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Selection of potential probiotic candidate bacteria from seawater and shrimp pond sediments for controlling the pathogenic *Vibrio parahaemolyticus*

Seleksi bakteri kandidat probiotik potensial asal air laut dan sedimen tambak udang untuk pengendalian bakteri patogen *Vibrio parahaemolyticus*

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ABSTRACT

Vibrio parahaemolyticus is one of the pathogenic bacteria that cause vibriosis. *V. parahaemolyticus* strain that expresses the PirA and PirB toxins is the main causative agent of AHPND (acute hepatopancreatic necrosis disease) which causes necrosis (cell death) suddenly in the hepatopancreatic organs of shrimp. The environmentally friendly approach to prevent bacterial infections in shrimp is through the application of probiotics. Probiotic isolates originated from the same environment as the host and pathogen were expected to have a better adaptation and competition capability. Probiotic selection begins with the process of searching for and screening the potential probiotic candidates. This study aims to obtain isolates and characterize the potential probiotic bacteria isolated from seawater and pond sediments as an inhibition effort *V. parahaemolyticus*. The research was conducted experimentally using a completely randomized design with 3 treatments and 2 replicates in the in vitro antagonistic test as well as the in vivo non-pathogenicity test. The results of the study successfully obtained 37 probiotic candidate isolates with 10 isolates having the greatest enzyme activity. LAZ-2 (*Acinetobacter radioresistens*) isolated from seawater and SAZ-22 (*Fusobacterium varium*) isolated from pond sediment have been selected as probiotic candidate bacteria that have beneficial enzymatic activities. Both isolates were capable of inhibiting the growth of the pathogenic *V. parahaemolyticus* cells population and both were not pathogenic to vannamei shrimp.

Keywords: probiotic, screening, vannamei shrimp, *Vibrio parahaemolyticus*

ABSTRAK

Vibrio parahaemolyticus merupakan salah satu bakteri patogen penyebab penyakit vibriosis. *V. parahaemolyticus* strain tertentu yang mengekspresikan toksin PirA dan PirB merupakan agen penyebab utama AHPND (*acute hepatopancreatic necrosis disease*) yang menyebabkan nekrosis (kematian sel) secara tiba-tiba pada organ hepatopankreas udang. Pendekatan yang ramah lingkungan untuk mencegah penyakit infeksi bakterial pada udang adalah melalui aplikasi probiotik. Isolat-isolat probiotik yang berasal dari lingkungan yang sama dengan inang dan patogen akan memiliki suatu kemampuan adaptasi dan kompetisi yang lebih baik dari yang lainnya. Seleksi probiotik diawali dengan proses pencarian dan skrining kandidat probiotik yang potensial. Penelitian ini bertujuan untuk mendapatkan isolat dan mengkarakterisasi bakteri probiotik potensial yang diisolasi dari air laut dan sedimen tambak sebagai upaya penghambatan *V. parahaemolyticus*. Penelitian dilakukan secara eksperimental menggunakan Rancangan Acak Lengkap (RAL) dengan 3 perlakuan dan 2 kali ulangan pada uji antagonistik in vitro dan uji non-patogenisitas. Hasil penelitian berhasil mendapatkan 37 isolat kandidat probiotik dengan 10 isolat yang memiliki aktivitas enzim terbesar. LAZ-2 (*Acinetobacter radioresistens*) yang diisolasi dari air laut dan SAZ-22 (Fusobacterium varium) yang diisolasi dari sedimen tambak terpilih sebagai bakteri kandidat probiotik yang memiliki aktivitas enzim yang menguntungkan. Kedua isolat tersebut mampu menghambat pertumbuhan populasi sel patogen *V. parahaemolyticus* dan keduanya tidak bersifat patogenik terhadap udang vaname.

Kata kunci: probiotik, seleksi, udang vaname, *Vibrio parahaemolyticus*

INTRODUCTION

Pacific white shrimp (*Litopenaeus vannamei*) is the most widely farmed shrimp species worldwide. *L. vannamei* dominates about 65% of the world's total shrimp production. *L. vannamei* production in Indonesia reached 752,819 tons in 2021, but decreased to 711,875 tons in 2022 (KKP, 2024). The decline in production can be caused by various factors, one of which is due to disease occurences from pathogenic agents such as viruses, parasites, fungi, and bacteria. Increased attention to disease outbreaks caused by bacteria in shrimp farming has occurred over the past decade.

One notable finding that has drawn attention is AHPND (acute hepatopancreatic necrosis disease), also known as EMS (early mortality syndrome). It is called "acute hepatopancreatic necrosis disease" because it causes sudden necrosis (cell death) of the hepatopancreatic organ. According to Kumar *et al*. (2021) the damaged hepatopancreas cells are R (resorptive), B (blister), F (fibrillar), and E (embryonic) cells. Hepatopancreas cell damage results in impaired digestion and nutrient absorption, which then causes lethargy, anorexia, slow growth, empty digestive tract, pale hepatopancreas and massive mortality in shrimp. AHPND disease can cause up to 100% mortality in postlarval shrimp within 20-30 days after stocking (Yuhana & Afiff, 2023).

The main cause of AHPND disease is *Vibrio parahaemolyticus* strains that harbor a pVA1-like plasmid containing the pirA and pirB genes (Lin *et al*., 2022). *V. parahaemolyticus* is a rod-shaped or curved Gram-negative bacterium that is motile, facultative aerobic, cannot form spores and commonly found in marine or estuarine environments. *V. parahaemolyticus* can cause pathogenic effects on shrimp through a quorum sensing. Quorum sensing (QS) is a method for bacteria to communicate and regulate genetic activity when they are in large enough numbers (Lin *et al*., 2022). Bacteria through QS mechanisms can regulate various processes, including the production of enzymes, toxins, or virulence factors to cause infection or disease.

The lowest concentration limit for pathogenicity of *V. parahaemolyticus* is 10^4 CFU/mL, causes 48% mortality in shrimp within three days (Saputra, 2023). The use of antibiotics as a form of treatment of pathogenic infections in shrimp farming activities causes various problems (Kusmarwati *et al*., 2017). Antibiotics can stop bacterial growth and kill bacteria by damaging cell walls and membranes. Antibiotic-resistant bacteria cannot be treated with the same antibiotic, making infections more difficult to treat and their spread difficult to control. Antibiotics can produce residues in the environment and shrimp bodies.

Antibiotic residues accumulated in shrimp when consumed by humans can cause health problems in humans. Aquaculture environments polluted with antibiotic residues take a long time to decompose because antibiotic residues accumulate in pond bottom sediments (Loo *et al*., 2020). According to Lai *et al*. (2015) *V. parahaemolyticus* in AHPND endemic areas is highly resistant to common antibiotics. There is a need for alternatives to antibiotics as an effort to improve sustainable aquaculture practices.

Probiotics are single cultures or mixtures of live microorganisms that are harmless and have various mechanisms of action to benefit the host. Various mechanisms of action of probiotics according to Yuhana (2010) include, they play a role in improving the water quality of rearing media, improve gastrointestinal health, produce antimicrobial compounds, are able to compete for nutrients and space with pathogens, and can stimulate host immune responses. Probiotics used in aquaculture generally come from Gram-positive and negative bacteria. Gram-positive bacteria used as probiotics include *Lactobacillus, Lactococcus, Enterococcus, Micrococcus, Clostridium* and *Bacillus*.

Yuhana *et al*. (2024) studied that *Bacillus cereus* BR2 effectively improved growth performance, digestive enzymes activity, immune-related gene expression and resistance of African catfish to *Edwardsiella tarda* ETS1. Gram-negative bacteria such as *Pseudoalteromonas, Vibrio* and *Aeromonas* can also be probiotic microbial agents. Research by Wang *et al*. (2018) showed that the application of probiotic *Pseudoalteromonas* was able to reduce the density of *Vibrio* in the gut of vannamei shrimp. The use of active and inactive probiotics (so called para-probiotic) (Yuhana *et al*., 2024) is increasingly applied to fish and shrimp farming activities as an environmentally friendly substitution for antibiotics.

The use of probiotics as treatment or prevention of infection requires careful consideration. Probiotic microbes used should be isolated from the same environment as the host and pathogen. Pathogenic bacteria *V. parahaemolyticus* and *L. vannamei* live and thrive in high salinity environments ranging from 15-30 ppt (Namadi

& Deng, 2023). The source of probiotic isolates can be obtained from marine ecosystems such as water, sponges, sediments and marine organisms. Probiotic microbes isolated from the same habitat as the host and pathogen are expected to be more adaptable, multiply and survive in sufficient numbers in the environment to provide sustainable benefits to the host. The aim of this experiment was focusing on screening of isolates characterizing, and selecting the potential probiotic bacteria isolated from seawater and pond sediments as an inhibition effort to *V. parahaemolyticu*s.

MATERIALS AND METHODS

Isolation of bacteria

The samples of seawater and shrimp pond sediment originated from Pinang Gading shrimp farm, Lampung Province. Sampling of seawater at a depth of 20 cm from the surface of the pond while sediment samples were obtained from the bottom of the pond using the Ekman grab tool. Samples that have been obtained are collected using sterile bottles and then stored in a coolbox and taken to the Aquatic Organisms Health Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine Science, IPB University. The stock solution and the dilution results of seawater and sediment samples were then spread as much as 0.025 mL on a Petri dish containing SWC (seawater complete) medium using the spread plate method. The results of the distribution were then incubated for 24 hours at a temperature of 27-30°C in an incubator. Bacteria that grow and have different morphological profiles were selected and then grown on new SWC medium.

Enzymatic activity

Enzymatic activity is performed to detect amylase, protease, and lipase activity in probiotic candidates. According to Idiawati *et al.* (2019) for the detection of amylase activity, the candidates were inoculated into SWC medium supplemented with 2% of starch. Amylolytic activity was characterized by the formation of a clear zone around the colony after the addition of 1% potassium iodide (KI) reagent. To detect protease activity, the probiotic candidates were inoculated into SWC media supplemented with 2% skim milk (Remijawa *et al*., 2020). The zone of clearance was observed and measured after 24 h incubation. For detection of lipase activity, the candidates were inoculated on to SWC medium supplemented with 2% of olive oil. Lipase activity was characterized by the formation of a green zone around the colony after the addition of $CuSO4$ reagent (Marlida & Elrifadah, 2017).

Preparing bacteria for spontaneous mutations

Bacterial tagging with spontaneous mutations was performed on pathogenic bacteria and probiotic candidates. One isolate of probiotic candidate and pathogenic isolate were inoculated on SWC medium containing antibiotics. The types of antibiotics used were *rifampicin, oxytetracycline, tetracycline* and *enrofloxacine* with a concentration of 50 µg/mL. The medium was then incubated at 28°C for 24 h. Colonies that grow on SWC medium containing antibiotics indicate bacterial resistance to the type of antibiotic tested. Sensitive bacteria to antibiotics were characterized by the absence of colony growth on the media (Firdaus, 2012).

The antagonistic test

In vitro antagonistic tests were carried out by two methods, the Kirby-Bauer method and co-culture. Kirby Bauer test was performed by spreading the *V. parahaemolyticus* on SWC using a sterile cotton swab. Whatman number 42 sterile paper disk was placed on SWC. A total of 5 µL of probiotic candidate cells suspension was dripped on the paper disk. The results were observed through the clear zone formed around the paper disk (Jalali *et al*., 2016).

The co-culture method applied different cell dencities. Probiotic candidates inoculated in a density of 10^6 CFU/mL while the pathogenic bacteria inoculated in a cell density of 10^4 CFU/mL. A total of 100 µL and pathogenic bacteria *V. parahaemolyticus* were inoculated into 10 mL of SWC broth. Probiotic candidate bacteria were replaced by sterile PBS (phosphate-buffered saline) as a control that would be cultured together with pathogenic bacteria. The growth of *V. parahaemolyticus* co-cultured with probiotic cells and PBS was counted cells the total plate count method on TCBS medium containing 50 µg/mL rifampicin antibiotic. The TPC results of bacteria co-cultured with probiotic candidates and PBS as a control were then compared.

Non-pathogenicity test

Non-pathogenicity test is measured based on vannamei shrimp survival for seven days after being injected with probiotic candidate bacteria. Probiotic candidate bacteria used the same density as in the co-culture which was 10^6 CFU/

mL. As a control, vannamei shrimp were injected with sterile PBS solution instead of probiotic candidates. The survival rate value was calculated at the end of the rearing period (Dehaghani *et al*., 2015):

$$SR\ (\%) = \frac{Nt}{No} \times 100$$

Note:
Nt = Initial number of shrimp
No = Final number of shrimp

Identification of selected probiotic candidate bacteria

Bacteria was purified to obtain pure culture or single colonies. Each colony was identified by morphological (colour, shape, elevation, edges, and Gram staining) and biochemical characteristics (motility, catalase, oxidase, oxidative/fermentative (O/F), lactose, maltose, sucrose, glucose and fructose. Bacterial identification was performed according to Cowan and Steel (2003) as well as by PCR sequencing of the 16S rRNA gene of the isolate LAZ-2.

Data analysis

Data were analyzed using Microsoft Excel 2019. Statistical analysis of bacterial density data in the co-culture antagonistic test and survival rate was analyzed using the SPSS version 26.0 application through the single factor analysis of variance (ANOVA) method with a 95% confidence interval and continued with the Duncan's test if it showed significantly different results ($P<0.05$). Other data results were analyzed descriptively by displaying tables and images in the form of curves and graphs.

RESULTS AND DISCUSSION

Probiotic isolation

The results of isolation of probiotic candidate bacteria derived from seawater samples obtained of 9 bacterial colonies and derived from pond sediments of 28 bacterial colonies. Total number of isolates obtained were 37 isolates. Bacteria isolated from seawater are coded with the prefix letter L and the prefix letter S for isolates from sediments origin.

Enzymatic activity

The enzymatic activity test selected 20 bacterial colonies that showed proteolytic enzyme activity, consisting of 12 isolates obtained from shrimp pond sediments and 8 isolates from seawater. The amylolytic enzyme activity test showed positive result of 8 isolates, consisted of 7 isolates from shrimp pond sediments and 1 isolate was from seawater. Figure 1a shows an example of the positive results of the amylolytic activity test and Figure 1b shows the positive results of the proteolytic activity test. Further physiological test of lipolytic activity, however, none of those isolates produced lipolytic zone. Based on the enzymatic activity tests that have been carried out, Table 1 presents 10 probiotic candidate isolates with the best enzymatic activity.

Kirby-Bauer in vitro antagonistic test

The results of in vitro antagonistic tests using the Kirby-Bauer method on 10 probiotic candidate isolates obtained results in the form of inhibition zones in two isolates, namely LAZ-2 isolate code (Figure 2a) and SAZ-22 isolate (Figure 2b) while 8 other isolates did not produce inhibition zones. The measurement of the inhibition zone diameter

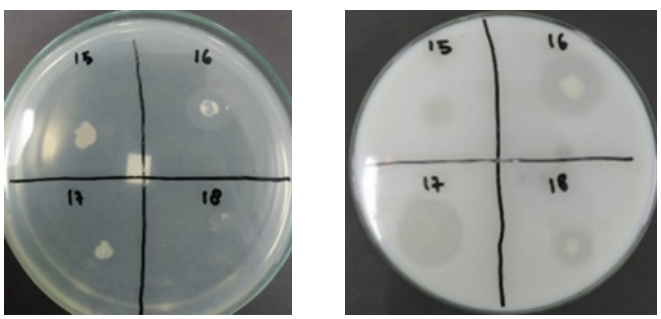

Figure 1. Enzyme activity test results. (1a) Amylolytic activity and (1b) Proteolytic activity.

of isolate LAZ-2 were 12.36 mm, 10.75 mm and 8.92 mm while isolate SAZ-22 were 10.15 mm, 9.37 and 8.12 mm. Isolates LAZ-2 and SAZ-2 were further tested to determine their ability to compete for nutrients through the co-culture method. For this method, *V. parahaemolyticus* needs to be marked with antibiotic resistance first.

In vitro antagonistic test using co-culture method

V. parahaemolyticus grew on media TCBS containing rifampicin after spontaneous mutation while LAZ-2 whereas SAZ-22 isolate did not grow on this medium. Probiotic candidate isolate that have the ability to inhibit the pathogen *V. parahaemolyticus* in the Kirby-Bauer antagonistic test were again tested in vitro with the co-culture method to measure their ability to compete for nutrients. The isolates tested were LAZ-2 and SAZ-22. Figure 3 shows that the colony density of *V. parahaemolyticus* (reflected the cells count) after co-cultured with probiotic candidate isolate (Figures 3a and 3b).

All co-culture tests resulted plate counts with much less *V. parahaemolyticus* colonies than that of the control plate (Figure 3c)**.** The cell density of pathogenic *V. parahaemolyticus* was further statistically analyzed to prove that there were

Table 1. Selected isolates with the greatest enzymatic activity.

Isolate	Diameter of amylolytic zone (mm)	Diameter of proteolytic zone (mm)
LAZ-2	0	8
LAZ-4	9	12
LAZ-5	0	10
SAZ-1	6	6
SAZ-7	7	13
SAZ-15	10	7
SAZ-16	13	15
SAZ-17	2	20
SAZ-18	10	9
SAZ-22	11	15

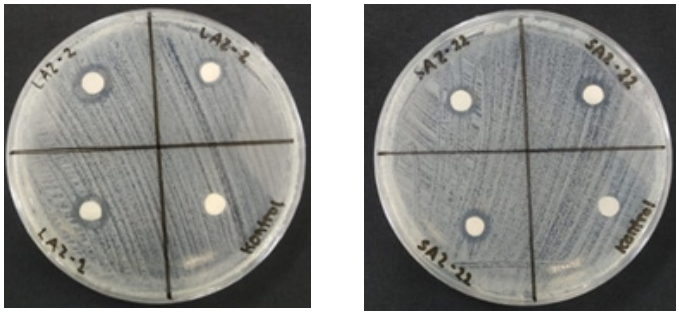

Figure 2. Zone of inhibition produced by probiotic candidate isolates against pathogenic *V. parahaemolyticus.*

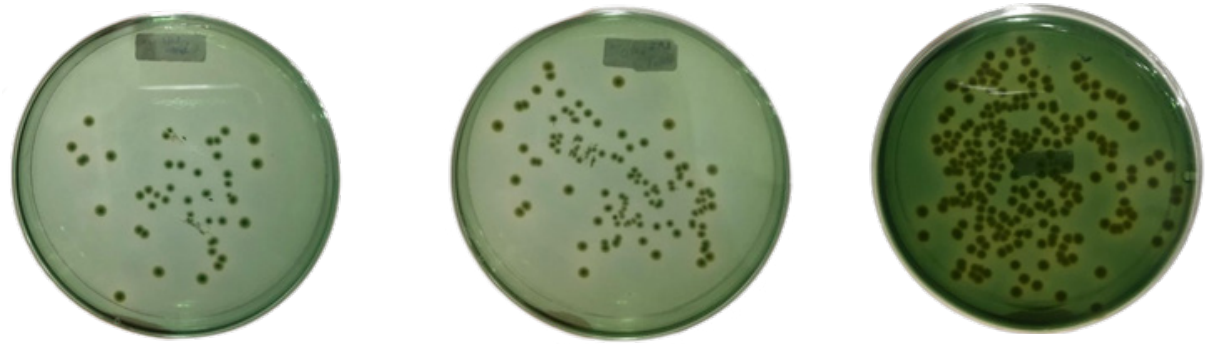

Figure 3. Total plate count results of *V. parahaemolyticus* co-cultured with probiotic candidate isolates. (a) isolate LAZ-2 (b) isolate SAZ-22, and (c) control PBS.

differences in results between treatments (Figure 4). The cells density of *V. parahaemolyticus* cultured with probiotic candidate bacteria is lower than the density in the control treatment. This result indicating that probiotic candidate was able to inhibit the cells growth of *V. parahaemolyticus*.

Non-pathogenicity test

Non-pathogenicity tests were conducted on probiotic candidate bacteria isolates LAZ-2 and SAZ-22. Parameters observed in this non-pathogenicity test were daily mortality and final SR of vannamei shrimp treated by injection. Figure 5 presents the daily mortality of each treatment during the non-pathogenicity test. The probiotic candidate isolate LAZ-22 began to show pathogenicity on the fifth day after injection characterized by the death of test animals. Survival data at the end of rearing vannamei shrimp in the non-pathogenicity test were statistically analyzed to see differences in results between treatments (Figure 6). After statistical tests, it was concluded that the LAZ-2 isolate did not have a significant difference from the control treatment and SAZ-22.

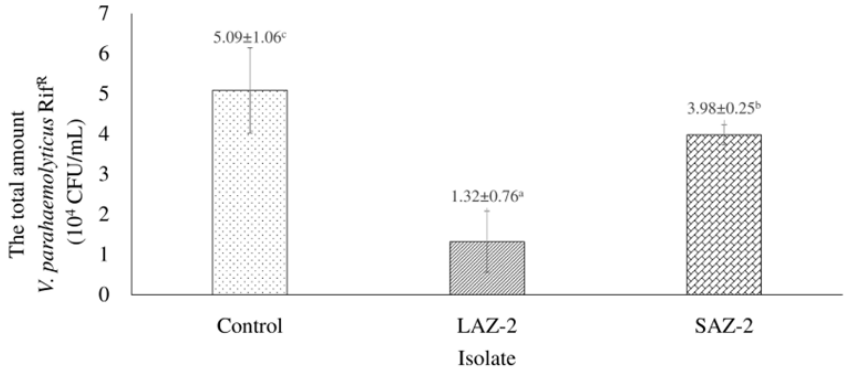

Figure 4. Cell density of *V. parahaemolyticus* from antagonistic in vitro culture with probiotic candidates LAZ-2 and SAZ-22. The same superscripts showed significantly different results according to Duncan test at a confidence level of α 0.05.

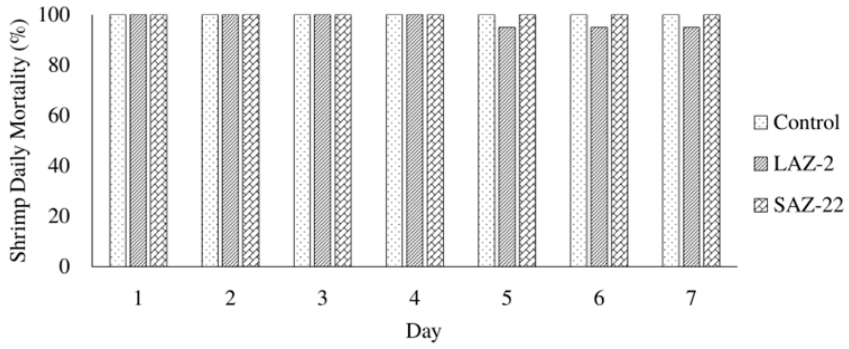

Figure 5. Daily mortality of vannamei shrimp during the non-pathogenicity test.

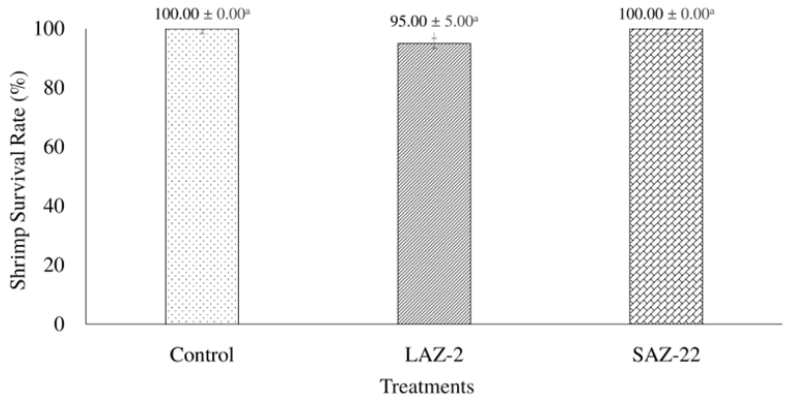

Figure 6. Shrimp survival rate (SR) during the non-pathogenicity test of probiotic candidates. The same superscripts showed significantly different results according to Duncan test at a confidence level of α 0.05.

Identification of probiotic candidate isolates

Isolates identification was carried out on probiotic candidate isolates with the code LAZ-2 and SAZ-22. Identification was done through morphological observation, Gram staining and biochemical tests (Table 2).

Discussion

Shrimp have a simple digestive system so that the level of digestibility of feed is relatively low. The low level of feed digestibility causes the nutritional value in the feed to be wasted after consumption (Ocktovian *et al*., 2024). The initial stage of probiotic selection can be started by screening process of the isolates of their capability in producing amylase, protease and lipase exogenous enzymes. Selection of probiotic candidate bacteria in this study resulted isolates capable of secreting protease and amylase enzymes as many as ten and eight best isolates, respectively. Isolate SAZ-17 is a proteolytic isolate that has the largest hydrolysis area of casein substrate compared to other isolates. Proteolytic strains are capable of secreting protease enzymes by breaking down proteins into amino acids, making it useful for increasing crude protein digestibility (Rahayu, 2022).

Isolate SAZ-16 is the best amylolytic strain because it is able to secrete the enzyme amylase. One of the functions of the amylase enzyme is to degrade starch into maltose and glucose which are then transported into the cell cytoplasm and used as a source of carbon and energy. Probiotic candidates that have amylase enzyme activity can break down amylum contained in the remaining feed (Fitriadi *et al*., 2023). Antibiotic resistance markers are carried out on probiotic candidate bacteria and indicator pathogens with the aim of facilitating the detection of bacteria if introduced in the shrimp living environment, so that they can be distinguished from native bacteria in the environment. This marker mechanism is used to facilitate the evaluation process of the ability of probiotic candidate bacteria that have been obtained in both in vitro and in vivo antagonistic tests (Novita, 2015).

The existence of gene mutations in the chromosome or plasmid acquisition of probiotic candidate bacteria and pathogenic bacteria causes the bacteria to be resistant so that they can grow on media that already contain antibiotics (Machado *et al*., 2022). The indicator pathogenic bacteria *V. parahaemolyticus* was marked with rifampicin-resistant, in a concentration of 50 µg/mL. In contrast, the probiotic candidate strain should be still sensitive to the antibiotic rifampicin. In vitro antagonistic tests were used to evaluate the ability of bacteria to inhibit pathogen growth through various mechanisms such as producing antimicrobial compounds, competition for nutrients and other interactions between the bacteria involved (Larasaty *et al*., 2021). The most commonly used method is the Kirby-Bauer method. Kirby-Bauer or also known as the disc diffusion method is one of the antagonistic tests whose positive results are indicated by the

Table 2. Morphology and biochemical tests of probiotic candidate isolates.

Test	Parameter	LAZ-2	SAZ-2
Colony morphology	Color	Yellowish white	Reddish white
	Shape	Circular	Irregular
	Elevation	Raised	Flat
	Tepi	Smooth	Smooth
Gram staining	Gram	-*	-
KOH 3%	Gram	-*	-
Cell shape	Form	Coccobaccili	Bacilli
Biochemical	Motility	(-)	(-)
	Catalase	(+)	(-)
	Oxidase	(-)	(-)
	Oxidative Fermentative	F	F
Result	Genus	*Acinetobacter*	*Fusobacterium*

Note: *the determination of Gram staining of isolate LAZ-2 was variative, however further confirmation was proceeded with 3% KOH test, where the cells revealed Gram negative result.

presence of a clear zone of inhibition around the disc paper (Schaeck, 2016).

The inhibition zone formed indicates that probiotic candidate bacteria are able to produce antimicrobial compounds that inhibit the growth of pathogens. Isolates LAZ-2 and SAZ-22 can inhibit the growth of pathogenic bacteria *V. parahaemolyticus* with a marked inhibition zone around the disc paper. Antimicrobial compounds produced through the Kirby-Bauer method can only be evaluated qualitatively, for this reason an in vitro antagonistic test of the co-culture method was carried out so that antimicrobial compounds can be evaluated quantitatively. The co-culture method is an antagonistic test method of probiotic candidates performed by growing probiotic candidate bacteria and indicator pathogenic bacteria on the same liquid medium. The density of probiotic candidate bacteria used is higher than the density of pathogenic bacteria to see the potential of probiotics as a prevention of infection with pathogenic bacteria (Widanarni *et al*., 2003).

The density of *V. parahaemolyticus* bacteria used in the Shared Culture method was 10^4 CFU/mL. The density of pathogenic bacteria used in the Shared Culture method refers to the research of Saputra *et al*. (2023) which says that the lowest concentration of *V. parahaemolyticus* that can cause pathogens against vannamei shrimp is a density of 10^4 CFU/mL. *V. parahaemolyticus* 10^4 CFU/mL is able to cause death in vannamei three days post-infection. Probiotic candidate bacteria using a density of 10^6 CFU/mL in accordance with Ministry of Marine Affairs and Fisheries Republic of Indonesia recommendations that the lowest density of probiotic bacteria used in aquaculture activities is 10^6 CFU/mL. The results of this co-culture method are seen from the density value of *V. parahaemolyticus* bacteria that grow on selective vibrio media, namely TCBS (thiosulfate citrate bile salts sucrose) media which contains rifampicin antibiotics.

According to Prabhakaran *et al*. (2020) *V. parahaemolyticus* is non-sucrose fermenting green colonies on TCBS media. Probiotic candidate bacteria LAZ-2 and SAZ-22 have been identified as not belonging to the vibrio group so they cannot grow on TCBS media. Probiotic candidate bacteria LAZ-2 and SAZ-22 were able to suppress the growth of *V. parahaemolyticus*, as it has been prooved by the density of *V. parahaemolyticus* cells cocultured with isolate LAZ-2 and/or SAZ-22 resulted in lowered cells density of $(1.32 \pm 0.76)\times10^4$ CFU/mL and $(3.98 \pm 0.25)\times10^4$ CFU/mL, respectively, compared to cells density in the control treatment, i.e. $(5.09 \pm 1.06) \times 10^4$ CFU/mL. Non-pathogenicity test is a screening stage that must be carried out on probiotic candidate bacteria. Given, the requirements that must be possessed by probiotic bacterial candidates should be extremely safe and should not pose any risk to the host (Pradhan *et al*., 2020).

Administration of probiotic candidate bacteria to test animals, namely vannamei shrimp, through injection in a relatively thin carapace. Clinical symptoms and signs of disease that appear in shrimp are observed every day. Parameters observed in this non-pathogenicity test were daily mortality and survival rate (SR). Vannamei shrimp injected late with probiotic candidate bacteria showed no symptoms of disease during the rearing period. The final SR value obtained in probiotic candidate isolates with LAZ-2 code is 95% and SAZ-22 code is 100%. Based on these data, probiotic candidate are categorized as safe and do not cause disease in experimented shrimp.

The last stage carried out in this study was the identification of probiotic candidate isolates, which was carried out on bacteria code LAZ-2 and SAZ-22. The identification results obtained through conventional identification by comparing the characteristics of bacteria in the Cowan and Steel table. The first characteristic observed in isolate LAZ-2 is in terms of morphology. LAZ has a round shape, smooth edges, convex elevation and yellowish white color on SWC media. LAZ has a round shape, smooth edges, convex elevation and yellowish white color on SWC media. The characteristics of LAZ-2 was Gram variative. This results due a difficulty in destaining process i.e tendency of the cells to retain crystal violet (the primary stain), furthermore, this ambiguity can lead to incorrect identification as Gram positive cocci (Doughari *et al*., 2011).

Actually *Acinetobacter* sp. belongs to Gram negative group, however the cells marked by purple color after Gram staining. However, the colonies becomes sticky and slimy after 3% KOH test, the confirmation test as Gram negative group of bacteria. Observation under a microscope showed that LAZ-2 was coccobacilli-shaped. Biochemical tests showed that LAZ-2 is a non-motile, catalase positive, oxidase negative and fermentative bacteria. The results of glucose, lactose, sucrose and mannose fermentation tests on LAZ-2 isolates are characterized by positive results while the results of maltose testing are

negative. Isolate SAZ-22 has morphology in the form of irregular shape, flat elevation, and clear white color on SWC media.

The characteristics of SAZ-22 are Gram negative characterized by a red color after Gram staining and not slimy after 3% KOH testing. Observation under a microscope shows LAZ-2 is coccobacilli -shaped. Biochemical tests showed that isolate SAZ-22 was a non-motile bacterium, negative for catalase and oxidase and fermentative. The results of glucose, mannose and sucrose fermentation tests on SAZ-22 isolates are characterized by positive results while the results of lactose and maltose tests are negative. The biochemical test results were matched with Cowan and Steel tables and then concluded that isolate LAZ-2 was identified as *Acinetobacter radioresistens* species and isolate SAZ-22 was identified as *Fusobacterium varium* species.

Identification up to species using the Cowan and Steel tables still has weaknesses, therefore it is necessary to carry out molecular identification. One of the most commonly used bacterial identification methods is the 16S rRNA gene marker (Noer, 2021). Isolates LAZ-2 and SAZ-22 were selected as probiotic candidates that have the ability of enzymatic activity and are able to inhibit the growth of *V. parahaemolyticus.* LAZ-2 and SAZ-22 were isolated directly from seawater and pond sediments, respectively, so that these isolates are expected to survive in high salinity environment where the salinity can be ranged from 15-30 ppt. Probiotic microbial agents isolated from the same environment as pathogens and hosts have various advantages, among others: can adapt well to pond environmental conditions, such as temperature, salinity, and other water conditions, do not cause negative effects on the environment or other organisms because they are normal microflora in the environment, and are able to multiply rapidly and survive in sufficient numbers in the environment to provide sustainable benefits to the host.

Probiotics used in shrimp farming generally contain microbial agents such as *Lactobacillus* sp, *Bacillus* sp and *Saccharomyces cerevisiae*. These microbial agents have the potential to not benefit the host if they cannot survive when applied to the pond environment. According to Liu *et al.* (2018) the use of multispecies probiotics that are not isolated directly in the environment where probiotics are applied, for example isolated from foodstuffs, feces or fresh water, may not benefit the host because they cannot survive in the environmental conditions where the probiotics are applied.

Isolate LAZ-2 was identified as *Acinetobacter radioresistens* species. *Acinetobacter* is a genus of bacteria that has been reported as a probiotic microbial agent in aquaculture environment since its capability in reducing the organic carbon waste as it belongs to heterotrophic bacteria (Liu *et al*., 2015). Moreover, *Acinetobacter radioresistens* is useful as bactoremediation agent for nitrogen waste removal in fish pond or the aquaculture environment (Liu *et al*., 2015; Rossiana *et al*., 2024). This indicates that the LAZ-2 isolate has the potential as a probiotic microbial agent and current research activity is being carried out emphasizing its effectiveness against *V. parahaemolyticus* in vannamei shrimp. SAZ-22 isolate was identified as *Fusobacterium varium*.

Information on the *Fusobacterium* sp. bacterial group is still very limited in aquaculture, moreover there is no information on the use of *Fusobacterium* as a probiotic microbial agent. According to Chen *et al.* (2022) *Fusobacterium* sp. is a pathogenic bacteria of periodontal disease. The use of the non pathogenic *Fusobacterium* as a probiotic microbial agent in aquaculture is interesting because it belongs to a new finding. Further studiessuch as in vivo antagonistic tests need to be conducted to determine the effects of probiotic candidates in the aquaculture commodities.

CONCLUSION

Probiotic isolate from the sea water, namely LAZ-2 (*Acinetobacter radioresistens*) and isolate from the shrimp pond sediment, namely SAZ-22 (*Fusobacterium varium*) have been selected as the potential probiotic candidate that have beneficial enzymatic properties especially for improving feed digestibility. Both isolates were capable of inhibiting the growth of pathogenic *Vibrio parahaemolyticu*s cells population and both were not pathogenic to vannamei shrimp.

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