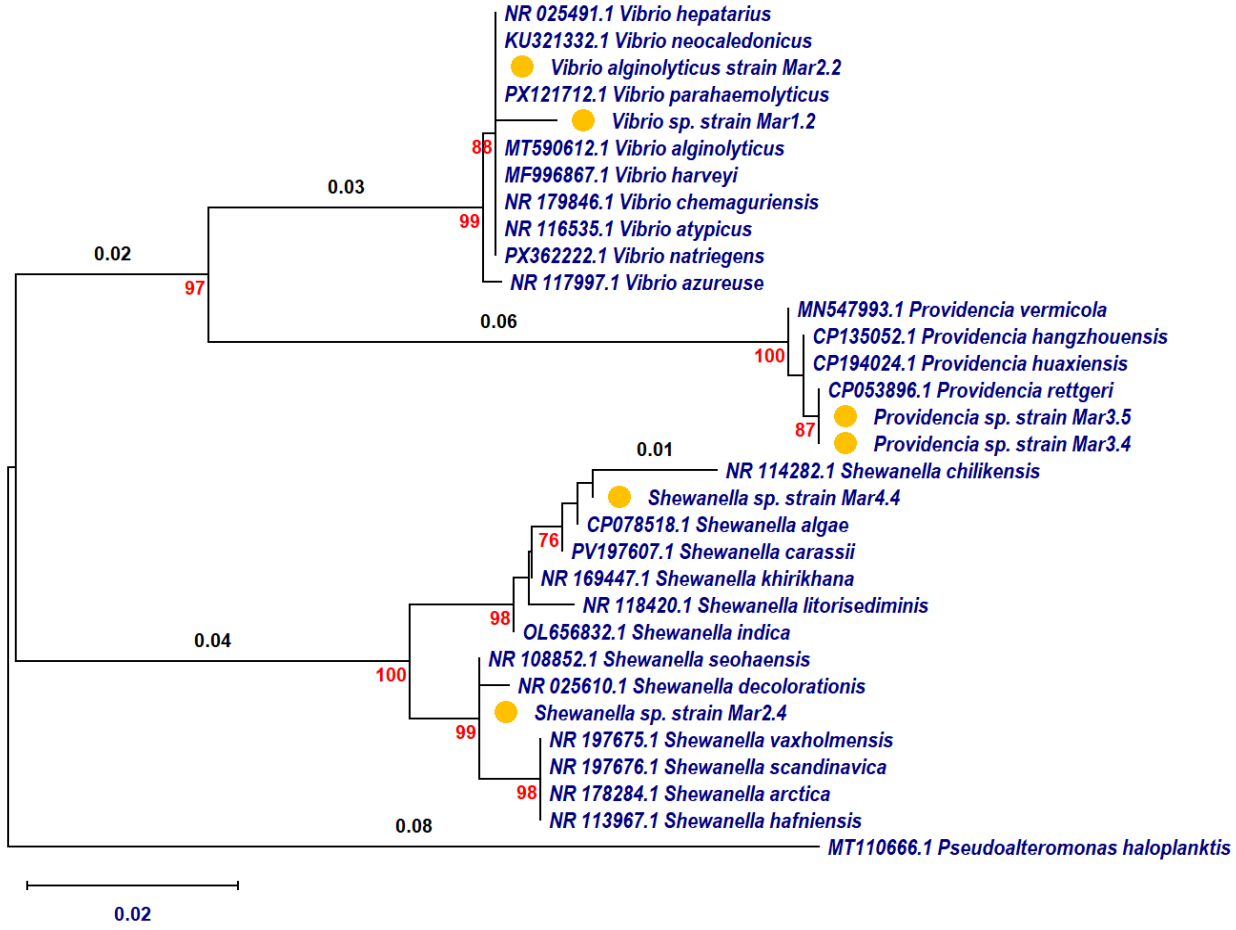

Figure 5. 16S rRNA gene-based maximum-likelihood phylogeny of bacterial isolates (Mar1.2, Mar2.2, Mar2.4, Mar3.4, Mar3.5, Mar4.4) recovered from the gut of white leg shrimp, with *Pseudoalteromonas* haloplanktis as the outgroup (K2+G; bootstrap = 1,000).

CONCLUSION

The present study successfully isolated 18 bacterial strains from TCBS agar from shrimp samples collected at four traditional markets in Can Tho City and subsequently evaluated their antibiotic susceptibility profiles and biofilm-forming capacities. The results demonstrated that most isolates exhibited resistance to the tested antibiotics, particularly ampicillin (15/18) and cefotaxime (18/18). Bacterial isolates exhibited multidrug-resistant phenotypes, including Mar2.2 (genus *Vibrio*), Mar3.4, and Mar3.5 (genus *Providencia*). Strong biofilm-forming was demonstrated for Mar1.2 (assigned to the genus *Vibrio*), as well as Mar2.4 and Mar4.4 (assigned to the genus *Shewanella*). Notably, the detection of *Shewanella* and *Providencia* species on TCBS agar, which is conventionally used for the selective isolation of *Vibrio*, highlights the microbial diversity present in shrimp-associated samples and indicates potential limitations and biases associated with relying solely on selective media for bacterial isolation. The present findings emphasized the necessity of integrating molecular identification methods to improve taxonomic accuracy. However, the present study represented only an initial assessment of the presence of antibiotic-resistant, biofilm-forming bacteria in shrimp intestinal samples collected from selected markets in Can Tho City. Therefore, further large-scale investigations are required to comprehensively evaluate antimicrobial resistance profiles across the entire production chain, from aquaculture farming systems to commercial shrimp products. Such efforts will provide essential scientific evidence to support food safety management strategies and enhance disease control practices in aquaculture.

DECLARATIONS

Authors' contributions

Hau Huu Tran performed methodology, investigation, data analysis, and original draft writing. Nhi Thao Huynh conducted methodology, investigation, and data analysis. Nam Van Be Tran provided sampling and data analysis. Thanh Uyen Le analyzed the data. Tam Ngoc Thanh Huynh contributed to laboratory analysis, statistical data, and review. All authors confirmed the final edition of the manuscript before submission to the journal.

Competing interests

The authors declare that they have no competing interests.

Ethical considerations

The authors confirm that this manuscript was prepared by the authors and has not been published elsewhere. Its content is based on original scientific findings generated by the authors. No artificial intelligence (AI) tools were used in the writing, preparing, or editing of this manuscript.

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Availability of data and materials

The data that support the present study's findings are available upon reasonable request from the corresponding author.

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