

In vitro selection and molecular characterization of indigenous yeasts from *Litopenaeus vannamei* with probiotic potential to prevent *Vibrio parahaemolyticus* infection

Mohammad Faizal Ulkhaq^{1,2}, Widanarni^{3,*},
Dinamella Wahjuningrum³, Munti Yuhana³, Jiun-Yan Loh⁴

1. Doctoral Program in Aquaculture Science, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University, Bogor, West Java 16680, Indonesia.
2. Research Group of Sustainable Aquaculture and Environment, Faculty of Health, Medicine and Life Science, Universitas Airlangga Banyuwangi, East Java 68425, Indonesia.
3. Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University, Bogor, West Java 16680, Indonesia.
4. Tropical Futures Institute, James Cook University Singapore, 149 Sims Drive, 387380, Singapore.

*** Corresponding author at:**

Department of Aquaculture,
Faculty of Fisheries and Marine
Sciences, IPB University, Bogor,
West Java 16680, Indonesia.

E-mail:

widanarni@apps.ipb.ac.id
(Widanarni).

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ABSTRACT. This study aimed to identify indigenous yeast strains from whiteleg shrimp (*Litopenaeus vannamei*) exhibiting probiotic potential and antagonistic activity against *Vibrio parahaemolyticus*. Samples were collected from shrimp intestines, sediment, and rearing water in shrimp ponds located in Banyuwangi, Jember, and Gresik. Subsequent screening was conducted based on anti-*V. parahaemolyticus* activity, biofilm inhibition, tolerance to temperature, pH, and bile salts, co-aggregation ability, hemolytic activity, pathogenicity in shrimp, and growth phase. Candidate probiotic yeast isolates were further identified using the Sanger method, with species identification based on sequence analysis against the GenBank NCBI database. A total of 120 yeast isolates were successfully obtained from the samples and screened, resulting in the identification of three candidate probiotic yeast isolates, i.e., GU12, GU13, and BA9. Molecular identification revealed that isolate GU12 exhibited 99.46% similarity to *Kodamaea ohmeri* (MK394144.1), whereas isolates GU13 and BA9 were identified as *Candida parapsilosis* (PX025649.1), with 100% similarity. Further studies are recommended to conduct additional biosafety evaluations and in vivo validation before applying these isolates in shrimp aquaculture as safe and environmentally sustainable alternatives for the prevention of bacterial diseases.

KEYWORDS: probiotic candidate, screening, sustainable aquaculture, *Vibrio parahaemolyticus*, yeast

INTRODUCTION

Infection by *Vibrio parahaemolyticus* has emerged as a significant constraint in global shrimp aquaculture due to the high mortality rates it induces. This bacterial infection can lead to sudden mass mortality of up to 100% within 30–35 days post-stocking [1]. Moreover, substantial economic losses,

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amounting to billions of dollars annually, are incurred by major shrimp-producing countries, such as China, Vietnam, Malaysia, Thailand, the Philippines, and Mexico, due to disease outbreaks [2]. Recently, preventive strategies against *V. parahaemolyticus* infection have shifted toward the use of environmentally friendly materials as substitutes for antibiotics, which are associated with significant negative impacts. The application of probiotics as an alternative to antibiotics has been extensively implemented in fish and shrimp aquaculture to mitigate the occurrence of pathogenic bacterial resistance [3].

Currently, the use of yeast as probiotics has been extensively investigated, primarily owing to its well-documented immunomodulatory properties [4]. Moreover, yeast has demonstrated various characteristics that support its suitability as a probiotic candidate, including tolerance to gastrointestinal conditions, adhesion to intestinal surfaces, antagonistic activity against pathogens, and antioxidant properties [5]. Furthermore, several bioactive compounds produced by yeast cells, such as glucans, glutathione, enzymes, and vitamins, have been reported to provide beneficial applications in aquaculture, as well as in the chemical, cosmetic, food, and pharmaceutical industries [6]. Several yeast genera commonly used as probiotics include *Saccharomyces*, *Debaryomyces*, *Hanseniaspora*, *Pichia*, *Meyerozyma*, and *Torulaspora* [7]. Within aquaculture, probiotic yeast species include *Candida* sp., *C. utilis*, *C. sake*, *C. tropicalis*, *Debaryomyces* sp., *D. hansenii*, *Hanseniaspora* sp., *Kloeckera* sp., *Kluyveromyces* sp., *Leucosporidium* sp., *Metschnikowia* sp., *Pichia* sp., *Rhodotorula* sp., *R. rubra*, *R. glutinis*, *Saccharomyces* sp., *S. cerevisiae*, *Sporobolomyces* sp., *Trichosporon* sp., and *Yarrowia* sp. [8].

Yeast can be isolated from natural environments as well as from fermented products [9], including kefir [10], sourdough [11], and other fermented food products [12]. In addition, yeast isolates can also be obtained from the gastrointestinal tracts of fish [13] and from aquaculture environments [14]. The selection of optimal probiotic candidates is accomplished through screening processes based on their ability to grow and thrive in their native environments [15]. This supports the premise that indigenous microorganisms possess a greater capacity to colonize, compete with pathogenic microbes, and enhance host resistance to disease [16]. Furthermore, indigenous microorganisms play an important role in promoting host health by strengthening immune responses, inhibiting opportunistic pathogens through the production of antimicrobial compounds, and facilitating nutrient conversion [17].

Previous studies have successfully isolated yeast from fish intestines and aquatic environments with potential as probiotic agents, including *R. mucilaginosa* and *Wickerhamiella infanticola* from the gastrointestinal tract of gilthead seabream (*Sparus aurata*) [18], as well as *D. hansenii* and *R. mucilaginosa* from the gastrointestinal tract of carnivorous fish [19]. Other notable isolates include *S. cerevisiae* and *D. hansenii* from the gastrointestinal tract of zebrafish (*Danio rerio*) [20]; *C. haemulonii* and *D. hansenii* from the gastrointestinal tract of cobia (*Rachycentron canadum*) [13]; *Cryptococcus albidus* from the Red Sea [21]; *P. kudriavzevii* HJ2 from mangrove sediment in the Beibu Gulf, South China Sea [22]; and *Naganishia diffluens* and *Apiotrichum mycotoxinivorans* from Toledo River water, Brazil [23]. However, limited information is available on indigenous probiotic yeasts from *L. vannamei* with antibacterial activity against *V. parahaemolyticus*, and their potential advantages over non-host-associated isolates remain insufficiently explored. This study aimed to molecularly identify indigenous yeasts from whiteleg shrimp and evaluate their probiotic characteristics and antagonistic activity against *V. parahaemolyticus*. The findings provide potential host-adapted probiotic candidates and enhance understanding of their application for effective, efficient, and environmentally sustainable disease prevention in shrimp aquaculture, supporting sustainable shrimp production.

MATERIALS AND METHODS

Ethical approval

The handling of experimental animals in this study was approved by the Ethics Commission of Universitas Airlangga under certificate number 1011/HRECC.FODM/IX/2025.

Study period and location

This study was conducted from May to August 2025 at the Integrated Laboratory, Faculty of Health, Medicine, and Life Sciences, Universitas Airlangga, for yeast probiotic isolation and selection. Sampling of shrimp, sediment, and aquaculture water from whiteleg shrimp ponds was carried out at shrimp farms in the coastal regions of East Java, namely Banyuwangi (S1), Jember (S2), and Gresik (S3) (Figure 1). The sampled ponds were operated under different culture systems, including traditional (S3), semi-intensive (S1), and intensive (S2) systems, with salinity ranging from 25–30 ppt. Shrimp appeared healthy at the time of sampling, and no disease outbreaks had been reported during the production cycle.

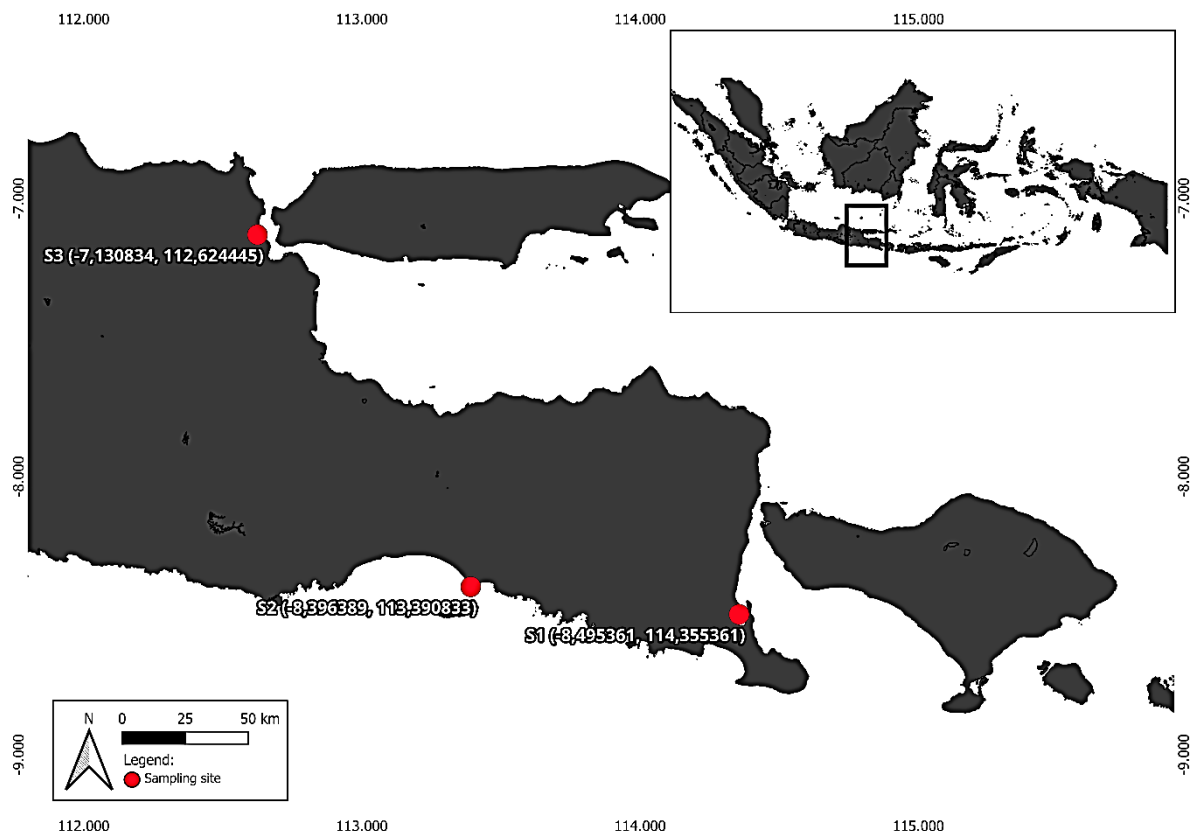


Figure 1. Sampling locations.

Yeast isolation

The procedures used for yeast isolation from the samples were based on previously reported methods [21–23]. A total of 30 healthy whiteleg shrimp, with an average weight of 23.35 ± 2.9 g and length of 14.82 ± 2.2 cm, were collected from each location (S1–S3). At each location, two ponds were randomly selected, and 15 healthy shrimp were collected from each pond. Samples from the same pond were pooled and dissected to obtain the gastrointestinal tract. A portion of 0.1 g of intestinal tissue was weighed and homogenized with sterile PBS solution at a volume 10 times the intestinal weight. A total of 1 g of pond sediment and 1 mL of pond water were each homogenized with 10 mL of PBS solution. Sediment and water samples were pooled from the central area of each location (S1–S3). Water samples were obtained from approximately 10–15 cm below the water surface using sterile ziplock bags. Furthermore, sediment samples were collected from the upper 5 cm layer using a sterile spatula. All suspensions were then serially diluted tenfold up to a 10^2 dilution, and 0.1 mL from each dilution was plated on yeast glucose chloramphenicol (YGC) agar (HiMedia, India) and incubated at 27°C for 48 h. All yeast colonies that grew and exhibited distinct colony morphologies were aseptically re-cultured on fresh YGC medium. The screening process for probiotic yeast candidates was conducted using a sequential screening method [25, 26].

Antagonistic activity against *V. parahaemolyticus*

This assay was conducted to determine the ability of probiotic yeast isolates to inhibit the pathogen *V. parahaemolyticus*. The *V. parahaemolyticus* strain used was obtained from the Integrated Laboratory, Faculty of Health, Medicine, and Life Sciences, Universitas Airlangga, and had been designated as rifampicin-resistant (Rif^R). Prior to use, the *V. parahaemolyticus* Rif^R strain was identified biochemically and molecularly and then recultured on thiosulfate citrate bile salts sucrose (TCBS) agar and tryptic soy broth (TSB) (Merck, Germany) supplemented with 3% NaCl, followed by enumeration using the plate count method.

The anti-*V. parahaemolyticus* assay was performed using a co-culture method [27] with several modifications. A total of 0.1 mL of a 48-h probiotic yeast isolate (density, 10^6 CFU mL⁻¹) was inoculated into 10 mL of brain heart infusion broth (BHIB) (Merck, Germany), followed by the addition of 0.1 mL of a 24-h *V. parahaemolyticus* Rif^R isolate (density, 10^2 CFU mL⁻¹). The positive control consisted of 0.1 mL of the *V. parahaemolyticus* Rif^R isolate (10^2 CFU mL⁻¹) inoculated into 10 mL of BHIB. All test tubes were incubated for 24–48 h at 27°C, and *V. parahaemolyticus* Rif^R counts were determined using the plate count method. A volume of 0.1 mL of microbial suspension was collected and homogenized with 0.9 mL of PBS solution, followed by serial dilution and plating on TCBS agar supplemented with 50 ppm rifampicin for enumeration of total *V. parahaemolyticus* Rif^R (TVpC). The antibacterial activity of probiotic yeast was indicated by a reduction in the number of *V. parahaemolyticus* Rif^R in co-culture with probiotic yeast compared with the positive control [28]. Currently, no universally accepted quantitative threshold is available for classifying the antagonistic activity of probiotic yeasts against *V. parahaemolyticus*. Therefore, isolate selection was based on relative inhibitory performance rather than an absolute cut-off value [29].

Antibiofilm activity

Antibiofilm activity indicates the ability of probiotic yeast to prevent the formation of biofilm layers by *V. parahaemolyticus* as an initial stage of colonization in shrimp [25, 26]. The antibiofilm assay was conducted based on a previously described method [32], with modifications. A total of 15 µL of a 48-h yeast isolate (density, 10^6 CFU mL⁻¹) was inoculated into a microplate containing 200 µL of BHIB medium. Subsequently, 15 µL of a 24-h *V. parahaemolyticus* culture (density, 10^2 CFU mL⁻¹) was added, followed by incubation at 27°C for 24 h. BHI broth without microbial isolates was used as the control. After incubation, the wells were washed twice with PBS and air-dried for 30 min, then stained with 0.1% (v/v) crystal violet for 5 min and washed with PBS up to three times. After air-drying for 30 min, 33% (v/v) glacial acetic acid was added for 10 min. Biofilm biomass was quantified by measuring absorbance at 620 nm using a microplate reader (Thermo, Germany). Yeast isolates were then classified as strong ($OD_{620} < 2 \times OD_{\text{control}}$), moderate ($2 \times OD_{\text{control}} < OD_{620} \leq 4 \times OD_{\text{control}}$), weak ($4 \times OD_{\text{control}} < OD_{620}$), and negative ($OD_{620} \geq 4 \times OD_{\text{control}}$) [33], as described in previous biofilm studies.

Tolerance to temperature, pH, and bile salts

Temperature resistance testing was conducted to evaluate the survival ability of each probiotic yeast isolate across a wide temperature range. This test was performed by inoculating 0.1 mL of a 48-h yeast isolate (density, 10^6 CFU mL⁻¹) into YPD broth (HiMedia, India) and incubating for 48 h at 10°C, 27°C, and 37°C. Tolerance to pH and bile salts was assessed to evaluate the ability of probiotic yeast to survive under gastric and intestinal conditions characterized by varying pH levels and high bile salt concentrations. The pH tolerance test was conducted by inoculating 0.1 mL of a 48-h yeast isolate (density, 10^6 CFU mL⁻¹) into YPD broth adjusted to acidic (pH 1.5, 2, and 3), neutral (pH 7), and alkaline (pH 9) conditions using HCl and NaOH [34–36]. Meanwhile, bile salt tolerance was evaluated by inoculating 0.1 mL of a 48-h yeast isolate (density, 10^6 CFU mL⁻¹) into YPD broth supplemented with bile salts (Loba Chemie, India) at concentrations of 0.5%, 1%, and 2% (w/v) [37]. All media were then incubated at 27°C for 48 h [38]. YPD broth without yeast isolates was used as the control. Following incubation, both treatments and controls were measured for absorbance using a

spectrophotometer (Shimadzu, Japan) at a wavelength of 620 nm [11]. The criteria for selecting yeast as probiotic candidates were isolates capable of growing across a wide range of temperatures and pH levels and demonstrating resistance to bile salts [12].

Co-aggregation test

This test was conducted to determine the ability of yeast isolates to aggregate pathogenic bacteria. A total of 0.5 mL of a 24-h yeast isolate (density, 10^7 CFU mL⁻¹) in sterile PBS was mixed with 0.5 mL of *V. parahaemolyticus* Rif^R (density, 10^9 CFU mL⁻¹), followed by incubation for 2 h at 27°C. The incubation mixture was then centrifuged at 5,000 rpm for 5 min. The supernatant was discarded, and Gram staining was performed to observe the formation of aggregates. The degree of aggregation was observed in 10 different fields of view under a light microscope and categorized as follows: (-), no aggregation; (+), low aggregation activity; (++) , moderate aggregation activity; and (+++) , high aggregation activity [38]. Selected probiotic yeast isolates should be capable of forming aggregates with pathogenic bacteria to prevent intestinal colonization and facilitate pathogen elimination [24].

Hemolytic activity

This assay was conducted to determine the ability of yeast isolates to lyse red blood cells [39]. A 48-h yeast culture was grown on blood agar (HiMedia, India) supplemented with 5% sheep blood and incubated at 27°C for 48 h. *Staphylococcus aureus* was used as a positive control because of its ability to produce β -hemolysis. The results were classified as α -hemolysis (greenish zone surrounding the colony), β -hemolysis (clear zone surrounding the colony), and γ -hemolysis (no hemolysis). Yeast isolates that did not cause any change in the agar medium (γ -hemolysis) were selected as safe probiotic candidates [5].

Pathogenicity in shrimp

The pathogenicity test was conducted to determine whether the probiotic yeast candidates exhibited pathogenic properties [40]. A total of 110 shrimp (average weight, 1.728 ± 0.23 g) were distributed into 22 plastic containers representing 11 treatments with two replicates, containing 10 L of water and equipped with aeration and filtration systems. A 48-h yeast isolate was introduced into water with a salinity of 15 ppt to achieve a final density of 10^8 CFU mL⁻¹ as a high-dose preliminary screening [41]. As a positive control, shrimp were exposed to *V. parahaemolyticus* Rif^R at a density of 10^5 CFU mL⁻¹, whereas the negative control received PBS at the same volume and via the same administration route. During the experiment, shrimp were fed a commercial pellet diet (FengLi, Suri Tani Pemuka, Indonesia) containing 30% protein, 7% lipid, 3% fiber, 13% ash, and 10% moisture. The diet was administered six times daily (07:00, 10:00, 13:00, 16:00, 19:00, and 22:00) at a feeding rate of 5%. No siphoning or water exchange was performed. Water quality parameters (temperature, pH, salinity, dissolved oxygen, ammonia, nitrate, nitrite, and phosphate) were monitored and maintained at optimal levels. Clinical symptoms and daily mortality rates were observed over a 7-day rearing period. Probiotic yeast candidates selected should be non-pathogenic to the host [38].

Growth curve of probiotic yeast

Growth curves were determined primarily to identify the exponential growth phase and establish the optimal cultivation time for subsequent probiotic characterization assays rather than to perform detailed kinetic modeling. A total of 0.1 mL of a 24-h yeast isolate (density, 10^6 CFU mL⁻¹) was inoculated into 10 mL of YPD broth and incubated for 72 h at 27°C [42]. Measurements were taken every 6 h using the plate count method on YGC agar medium [13]. The yeast population was then expressed in colony-forming units (CFU) and calculated using the following equation:

$$\text{Yeast population (CFU / mL)} = \frac{\text{Number of colony} \times \text{dilution factor}}{\text{volume of inoculum (mL)}} \dots\dots\dots (1)$$

Molecular identification and phylogenetic tree construction

Molecular identification of selected probiotic yeast isolates was performed by amplification of the ITS (internal transcribed spacer) gene region, followed by sequence analysis. Genomic DNA from yeast isolates was extracted using the Quick-DNA MagBead Plus Kit (Zymo Research, D4082). The ITS gene region of probiotic yeast was amplified by PCR using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers [38]. The PCR master mix (25 µL total volume) consisted of 12.5 µL of MyTaq HS Red Mix (2×) (Bioline, BIO-25048), 1 µL of ITS1 primer, 1 µL of ITS4 primer, 1 µL of DNA template, and 9.5 µL of ddH₂O. Amplification was performed using the following program: pre-denaturation at 95°C for 1 min, followed by 35 PCR cycles consisting of denaturation at 95°C for 10 s, annealing at 52°C for 15 s, and extension at 72°C for 15 s. PCR products were visualized using a gel imaging system following 1% agarose gel electrophoresis in TBE buffer (100 mM). Amplified DNA was sequenced bidirectionally using the Sanger method via capillary electrophoresis. Forward and reverse sequences of each isolate were aligned and compared with reference sequences in the NCBI GenBank database using the Basic Local Alignment Search Tool (BLAST), and the percentage identity of each sequence was determined. Phylogenetic relationships were reconstructed using the maximum likelihood method after selecting the best-fit nucleotide substitution model with the Find Best DNA Model test available in MEGA X. The reliability of the resulting phylogenetic tree was assessed using 1,000 bootstrap replicates [24].

Statistical analysis

Data obtained from the selection and identification of probiotic yeast isolates were tabulated and presented graphically using Microsoft Excel 2021. Antagonistic activity and tolerance assays were statistically analyzed using one-way ANOVA at the 95% confidence level and compared with the control. Furthermore, antibiofilm activity was analyzed using the chi-square goodness-of-fit test at the 95% confidence level, whereas co-aggregation activity was analyzed using an exact binomial test at the 95% confidence level. Pathogenicity data were statistically analyzed using ANOVA at the 95% confidence level, followed by Tukey's HSD post hoc test. Nucleotide sequencing data and the phylogenetic tree were analyzed bioinformatically using MEGA X software.

RESULTS

Yeast isolation

A total of 120 yeast isolates were successfully obtained from water, sediment, and shrimp intestine samples collected from three different sampling stations. Of these, 54 isolates (45%) were derived from the intestine, 39 isolates (32.5%) from water, and 27 isolates (22.5%) from sediment samples. In general, 47 isolates (39.2%) were obtained from S1 (Banyuwangi), 28 isolates (23.3%) from S2 (Jember), and 45 isolates (37.5%) from S3 (Gresik) (Table 1).

Table 1. Number of yeast isolates obtained from each sampling location

Station	Source			Total
	Water	Sediment	Intestine	
S1 (Banyuwangi)	16	2	29	47
S2 (Jember)	9	12	7	28
S3 (Gresik)	14	13	18	45
Total	39	27	54	120

Antagonistic activity against *V. parahaemolyticus*

Antagonistic activity against *V. parahaemolyticus* was the primary criterion for selecting probiotic candidates in this study. Based on the co-culture assay results, 58 of the 120 yeast isolates exhibited anti-*V.*

parahaemolyticus activity, consisting of 19 isolates (32.76%) from S1 (Banyuwangi) and 39 isolates (67.24%) from S3 (Gresik). Of these 58 isolates, 29 isolates (50%) were derived from the intestine, 18 isolates (31.03%) from water, and 11 isolates (18.96%) from sediment (Figure 2). Analysis of variance revealed significant differences in antagonistic activity among the isolates compared with the positive control ($F = 3.12$, $p < 0.05$). Interestingly, none of the yeast isolates recovered from S2 (Jember) exhibited anti-*V. parahaemolyticus* activity.

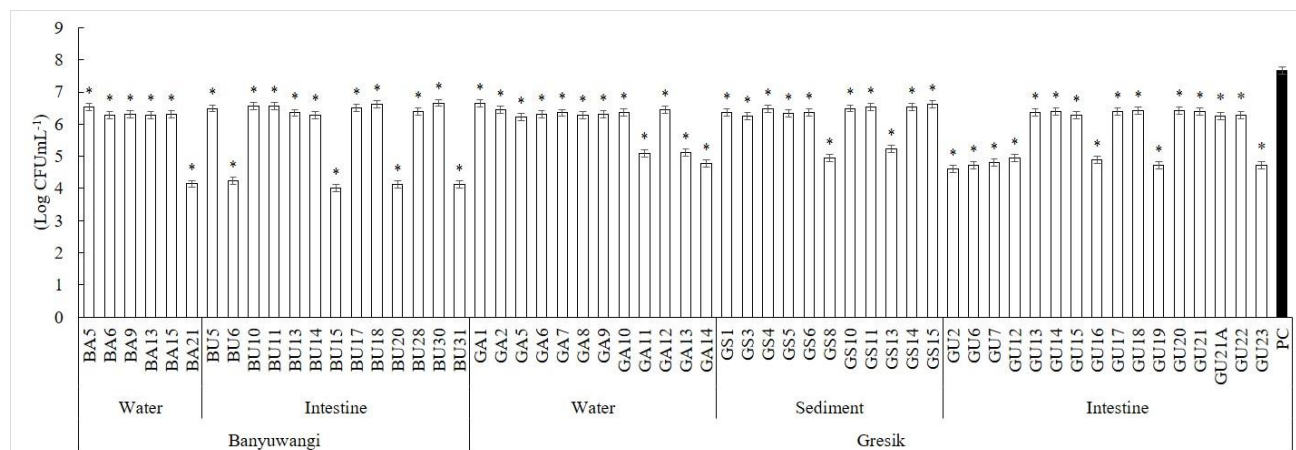


Figure 2. Number of *V. parahaemolyticus* colonies after the co-culture assay with selected yeast isolates. PC = positive control. Data are presented as mean $\pm$ SD of three replicates. Asterisks (*) indicate significant differences ($p < 0.05$).

Antibiofilm activity

Antibiofilm activity indicates the ability of probiotic isolates to inhibit biofilm formation. Based on the biofilm inhibition assay, all tested isolates exhibited antibiofilm activity with varying degrees of activity. A total of 39 isolates (67.24%) showed strong activity, 15 isolates (25.86%) showed moderate activity, and 4 isolates (6.89%) exhibited weak antibiofilm activity, namely BU5, BU13, BU15, and BU31; these isolates were subsequently excluded from further testing. A chi-square goodness-of-fit test indicated that the distribution of isolates among the antibiofilm activity categories differed significantly from an equal distribution ($\chi^2 = 33.13$, $df = 2$, $p < 0.001$).

Tolerance to temperature, pH, and bile salts

Based on the temperature tolerance test (Figure 3A), all tested isolates (54 isolates) demonstrated the ability to survive at 10°C, 27°C, and 37°C, as indicated by OD values ranging from 0.068 to 0.982. One-way ANOVA demonstrated that temperature had a significant effect on the OD values of the isolates ($F = 5.89$, $p < 0.05$). In the pH tolerance test (Figure 3B), only 43 of the 54 isolates (79.63%) were able to grow across a wide pH range (1.5–9); thus, only these 43 isolates were selected for subsequent bile salt tolerance testing. The ANOVA results indicated that pH significantly affected the OD values of the isolates ($F = 7.94$, $p < 0.05$). In the bile salt tolerance assay (Figure 3C), among the 43 selected isolates, only 22 isolates (51.16%) were able to tolerate bile salt concentrations of up to 2% and were subsequently selected for the next screening procedures. Statistical analysis using one-way ANOVA showed significant differences in the OD values of the isolates compared with the control ($F = 23.16$, $p < 0.05$).

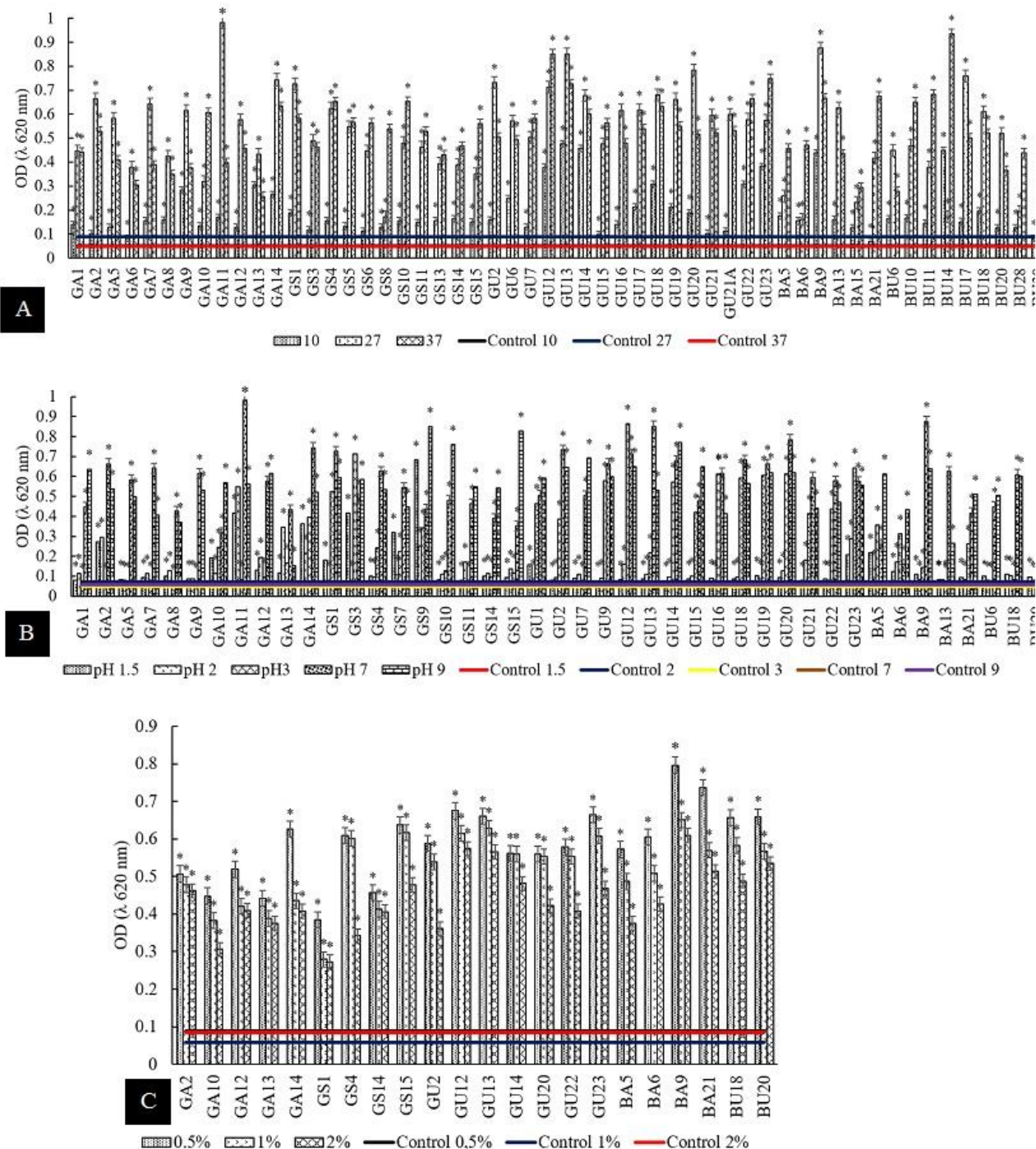


Figure 3. OD values of selected yeast isolates after incubation at different (A) temperatures, (B) pH levels, and (C) bile salt concentrations. Data are presented as mean $\pm$ SD of three replicates. Asterisks (*) indicate significant differences ($p < 0.05$).

Co-aggregation test

The ability to aggregate *V. parahaemolyticus* was one of the selection criteria for probiotic yeast candidates in this study. Based on the co-aggregation test, varying aggregation abilities were observed among the 22 tested isolates, with 10 isolates (45.45%) showing a high degree of microscopic co-aggregation ability (Figure 4A) and 12 isolates (54.54%) showing low aggregation ability (Figure 4B). An exact binomial test showed that the distribution of isolates between the high- and low-aggregation groups did not differ significantly from an equal distribution ($p = 0.832 > 0.05$). The 10 isolates with high aggregation ability were selected for further testing.

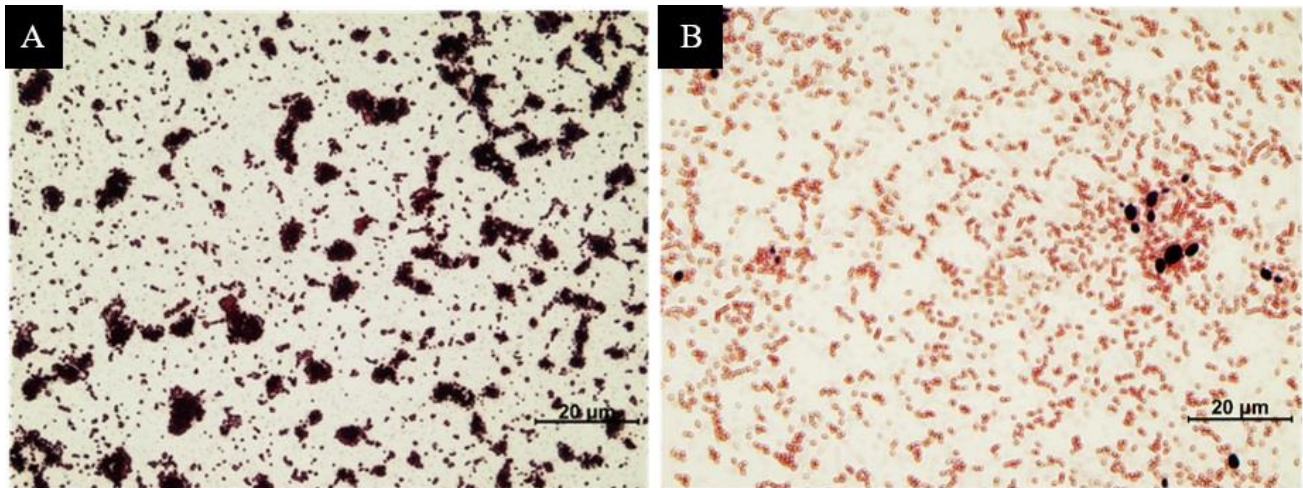


Figure 4. Co-aggregation test results of selected yeast isolates. (A) High aggregation ability; (B) low aggregation ability.

Hemolytic activity

The hemolytic activity assay indicates the ability to lyse erythrocytes, which is a virulence factor of *V. parahaemolyticus*, and was used to determine the non-pathogenic nature of the yeast isolates. Among the 10 isolates selected from the previous test, only one isolate (GA14) exhibited moderate hemolysis (α -hemolysis), whereas the remaining nine isolates, i.e., GU12, GU13, BA9, GA13, BA21, BA6, GA10, GU14, and GU23, showed γ -hemolysis (no erythrocyte lysis) (Figure 5A) and were selected for further screening. The positive control, *Staphylococcus aureus*, demonstrated strong hemolytic activity (β -hemolysis) (Figure 5B).

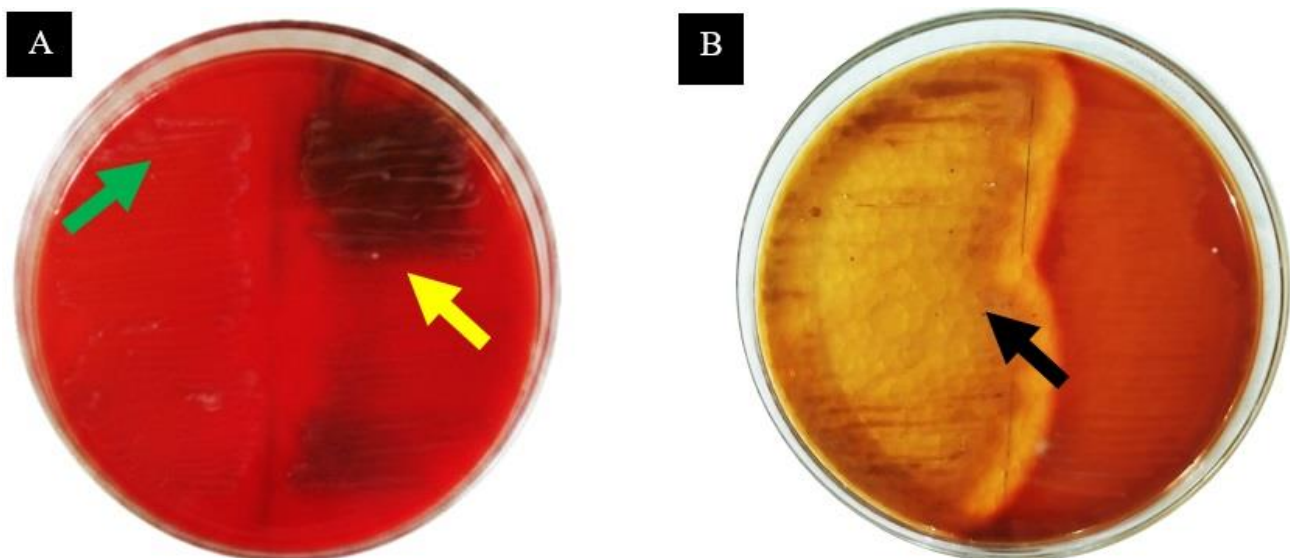


Figure 5. Hemolytic activity test results of probiotic yeast isolates. (A) α -hemolysis in isolate GA14 (yellow arrow) and γ -hemolysis in isolate GU12 (green arrow); (B) β -hemolysis in *S. aureus* (black arrow).

Pathogenicity in shrimp

The pathogenicity test was conducted to determine the pathogenic characteristics of the selected yeast isolates in shrimp. The results (Figure 6) showed that, among the nine isolates tested over a 7-day period, only six isolates demonstrated survival rates above 90%, namely, GU12 (100% $\pm$ 0.00), GU13 (100% $\pm$ 0.00), BA9 (100% $\pm$ 0.00), GA13 (96.67% $\pm$ 1.33), BA21 (95.00% $\pm$ 2.00), and BA6 (95.00% $\pm$ 1.67). These isolates had significantly higher survival rates than the positive control (Tukey's HSD post hoc test, $p < 0.05$). Shrimp mortality differed significantly among the treatments (one-way ANOVA, $F = 37.63$, $p < 0.05$). Daily qualitative

clinical monitoring revealed no overt physical distress or abnormal behavioral changes. All shrimp in all groups maintained normal physical activity, without signs of external lesions. The remaining three isolates, i.e., GA10, GU14, and GU23, showed survival rates below 90%, with values of $75.00\% \pm 3.50$, $66.67\% \pm 3.00$, and $65.00\% \pm 2.66$, respectively, and were therefore excluded from further testing.

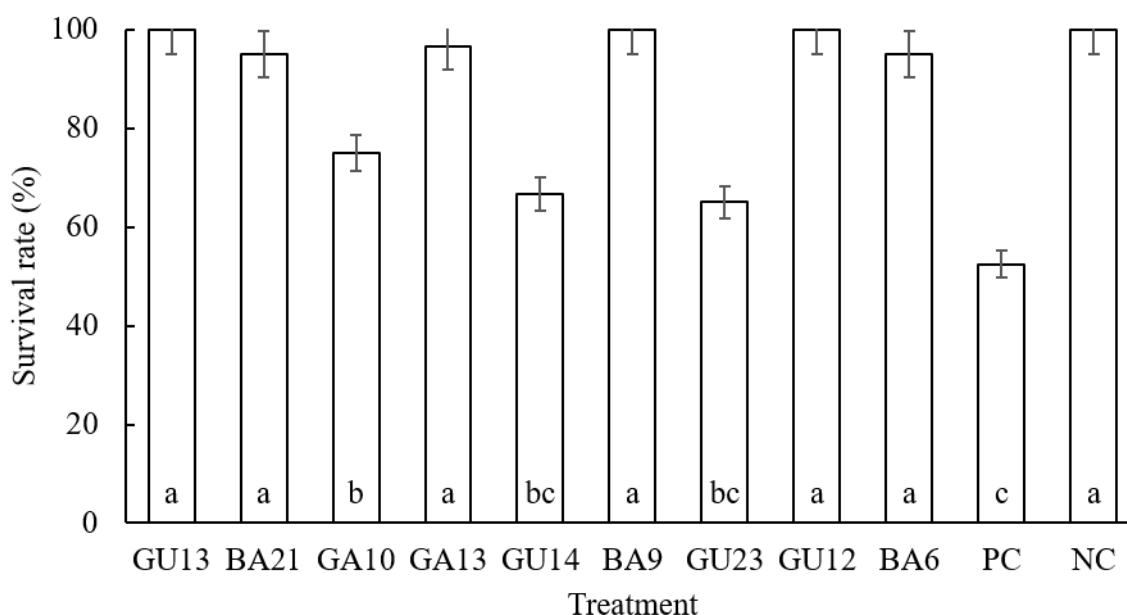


Figure 6. Shrimp survival rate (%) in the pathogenicity test of probiotic yeast candidates.

PC (positive control) = immersion with *V. parahaemolyticus* Rif^R at 10^5 CFU mL⁻¹; NC (negative control) = immersion with PBS. Data are presented as mean $\pm$ SD of two replicates.

Growth curve of probiotic yeast

Observation of the growth curve was conducted to determine the incubation time required for the yeast isolates to reach the exponential phase. In this test, only three yeast isolates with the highest survival rates in the shrimp pathogenicity test were used, i.e., GU12, GU13, and BA9. The results showed that isolate GU12 entered the exponential phase earlier than the other isolates (Figure 7); however, quantitative growth parameters were not calculated. Isolate GU12 reached the exponential phase after 12 h of incubation, with a density of 10.75 ± 0.54 log CFU mL⁻¹, whereas isolates GU13 and BA9 reached their highest densities at 24 h, with values of 9.32 ± 0.79 log CFU mL⁻¹ and 9.82 ± 0.96 log CFU mL⁻¹, respectively. Subsequently, a decline in cell density was observed until the end of the observation period (72 h), with densities ranging from 7.03 ± 1.05 to 7.18 ± 1.14 log CFU mL⁻¹.

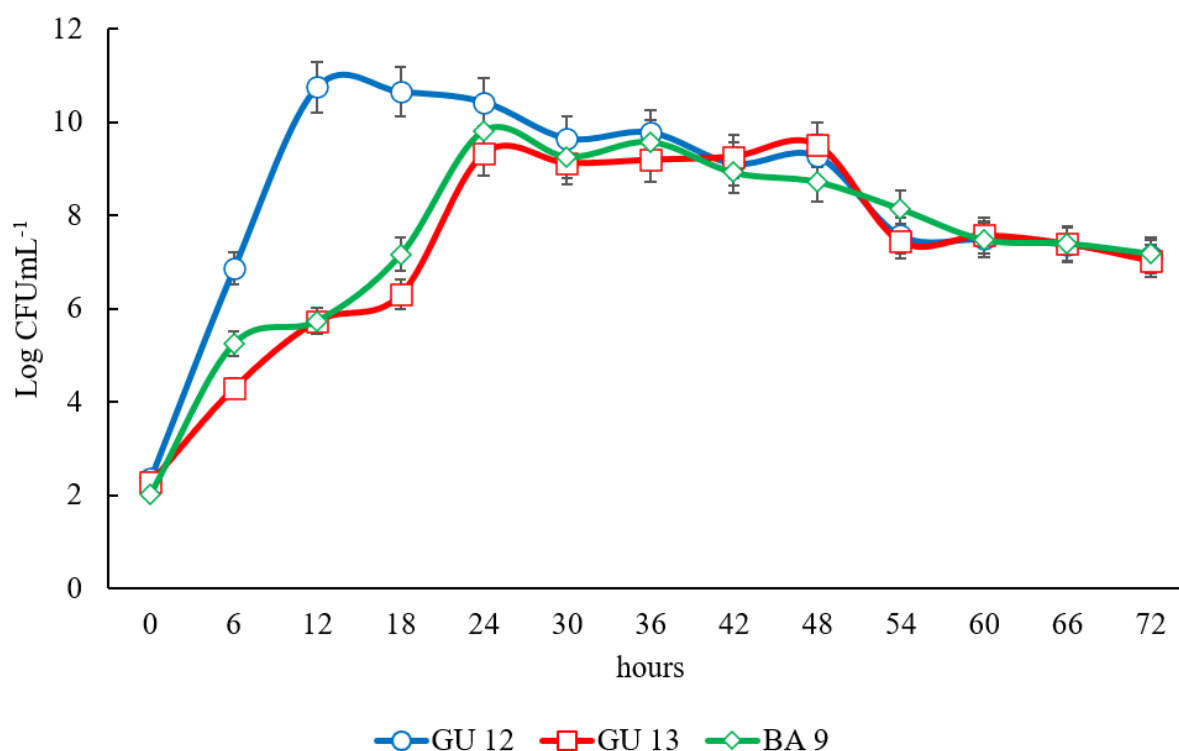


Figure 7. Growth curves of probiotic yeast candidates during 72 h of observation.

Molecular identification

Molecular identification of the yeast isolates was performed based on sequencing results of the ITS1 and ITS4 regions to the species level. The obtained sequences were subsequently subjected to homology analysis using BLAST against the NCBI database. The ITS region identification results (Table 2) showed that isolate GU12 exhibited 99.46% similarity to *Kodamaea ohmeri* (PZ742271), whereas isolates GU13 and BA9 were identified as *C. parapsilosis* (PZ742277 and PZ744513, respectively), with 100% similarity. All ITS nucleotide sequences generated during this study have been deposited in the NCBI GenBank database and are publicly available through the GenBank database. The ITS phylogenetic tree confirmed that isolate GU12 grouped with *K. ohmeri* (Figure 8), whereas isolates GU13 and BA9 clustered with *C. parapsilosis*, supported by high bootstrap values (>95%) (Figure 9).

Table 2. Percentage similarity of probiotic candidate yeasts based on NCBI BLAST analysis

Code isolates	Identity (%)	Species	Query value	E-value	GenBank Accession number (this study)
GU12	99.46%	<i>K. ohmeri</i>	100%	0.0	PZ742271
GU13	100%	<i>C. parapsilosis</i>	100%	0.0	PZ742277
BA9	100%	<i>C. parapsilosis</i>	100%	0.0	PZ744513

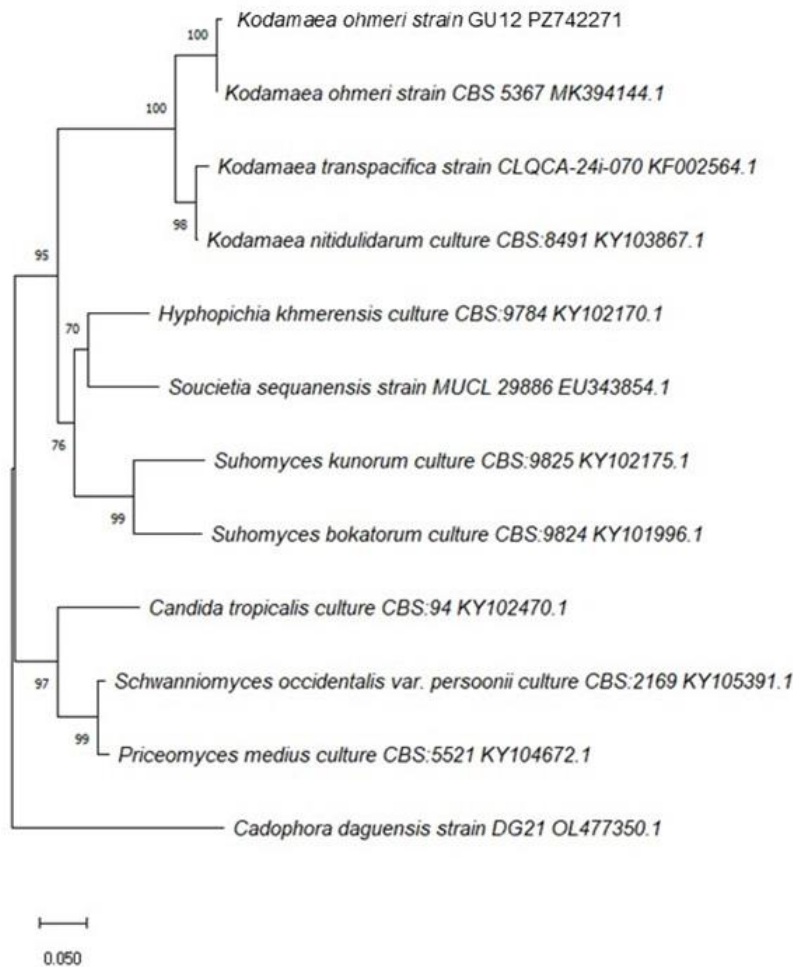


Figure 8. Phylogenetic tree of *K. ohmeri* GU12 (PZ742271).

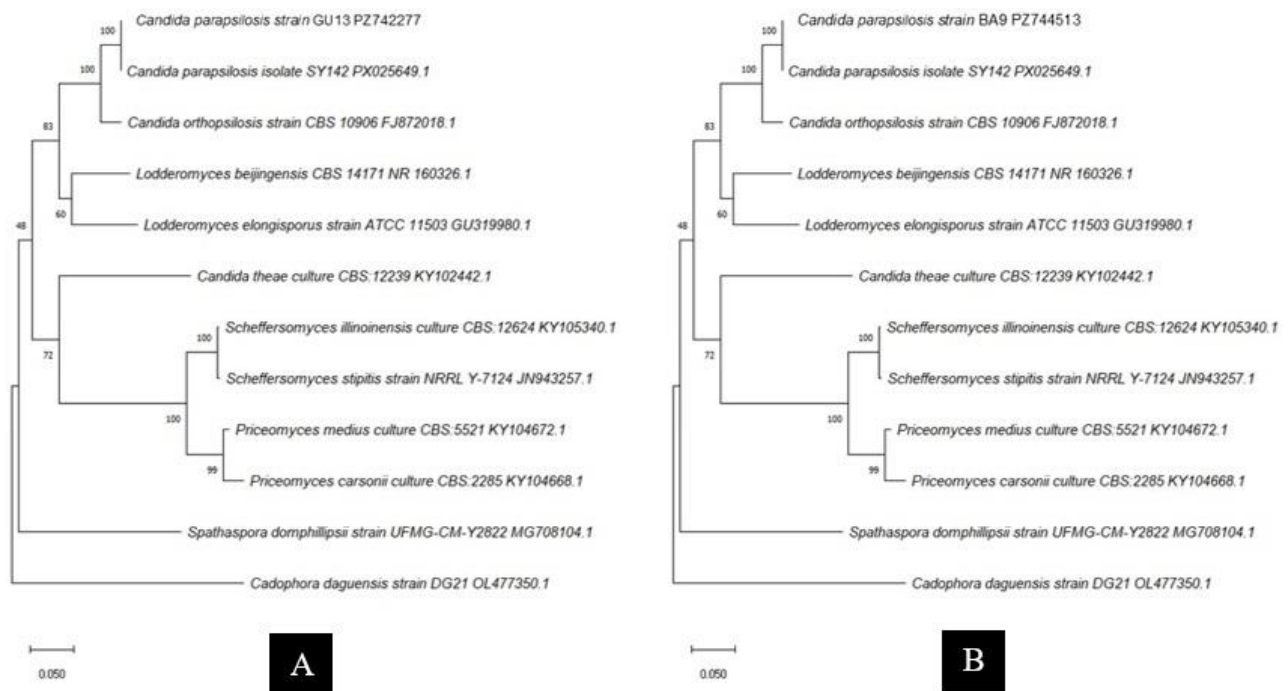


Figure 9. Phylogenetic trees of (A) *C. parapsilosis* GU13 (PZ742277) and (B) *C. parapsilosis* BA9 (PZ744513).

DISCUSSION

Currently, the application of probiotics in aquaculture is increasing due to their established positive effects on fish growth and overall health status. Yeast has strong potential as a probiotic candidate owing to its rich nutrient profile, which includes amino acids, vitamins, and minerals, as well as cellular components such as β -glucans and nucleotides that provide beneficial effects to the host [43]. Several previous studies have successfully isolated probiotic yeast candidates, including *C. haemulonii* and *D. hansenii* from the gastrointestinal tract of cobia (*R. canadum*) [13], *R. mucilaginosa* from Antarctic waters [44], and *S. cerevisiae* from aquaculture environments [45].

The findings of this study showed that 120 yeast isolates were successfully obtained from three different shrimp farming locations, with the majority originating from S1 (Banyuwangi) and S3 (Gresik), accounting for 39.2% and 37.5%, respectively. This may be because both ponds (S1 and S3) had earthen bottoms, which possibly support high microbial biodiversity. Yurkov *et al.* [46] reported 40 yeast species, including several novel species, isolated from forest and grassland soils. Furthermore, the high ecological diversity and widespread distribution of microorganisms in natural environments, such as soil, freshwater, and marine ecosystems, make them important subjects for metabolomic approaches aimed at preserving microbial biodiversity *ex situ* [47]. Of the 120 yeast isolates, nearly half (45%) were obtained from shrimp intestines, suggesting high microbiota diversity within the gastrointestinal tract. The intestine serves as a favorable habitat for microbial communities, including yeast, which are essential for competing with pathogens, modulating the immune system, and contributing to nutrient metabolism [48]. The secretion of polyamine compounds and structural components such as β -glucans and mannoproteins by yeast contributes positively to gut health, enhances immune responses, and provides antioxidant activity within the host [49]. The absence of anti-*V. parahaemolyticus* activity among isolates from S2 (Jember) may reflect differences in pond bottoms and farming practices. The culture system used in S2 was intensive, with plastic-lined ponds. Intensive aquaculture is recognized as exposing shrimp to environmental stressors, including high stocking densities, increased organic loads, and fluctuating water quality, which can alter the composition and stability of the shrimp-associated microbiota [50]. Such an imbalance in the microbiota can reduce the abundance of beneficial microorganisms and increase the dominance of opportunistic taxa [51].

The sequential elimination method used in this study was selected because it efficiently reduced the number of isolates while selecting candidates that fulfilled multiple probiotic criteria [25]. This approach has been widely adopted in preliminary probiotic screening to prioritize isolates with broad functional potential [52]. Nevertheless, this approach may overlook isolates with favorable characteristics that fail to meet a single selection criterion. The selection of probiotic candidates based on their ability to inhibit pathogens is a key requirement in this study. Based on the antagonistic assay, 58 yeast isolates (48.3%) were able to inhibit the growth of *V. parahaemolyticus* Rif^R in co-culture testing. This antibacterial activity may be associated with multiple mechanisms, including competitive exclusion, secretion of antimicrobial peptides (AMPs), or a combination of these mechanisms [29]. These AMPs can inhibit the growth of both Gram-positive and Gram-negative bacteria, as well as fungi, making them potential agents for controlling infectious diseases [53]. Several AMPs produced by yeast with antibacterial properties include CU (*C. utilis*), produced by *Candida* sp. [54], as well as K1 and K28 toxins produced by *S. cerevisiae* [55]. These compounds function by inhibiting microbial growth, disrupting cell surface structures, and reducing membrane permeability in pathogenic cells [56]. However, the present study did not identify or characterize the compounds responsible for the observed inhibition.

The ability of *V. parahaemolyticus* to form biofilm layers is a key virulence factor that protects cells from environmental stress and facilitates colonization within the shrimp host. Once colonization and quorum sensing are achieved, *V. parahaemolyticus* produces toxins and other virulence factors that lead to disease in shrimp [57]. In this study, 54 yeast isolates (93.1%) demonstrated the ability to inhibit biofilm formation by *V. parahaemolyticus* Rif^R. This antibiofilm activity may be attributed to the ability of yeast to compete for space

and nutrients with pathogens, thereby suppressing their growth and virulence [58]. In addition, several yeast genera, including *Candida*, *Pseudozyma*, *Saccharomyces*, *Rhodotorula*, *Yarrowia*, *Kluyveromyces*, *Wickerhamomyces*, and *Debaryomyces*, have been reported to produce natural biosurfactants with antibiofilm potential [56]. Furthermore, several yeast species, including *C. bombicola*, *C. apicola*, *C. stellata*, *R. babjevae*, *D. hansenii*, and *Wickerhamiella domercqiae*, have been reported to secrete sophorolipid (SLP) molecules capable of metabolizing lipids, which are key components of biofilm structures [59]. Although the adopted classification facilitates interpretation of inhibition levels, reporting quantitative inhibition percentages remains essential for objective comparisons among studies.

The tolerance of probiotic candidates to environmental factors is an essential criterion in the selection process for ideal probiotics. High tolerance to environmental conditions indicates the ability to survive under diverse circumstances [60]. The results showed that only 22 yeast isolates (40.7%) exhibited broad tolerance to temperature, pH, and bile salt conditions, indicating their potential as probiotic candidates. Several mechanisms may facilitate the adaptation of yeast cells to low-pH conditions, including regulation of cell wall integrity pathways, modification of cell wall structure, and regulation of calcium metabolism [61]. Acid tolerance at pH 1.5 and 2.0 has been widely used as a preliminary screening criterion for shrimp probiotics [35, 36]. In this study, the assay was performed to facilitate comparison with previous studies and to assess isolate tolerance to severe acidic stress. However, these pH conditions do not represent the digestive physiology of *Litopenaeus vannamei*. Therefore, acid tolerance should be interpreted as an indicator of physiological robustness rather than direct evidence of survival in the shrimp gastrointestinal tract. Future studies should employ host-specific gastrointestinal models that better reflect shrimp digestive conditions. Furthermore, other mechanisms could contribute to bile salt resistance, including the production of bile salt hydrolase (BSH) enzymes in *S. boulardii* [62], Ena1 P-type ATPase in *S. cerevisiae* [63], and glycogen mobilization as a response to environmental stress [64]. Shrimp have a limited ability to synthesize cholesterol *de novo*, which is a precursor of bile acids (BA). Instead, shrimp utilize cholesterol from their diet to produce BA as part of their nutrient metabolism [65]. Nevertheless, future studies should establish more shrimp-specific screening methods that better reflect the physicochemical conditions of the shrimp digestive tract.

The aggregation of pathogens by yeast cells serves to prevent pathogen colonization in the intestine and to form yeast–bacteria complexes that facilitate pathogen elimination by the host immune system [66]. The results showed that 10 yeast isolates exhibited a high degree of microscopic co-aggregation ability against *V. parahaemolyticus* Rif^R. Yeast cell coagulation activity is potentially associated with the presence of mannans and polysaccharides in the outer layer of the yeast cell wall [67]. In addition, agglutinin proteins also play a role in determining the efficiency of yeast cells in aggregating pathogens [68]. Co-aggregation activity against several freshwater pathogenic bacteria has been reported in *Cutaneotrichosporon jirovecii*, *D. nepalensis*, *Blastobotrys proliferans*, and *Diutina catenulata* [24]. Similarly, *C. zeylanoides* and *Hyphopichia pseudoburtonii* have demonstrated comparable activity against marine pathogenic bacteria [38]. The co-aggregation assay in the present study was evaluated using qualitative microscopic observations. Although this approach is commonly used for preliminary screening [24, 38], it may introduce observer bias and does not provide quantitative measurements of aggregation efficiency. Future studies should employ quantitative methods, such as spectrophotometric assays or digital image analysis, to improve reproducibility and allow statistical comparisons among isolates.

Hemolytic activity in yeast cells indicates their ability to lyse red blood cells and suggests potential pathogenicity [5]. The results showed that isolate GA14 exhibited moderate hemolytic activity and was therefore not selected as a potential probiotic candidate. The hemolytic ability of yeast cells may be associated with the production of hemolysin toxins, which function to obtain iron compounds from hemoglobin, thereby supporting metabolism, growth, and host invasion [69]. Several yeast species from the genera *Candida* [70] and *Trichosporon* [71] have been reported to exhibit varying hemolytic activities.

One of the essential criteria for yeast as a probiotic candidate is that it must be non-pathogenic to the host [39]. The pathogenicity test results showed that three yeast isolates—GU12, GU13, and BA9—achieved a 100% survival rate. This high survival rate indicates a high level of safety, rendering them ideal probiotic candidates. These findings are consistent with previous reports demonstrating that *C. zeylanoides* and *H. pseudoburtonii* can function as safe probiotics for rainbow trout (*Oncorhynchus mykiss*) [38]. Additionally, *S. cerevisiae* and *D. hansenii* have been reported to be non-pathogenic in zebrafish (*D. rerio*) [20]. Evaluation of acute toxicity through short-term observational windows (7 days), focusing on mortality rates and clinical physical signs, serves as an established preliminary safety screening threshold. The 100% survival rate and complete absence of abnormal clinical symptoms, such as erratic swimming, lethargy, or body discoloration, observed in this study indicate that the indigenous yeast isolates lacked acute pathogenicity or systemic toxicity in experimental shrimp [29].

The observation of yeast growth phases was conducted to determine the growth rate and the duration of the stationary phase for each isolate. Generally, microbial growth encompasses the lag phase, exponential phase, and stationary phase [72]. The results showed that isolate GU12 reached the exponential phase faster than the other yeast isolates, specifically at 12 h, and remained in the stationary phase until 24 h. However, quantitative growth parameters such as the specific growth rate (μ), doubling time, and carrying capacity were not calculated. These metrics would provide a more objective comparison of growth performance among isolates and facilitate a better understanding of their physiological characteristics. Future studies should incorporate mathematical growth models to estimate these parameters and enable more comprehensive comparisons. Differences in growth time may be attributed to intrinsic factors such as strain type, physiological condition, and nutritional status [73, 74], as well as extrinsic factors including environmental conditions and the nutrient composition of the culture medium [63].

Molecular identification of probiotic yeast candidates was performed by DNA sequencing and comparison with the GenBank database at NCBI. The three selected yeast isolates showed that GU12 exhibited 99.46% similarity to *K. ohmeri* (PZ742271), whereas isolates GU13 and BA9 were identified as *C. parapsilosis* (PZ742277 and PZ744513, respectively), with 100% similarity. The phylogenetic analysis provided additional taxonomic support beyond BLAST similarity, increasing confidence in species assignment. *K. ohmeri* has been reported to be isolated from several fermented food products, such as tapai [75], kefir [76], and cheese [77]. Although some strains have been reported as opportunistic pathogens [78], other strains have shown promising probiotic potential due to their tolerance to low-pH conditions [79], ability to produce antimicrobial compounds [80], and secretion of digestive enzymes [67, 68].

Meanwhile, *C. parapsilosis* has been isolated from domestic animals, insects, sediments, marine environments [83], and fish feed [84]. Notably, *C. parapsilosis* isolated from the gastrointestinal tract of cobia (*R. canadum*) has demonstrated probiotic activity [13]. Furthermore, when combined with *Bacillus thuringiensis*, *C. parapsilosis* has been reported to prevent *V. parahaemolyticus* infection in shrimp (*Penaeus vannamei*) [85]. Although previous studies have reported that some strains of *K. ohmeri* and *C. parapsilosis* exhibit beneficial characteristics, including antagonistic activity against pathogens and adaptation to aquatic hosts [86, 87], both species are also recognized as opportunistic pathogens [88, 89]. Therefore, probiotic activity should be evaluated at the strain level through comprehensive biosafety assessments, including antifungal susceptibility testing and genomic analysis, before practical application and commercialization. In addition, [90] stated that although the selected yeast isolates exhibited favorable probiotic characteristics under *in vitro* conditions, these findings should be interpreted with caution because *in vitro* assays cannot fully replicate the complex interactions occurring in the shrimp gastrointestinal tract or aquaculture environment.

Commercial probiotics used in aquaculture are predominantly composed of well-characterized bacterial genera such as *Bacillus*, *Lactobacillus*, and the yeast *S. cerevisiae* [91, 92]. Therefore, benchmarking newly identified indigenous probiotic candidates against established commercial strains would provide a more robust assessment of their relative efficacy and practical significance for aquaculture applications [29]. Such

comparative evaluations should be incorporated into future studies before field-scale implementation and commercialization. Nevertheless, the identification of host-associated indigenous yeasts provides a valuable foundation for developing shrimp-specific probiotic candidates, which may possess ecological adaptations that facilitate colonization and persistence within the host gastrointestinal tract.

Beyond intrinsic probiotic traits, the ecological integration of candidate yeasts within the shrimp gut and culture environment is crucial for host resilience. Yeast cell wall components (β -glucans and mannan-oligosaccharides) act as prebiotic substrates that stimulate beneficial native microbiota while suppressing opportunistic pathogens through competitive exclusion [93–95]. Additionally, the metabolic activity of probiotic yeasts aids in lowering gut pH and degrading organic waste in the water column, thereby maintaining microbial diversity and pond ecosystem stability [96]. Although high-throughput metagenomic profiling remains a key objective for future long-term trials, these findings highlight the strong potential of indigenous yeast isolates to foster a balanced host–environment microbiome.

CONCLUSIONS

In this study, probiotic yeast candidates were successfully isolated, selected, and characterized from the intestines and aquaculture environments of whiteleg shrimp. These isolates were identified as *K. ohmeri* (PZ742271) and *C. parapsilosis* (PZ742277 and PZ744513, respectively), demonstrating anti-*V. parahaemolyticus* activity and tolerance to temperature, extreme pH, and high bile salt concentrations. In addition, the selected probiotic yeast candidates exhibited rapid growth and were considered non-pathogenic to the host. Further studies are recommended to conduct additional biosafety evaluations and in vivo validation before applying these isolates in shrimp aquaculture as a safe and environmentally sustainable alternative for the prevention of bacterial diseases.

DECLARATIONS

Availability of data and materials

All the generated data are included in the manuscript.

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Declaration of generative artificial intelligence (AI)

The authors declare that generative AI tools were used solely to improve the language quality of this manuscript.

Authorship contributions

Mohammad Faizal Ulkhaq: conceptualization, formal analysis, investigation, methodology, writing—original draft, writing—review and editing; Widanarni, Dinamella Wahjuningrum, Munti Yuhana: supervision, formal analysis, writing—review and editing; Jiun-Yan Loh: writing—review and editing.

Conflicts of interest

We certify that there is no conflict of interest related to any financial, personal, or other relationships with individuals or organizations associated with the material discussed in this manuscript.

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