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SCREENING OF PROBIOTIC CANDIDATES BACTERIA AS BIOCONTROL OF *AEROMONAS HYDROPHILA* PATHOGEN ISOLATED FROM MINA PADI CULTIVATION AREA

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Abstract

The agricultural activities in rice-farming system can have an impact on fish farming and the characteristics of bacterial community, especially pathogenic bacteria. Utilization of probiotics as environmentally friendly biotechnology products can be used to improve environmental quality and suppress the presence of pathogenic bacteria. This study aimed to select bacteria as probiotic agents from aquaculture ponds with the Rice-fish farming system. A total of 22 bacterial isolates were isolated from the water and sediment contained in the culture ponds. Based on the screening results, 15 isolates were confirmed as general non-pathogenic bacteria (Aeromonas sp.), 9 isolates had antibacterial activity against Aeromonas hyrophylla and 4 isolates showed high antibiotic sensitivity and were able to synergize. The results showed the Proteus mirabilis, Proteus penneri, Kurthia gibsonii and Bacillus cereus strains. Bacillus cereus strain LB8 has antibacterial activity that can inhibit the pathogen Aeromonas hydrophila with an inhibition zone of 8mm and has a very high sensitivity to antibiotics. These four isolates are able to work together synergistically and can be used as consortium probiotic bacterial agents to suppress the growth of pathogens.

Keywords*: Antibacterial; Antibiotics; Bacillus cereus; Rice-Fish Farming System; Probiotic Agent*

Introduction

Rice-farming is one of the integrated cultivation systems that is applied to produce fish and rice simultaneously [1]. Rice-farming can optimize land use through complementary use by utilizing the mutual relationship between fish and plants [2]. However, chemical fertilizers and pesticides are used in rice plant maintenance, which can have adverse effects on the environment. The main consideration of these impacts is pollution and changes in the microorganism structure of the environment for rice-farming cultivation. These agricultural activities can trigger the emergence of bacterial new characteristics that are difficult to control, especially pathogenic bacteria that can damage the balance of the bacterial community in the environment. Chemical

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compounds from agricultural activities can lead to the resistant properties of pathogenic bacteria so that they can harm one of the integrated cultivation of rice-farming, namely fish farming [3].

Bacterial community play a critical role in aquaculture ecosystems, including the breakdown of organic matter and environmental balance [4]. Bacterial communities in the environment have high diversity, so it is necessary to characterize them to determine their properties and abilities so that potential bacteria can be obtained that can be used as probiotic bacteria. Probiotic bacteria are bacteria that have beneficial properties for the host and the environment [5]. The probiotic bacteria produce extracellular enzymes to degrade organic compounds [6], improve fish health, suppress pathogen growth and synergize with other bacteria to maintain the balance of the environment [7]. Antagonistic ability against pathogenic bacteria is an excretion mechanism of antibacterial compounds produced from secondary metabolites as a form of defense in environmental competition. Probiotic bacteria must possess this requirement as a mandatory characteristic. Several probiotic bacteria that have been isolated and known to produce antibacterial compounds and can degrade pesticides include the genera *Bacillus* sp., *Flavobacterium* sp., *Alcaligenes* sp., *Arthrobacter* sp., *Achromobacter* sp. and *Diaphorobacter* sp. [8].

Water and sediment containing probiotic bacteria can be used as bioremediation agents and suppress pathogenic bacteria populations. Most pathogenic bacteria in water are opportunistic, which means they are common bacteria that live in the environment, but under certain conditions, they can be pathogenic to fish. For example, when there is a poor water environment and fish health declines, they can become pathogenic bacteria. Based on these properties and conditions, probiotics can be a biocontrol agent in the environment to maintain the balance of the bacterial community, especially in suppressing the growth of pathogens. Including the environment in mina rice cultivation, bacterial communities, especially pathogens, need to be emphasized in the growth of these communities so that the health and safety of fish remain stable.

Based on several studies, show that Bacillus sp. can play a role as a probiotic bacterium that can suppress pathogens and maintain the balance of bacterial communities in water, ome groups of Bacillus sp. produce the AHL lactonase compound which can inhibit pathogenic bacteria when carrying out the quorum sensing stage as a virulent factor [9, 10].

This paper describes the characterization of bacteria isolated from the rice-fish farming environment with the concern of obtaining bacteria with beneficial properties and potential for probiotic applications. Indigenous bacterial isolates are known to have advantages in adapting as an effort to optimize probiotic bacteria. Description of the bacterial potency by several test measures such as the selection of non-pathogenic test, antibacterial assay, synergism test and antibiotic resistance test were carried out as an initial process in the selection of probiotic candidates.

Materials and methods

Probiotic agent bacterial isolation

The sample of bacterial isolates were taken from the rice-fish farming system Mitra Kridoyuwono PNb fish pond, Panembangan Village, Banyumas Regency between November 2021 and December 2022. The types of samples taken were water and sediment with 2 samples each. Water and sediment sampling points were taken by random sampling method in 3 different ponds which were pooled in one container. Sediment samples were taken from sediments adjacent to the roots of the rice plants with a depth of ±5cm, while water samples were taken below the surface in the ditches surrounding the rice plants. Bacterial isolation was conducted with non-selective media to obtain as many bacterial strains as possible. Bacterial isolation was conducted with a modified procedure [11]. Water samples were diluted using five test tubes containing 4.5mL of sterile physiology. The water sample was diluted directly by inserting 0.5mL of the sample into the dilution tube until 5 times the dilution was obtained. Next the same steps for sediment samples. The sample solution at each dilution was cultured in TSA media,

which is a common bacterial growth medium using the pour plate technique [12]. Culture is carried out by adding 0.5mL of dilution sample solution to the growth medium then incubating at 28ºC for 24 hours. The bacteria on the solid growth media were counted and their colony morphology was recorded which included shape, edge, elevation, color and colony size. The selected bacterial isolates obtained were 22 isolates consisting of 7 isolates of water bacteria and 15 isolates for sediment bacteria. The bacterial isolates were purified by streak method on solid Growth media.

Probiotic agent bacterial screening

Common pathogen test

Common pathogens found in aquaculture pond ecosystems are *Aeromonas* and *Pseudomonas* bacteria. Glutamate Starch Phenol (GSP) selective media was used as a screening in detecting these pathogenic bacteria. Each bacterial isolate was streaked on GSP media and incubated for 24 hours at 28ºC. The indicator of bacterial pathogenicity was seen from the color of the colonies and the color change in the media. Bacterial colonies that produce color are classified in the *Pseudomonas* genus, while yellow colonies are classified in the *Aeromonas* group [13].

Antibacterial activity test

An antimicrobial activity test was conducted on TSA media. A 100µL suspension of *Aeromonas hydrophila* bacteria was dripped on TSA media after 24 hours of incubation on TSB media and allowed to stand for 5-10 minutes to allow the suspension to seep into the media. Paper disks with a diameter of 5mm were stored on the media and added with 0.5µL of suspension of the tested bacterial isolates that had been cultured on TSB media. Then, the TSA medium was incubated at 28ºC for 24 hours. The diameter of the inhibition zone formed was measured to determine the antimicrobial activity of bacterial isolates against *Aeromonas hydrophila*. The diameter of the inhibition zone was measured with the following formula:

$$D = \frac{Dv + Dh}{2} \quad (1)$$

where: D - Average diameter of inhibition zone; Dv - Vertical inhibition zone diameter – Paper disk diameter; Dh - Horizontal inhibition zone diameter – Paper disk diameter

Antibiotic sensitivity test

The sensitivity of bacterial isolates to antibiotics was conducted with Kirby-Bauer disk diffusion test method, by making a direct colony suspension. The bacterial isolate on liquid media was taken as much as 100µL and rejuvenated onto solid media using the spread plate method and allowed to stand for 5-10 minutes so the suspension could seep into the media. Tetracycline, Amoxicillin, Chloramphenicol and Gentamicin Antibiotic discs were attached to solid media and pressed slowly, then incubated at 28ºC for 24 hours. The diameter of the zone of inhibition of bacterial growth formed around the antibiotic disc was measured with a ruler in millimeters. The interpretation of the results was conducted regarding to the Clinical and Laboratory Standards Institute [14]. The sensitivity category (sensitive, intermediate and resistant) of bacterial isolates to antibiotics was determined by the size of the inhibition zone formed based on the CLSI standard recommendations.

Synergism test

Each bacterial isolate was streaked crosswise with each other so that the isolates would meet. The bacterial isolates were incubated for 24 hours at 28ºC and observed whether there was a clear zone or an inhibition zone between the two isolates that were in contact. An isolate is considered synergistic if there is no inhibition zone at the meeting area of the two isolates and antagonistic if there is an inhibition zone at the meeting area of the two isolates [15].

Molecular identification

Screening was used to select probiotic bacteria and 16S rRNA gene analysis was used to identify them. The first step that needs to be done is extracting bacterial DNA. The procedure for obtaining clean gDNA was followed according to the instructions of the PrestoTM Mini gDNA Bacteria Kit (Geneaid). Next, the results of each bacterial gDNA extraction were mixed

with a mastermix containing a pair of primers, nuclease free water and Mytaq HS Redmix 2x (DNA polymerase, Buffer $MgCl_2$ and dNTP) for PCR amplification. PCR amplification using Primus 25 Thermocycler PCR (Peqlab) with oligonucleutides used following research by *J.R. Marchesi et al.* [16]. The PCR program used follows the kit used in amplification with an annealing temperature of 55°C. PCR amplification results were visualized using electrophoresis with a 1.5% agarose gel in 1x TBE buffer. These results were then observed with the help of a UV transilluminator. Successful samples with a length of 1500bp were then sequenced. The complete sequence results were subjected to Basic Local Alignment Search Tool (BLAST) analysis using the online program NCBI (http://ncbi.nlm.nih.gov/) and phylogenetic analysis was carried out using the MEGA 10.1 program.

Data analysis

A detailed morphological analysis and results of testing probiotic candidates, including pathogenicity tests, synergistic tests, antibacterial tests and sensitivity tests to antibiotics, were presented to determine which bacteria had potential and compare the different systems used for cultivation of Rice-fish farming system bacteria. Bacterial identification data were analyzed by comparing the sample sequence homology with the sequence in GenBank and presented in an evolutionary tree. Data analysis results were discussed descriptively supported and compared with previous research.

Results

Common pathogen test

Generally, a pathogen test is conducted to detect Aeromonas and Pseudomonas, which are common pathogenic bacteria in aquaculture ponds. Detection of these pathogens can be seen using specific GSP media. Aeromonas and Pseudomonas pathogenic bacteria that grow on specific media can be seen from the color of the colonies and changes in the color of the media. Pathogenicity test results can be seen in Table 1.

Table 1. Test results of bacterial pathogens isolated from sediment and water

Sediment Bacteria	Pathogenicity	Water Bacteria	Pathogenicity
SB1	*Aeromonas* sp.	WB1	-
SB2	-	WB2	-
SB3	-	WB3	-
SB4	-	WB4	*Aeromonas* sp.
SB5	-	WB5	-
SB6	*Aeromonas* sp.	WB6	*Aeromonas* sp.
SB7	*Aeromonas* sp.	WB7	-
SB8	-		
SB9	*Aeromonas* sp.		
SB10	-		
SB11	-		
SB12	*Aeromonas* sp.		
SB13	-		
SB14	-		
SB15	-		

Description: Positive Pathogen (+), Negative Pathogen (-)

Based on the general pathogen test conducted on specific GSP media, 7 isolates of bacteria were positive for the pathogen, namely isolates WB4, WB6, SB1, SB6, SB7, SB9 and SB12. Meanwhile, 15 other isolates were negative for the pathogen. 6 bacterial isolates were thought to belong to the genus Aeromonas and Pseudomonas. The allegation was based on the selective media used, which are selective media for Aeromonas and Pseudomonas pathogens, where the color of bacteria and media will change if the bacteria are included in the pathogen in question.

Antibacterial activity test

From the results of the general pathogen test, 16 isolates were found that were not pathogenic. The isolates were continued with the bacterial activity test. Antibacterial activity test was conducted on *Aeromonas hydrophila* bacteria which is a common pathogen found in aquaculture waters. The presence of antibacterial activity was indicated by the formation of an inhibitory zone around the paper disk which was given a probiotic candidate isolate. The strong category was obtained by isolating WB 7. According to *W.W. Davis and T.R. Stout* [17], the bacterial inhibition zone was divided into four categories, namely weak (≤5.0mm), medium (6-10mm), strong (11-20mm) and very strong (≥20mm). Inhibition zone activity Antibacterial activity can be seen in table 2.

Table 2. Antibacterial Test Results of Probiotic Candidate Isolates Against *Aeromonas Hidrophylla*

Sample Code	Inhibition Zone Diameter (mm)			Category
	12 hours	24 hours	36 hours	
Water Bacteria				
WB1	-	2	3	weak
WB2	-	-	-	-
WB3	-	7	9	medium
WB4	-	-	-	-
WB5	-	6	6	medium
WB7	-	25	25	Very strong
Sediment Bacteria				
SB1	-	-	-	-
SB3	-	-	-	-
SB4	-	-	-	-
SB5	-	-	-	-
SB8	2	5	5	weak
SB10	-	6	6	medium
SB11	-	5	5	weak
SB13	-	8	8	medium
SB14	-	-	-	-
SB15	-	7	7	medium

Based on the *Aeromonas hydrophila* bacteria antibacterial activity test, nine isolates were found which showed inhibition zones against *Aeromonas hydrophila* bacteria. The bacterial isolates include WB1, WB3, WB5, WB7, SB8, SB10, SB11, SB13 and SB15. The diameter of the inhibition zone in this study was included in the weak, medium and strong categories. The weak category was obtained by isolates BA1, BS8 and BS11, which were 2-5mm. The medium category obtained by isolates BA3, BA5, BS10, BS13 and BS15.

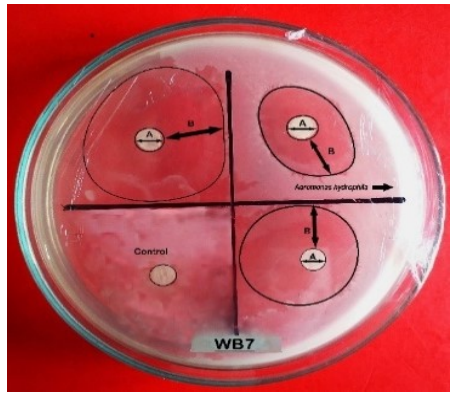

Fig. 1. Antibacterial Activity Test: (A) Isolate the test bacteria in paper disc; (B) inhibition zone formed against *Aeromoas hydrophila*

Antibiotic sensitivity test

Based on the antibacterial activity test, 9 isolates were found that had potential as probiotic agents. Then, the isolates were tested for sensitivity to antibiotics Tetracycline, Amoxicillin, Chloramphenicol and Gentamicin. The presence of an inhibition zone formed indicates the level of antibiotic resistance. Interpretation of antibiotic inhibition is divided into three categories according to [18], namely Resistant (≤14mm), Intermediate (15-18mm) and Susceptible (≥19mm). The results of the antibiotic sensitivity test can be seen in Table 3.

Table 3. Antibiotic Sensitivity Test Results

Sample Code	Antibiotic Sensitivity Test			
	Tetracycline (30 mcg)	Amoxicillin (25 mcg)	Chloramphenicol (30 mcg)	Gentamicin (10 mcg)
Water Bacteria				
WB1	S	R	S	S
WB3	S	R	I	I
WB5	R	R	R	R
WB7	R	R	R	I
Sediment Bacteria				
SB8	I	R	I	I
SB10	S	R	R	I
SB11	R	S	R	I
SB13	S	I	I	I
SB15	S	R	R	I

Description: Resistant (R), Intermediate (I) and Susceptible (S)

Based on the antibiotic resistance test, 4 isolates had the potential as probiotic agents, namely WB1, WB3, SB8 and SB13 (Fig. 2). These results refer to the level of interpretation of antibiotic inhibition in which the four isolates had moderately high intermediate and susceptible levels compared to other isolates.

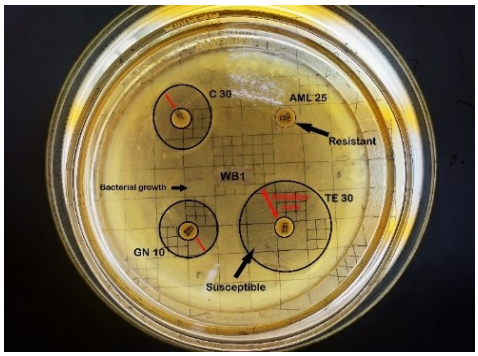

Fig. 2. Antibiotic Sensitivity Test: TE 30 (Tetracycline 30 mcg);
GN 10 (Gentamicin (10 mcg); C 30 (Chloramphenicol 30 mcg); AML (Amoxicillin 25 mcg)

Synergism test

Based on the results of the antibiotic sensitivity test, 3 isolates (WB1, WB3 and BS8) were found to be sensitive and intermediate to tetracycline, chloramphenicol and gentamicin antibiotics. One isolate (BS13) is sensitive and intermediate to all types of antibiotics tested. This shows that the four isolates showed non-resistant properties to antibiotics which have the potential as probiotic agents. Then, the isolates were tested for bacterial synergism. The synergism test is expected to be able to work together (a consortium) with each other. Basically, bacteria that will be used as probiotics must work together to improve host or environmental conditions. The results of the synergism test can be seen in Table 4.

Table 4. Synergism Test Results

Isolate code	WB1	WB3	SB8	SB13
WB1	+			
WB3	+	+		
SB8	+	+	+	
SB13	+	+	+	+

Description: Synergistic (+)

Based on the table above, the four bacterial isolates were able to synergize or work with each other. This can be seen from the absence of an inhibition zone formed from the meeting point of each cross-strike isolate.

Molecular identification of probiotic candidate bacteria

A total of four bacterial isolates were selected based on bacterial pathogenicity, bacterial synergism, the presence of antibacterial activity and the low level of sensitivity to antibiotics. The selected isolates were identified molecularly based on the 16s rRNA gene. Identification was conducted with amplification of the 16s rRNA gene with the help of a PCR machine to obtain a 1500bp amplicon product. The results of the amplicon products were sequenced to obtain sequences for blast analysis on GenBank. Analysis of blast results is shown in Table 5.

Table 5. Blast analysis data of 16s rRNA gene sequences of probiotic candidate bacteria

Isolate code	Reference Sequence (blast)	Query cover	Sequence Length (bp)	Accession number	Identity (%)
WB1	*Proteus mirabilis* strain MRKMSEC 72	99	942	KX384040.1	84.39
WB3	*Proteus penneri* strain CPrp_RA24	100	1419	MH788991.1	99.79
SB8	*Kurthia gibsonii* strain TY-06	100	1417	MN960342.1	82.81
SB13	*Bacillus cereus* strain LB8	100	983	MN087777.1	82.44

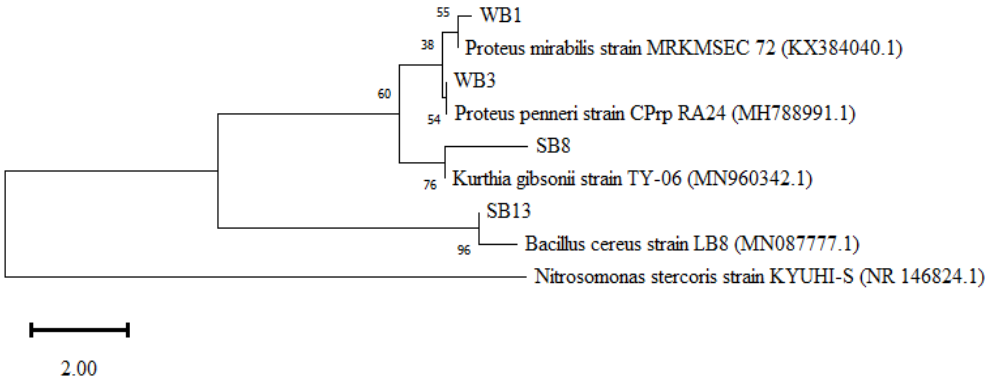

Fig. 3. Bacterial phylogenetic tree of probiotic agents. Evolutionary rate analysis settings were calculated using neighbor-joining and bootstrap repetition 1000 times. Branching in Nitrosomonas stercoris strain KYUHI-S as out-group

Analysis of the blast sequence of the 16s rRNA gene of probiotic candidate bacteria aligned with the sequence in GenBank identified three different groups, namely *Proteus* sp., *Kurthia* sp. and *Bacillus* sp. The analysis got the similarity of identity percentage 82.44%-99.79%. Bacteria with isolate codes BA1 and BA3 were identified as *Proteus* sp. namely *Proteus mirabilis* and *Proteus penneri*. Bacteria with isolate code BS8 were identified as *Kurthia* sp. namely *Kurthia gibsonii*. Also, bacteria with the isolated code BS13 were identified as *Bacillus* sp. namely *Bacillus cereus*. The evolutionary analysis found stable branching in both groups with 99% bootstrap value.

Discussion

This study found four isolates that have the potential as probiotic agents, based on the screening conducted at the beginning of the study. Pathogenicity tested used Glutamate Starch Phenol selective media was conducted as an initial step to select pathogenic gram-negative bacteria, namely *Aeromonas* and *Pseudomonas* [19, 20]. One of the conditions for bacteria to be used as probiotic agents is that they are non-pathogenic and non-toxic [21, 22]. According to *S. Mishra and S. Acharya* [23], probiotics are non-pathogenic live microorganisms which, when administered in adequate amounts, provide health benefits to the host.

Probiotic bacteria in aquaculture have mandatory criteria that are safe and beneficial for fish, environment and humans [24]. One of the detections related to food safety for humans is antibiotics. Antibiotics are of particular concern in aquaculture so the role of probiotics is one of the breakthroughs in suppressing the growth of environmentally friendly pathogens [25]. The advantage of probiotic bacteria is that they produce antibacterial compounds to inhibit the growth of both gram-positive and negative bacteria [26]. This inhibition mechanism forms the basis of antagonistic properties against pathogenic bacteria so that it can be used as a probiotic agent to suppress the presence of pathogens. In addition to the ability to produce antibacterial, probiotic bacteria are recommended to have the ability to synergize in an environment. The existence of these probiotic bacteria will interact with bacteria in the community so that the balance of the environmental bacterial community is maintained [27].

Generally, the variation of the inhibition zone formed is influenced by the concentration of bacteria, the bacterial species in producing antibacterial against the tested isolates. According to *J. Hudzicki,* [28] the higher the concentration of antibacterial agents, the larger the clear zone formed. The concentration of bacteria capable of producing higher antibacterial activity contains more active ingredients, making it more effective in inhibiting bacteria and creating a wider clear zone. Conversely, at low concentrations, antibacterial substances contain fewer antibacterial substances so they become less active [29]. This study used the paper disk method to drop-test bacteria of the same concentration so that differences in antibacterial compound production ability were attributed to different strains of bacteria. *J. Cleveland et al.* [30] stated that the bacterial species and the type of test bacteria affected the ability of bacteriocins to inhibit bacteria. The difference in inhibitory activity is because bacteriocins have inhibitory activity against certain bacteria and usually have a close phylogenetic relationship with bacteriocin-producing bacteria [31].

Currently, one of the requirements for the use of bacterial isolates as probiotics is not to show resistance to one or more antibiotics commonly used in humans and animals. Bacteria that are resistant to antibiotics are known to carry resistance genes that may pose a risk in the

environment, the host to humans. Probiotic bacteria must be tested for resistance in order to determine if they possess resistance abilities. There are several risks if probiotic bacteria have resistant genes that can carry out the genetic exchange between bacteria it can damage the balance of the community against pathogens [32]. Further, probiotic bacteria with resistant properties can pose a threat to humans because such bacteria can be transmitted to fish [33]. Based on this study, probiotic agent bacteria are recommended not to have antibiotic-resistance genes because they can cause serious impacts. This study found resistant bacteria caused by chemical fertilizers and pesticides used by farmers to combat rice pest attacks. According *to A. Nurawan et al.* [34], the use of synthetic chemicals or pesticides can cause resistance to bacteria, cause residues and environmental pollution. Test results suggest that rice paddy environment has been contaminated with antibiotics, so it needs to be considered when applying chemical fertilizers and pesticides to rice plantations.

Several bacterial species found in a substrate are generally able to influence and interact with each other. Bacterial synergy is a form of positive interaction that can occur in microbes such as bacteria, they take action to cooperate with each other in their habitat in the form of a consortium [35]. Bacterial consortium is a collection of bacteria that form a community to produce a product. Compatibility or synergy between two or more inoculated bacterial isolates is a very important factor for the success of bacterial collaboration [36]. As the results of this study, the synergy between each isolate is very positive, where the isolates meet and synergize with one another, or the bacteria do not inhibit each other. The synergistic relationship between bacteria is an important point in probiotic products, where generally a probiotic product contains several bacteria that work together to improve host or environmental conditions. Probiotics involve various forms of interaction between two or more microorganisms, namely neutralism, commensalism, synergism or protocooperation, mutualism (symbiosis), competition, predation and parasitism [37].

Identification of probiotic candidate bacteria based on the analysis of the 16s rRNA gene sequence has similarities with three groups of bacteria, namely *Proteus* sp., *Kurthia* sp. and *Bacillus* sp. The identity value obtained is quite high with 82.44%-99.79%. the isolates identified from the *Proteus* sp. namely WB1 as *Proteus mirabilis* and WB3 as *Proteus penneri*, while the *Kurthia* sp. and *Bacillus* sp. each only found 1 isolate with a SB8 code, namely *Kurthia gibsonii*. Bacteria with the isolate code SB13 were identified as the *Bacillus* sp. namely *Bacillus cereus*. *Proteus* sp. is an opporturistic pathogen that can be found in soil and water and is a normal flora in the digestive tract of humans and mammals [38]. *Proteus* bacteria generally include pathogenic bacteria that attack humans and mammals [39]. However, in aquaculture these bacteria have the potential as an indicator of environmental pollution and probiotic agents. The presence of *Proteus* sp. in water can show the level of pollution of an environment [40]. In this study, the bacteria *Proteus* sp. comes from water sources that pass through residential areas, so it is possible that household waste carried is the cause of this bacteria. Apart from being an indicator of environmental pollution, *Proteus* sp. also possible to be a candidate for probiotics. According to *Sabariah* [41] *Proteus* mirabilis can be used as a probiotic agent because it can increase the value of feed conversion, protein digestibility, total digestibility, protein retention and growth rate of jelawat fish. In addition, *Proteus* sp. is also able to produce biosurfactants that are able to degrade oil content in soil and water [42].

Kurthia Sp. is a group of bacteria that are non-pathogenic to fish and other organisms [43]. Kurthia is commonly found in the environment and in animal waste [44]. Stated that

Kurthia sp. is more commonly found in fish and rearing water than other bacteria. *Kurthia* bacteria is a normal flora in the waters of salmon fish *Scomberomus* sp. According to *M.J. Pelczar et al.* [45], bacteria that are always present in the culture environment can be grouped as normal flora. The role of normal flora for an organism is to contribute to the development of the digestive system, supply vitamins, stimulate the body's defense system and fight pathogens [46]. Based on its characteristics, *kurthia* can be used as a probiotic agent. According to *A. Agustina,* [47] *Kurthia gibsonii* isolated from the rearing environment and intestines of African catfish can be used as a probiotic candidate because it can increase the volume of leukocytes and the phagocytic index which is part of the immune system of fish.

Bacillus sp. is one of the most widely used gram-positive bacteria as probiotics. Several studies have stated that *Bacillus* sp. can colonize, capable of producing bacteriocin (antimicrobial compound), immunostimulant [48, 49], producing various secretory proteins, producing digestive enzymes, vitamins and carotenoids. *Bacillus* species are resistant to aggressive physical and chemical conditions, with various species exhibiting unusual physiological features enabling them to survive in a variety of environmental conditions including freshwater, marine sediments, desert sands, hot springs, arctic soils and the digestive tract of fish fins and shells. They can rapidly replicate and tolerate many environmental conditions, exerting various beneficial effects in the aquaculture sector [50, 51, 52]. Several species of *Bacillus* used as probiotic agents are *Bacillus cereus, Bacillus clausii, Bacillus pumilus* [53], *Bacillus subtilis, Bacillus coagulans* and *Bacillus licheniformis* [54, 55].

In recent years, *Bacillus cereus* has been studied as a potential probiotic agent. *Bacillus cereus* can produce amylase, cellulose and protease enzymes [56]. Extracellular enzyme activity produced by *Bacillus cereus* can improve the digestive system of fish and improve environmental water quality. In addition to enzymatic activity, *Bacillus cereus* has antibacterial activity against *Aeromonas hyrophylla*, *Vibrio* sp, *Escherichia coli* and *Staphylococcus aureus*. He antibacterial activity is indicated by the presence of an inhibition zone which indicates that Bacillus cereus can inhibit bacterial growth. According to J.C. Oscariz et al. [57] *Bacillus cereus* produces cerein-type bacteriocins that are active against all Gram-positive and some Gram-negative bacteria. The potential of *Bacillus cereus* as a probiotic is shown by *P. Prashantkumar et al.* [58], which stated that *Bacillus cereus* can prevent abundant blue green algae populations in pond waters. According to *M. Navin-Chandran et al.* [59], *Bacillus cereus* was able to increase the growth and immune system of tiger prawns.

Probiotics used in aquaculture play an important role in aquaculture productivity. These include increased feed consumption and nutritional value, stimulation of host responses to disease-causing pathogens and the ability to improve the environment [60, 61]. As a probiotic agent, bacteria must meet certain criteria that other bacteria do not have [62]. There are a number of criteria that need to be met, including not being pathogenic or disturbing the host, not being pathogenic to consumers (humans and animals), maintaining and reproducing easily, being able to survive and reproduce in the fish's intestines, being reared in a medium suitable for use and allowing introduction into the fish's intestines and living and thriving in fish rearing containers' water [63, 64, 65]. Another requirement that must be possessed by probiotic bacteria is the ability to produce antibacterial substances so that they can suppress the growth of enteric pathogens [66]. Various types of antimicrobial substances produced by probiotic bacteria are organic acids, hydrogen peroxide, diacetyl and bacteriocins which are thought to be proteins or polypeptides that have antimicrobial properties [67].

Conclusion

Bacterial isolates as probiotic agents were isolated from water and sediment contained in aquaculture ponds using the Rice-fish farming system. Screening of bacteria as probiotic agents based on tests of common pathogens, antibacterial activity, antibiotic sensitivity and bacterial synergism, obtained 4 isolates that have potential as probiotic agents, namely WB1, WB3, SB8 and SB13. The four isolates were identified from the *Proteus* sp. group, *Kurthia* sp. and Bacillus sp. with an identity of 82.44%-99.79%. The strains *Proteus* mirabilis, *Proteus* penneri, *Kurthia gibsonii* and *Bacillus cereus* have an antibacterial activity that can inhibit *Aeromonas hydrophila* pathogens, have very high sensitivity to antibiotics and the four isolates can synergize with each other which is included in the criteria as probiotic bacteria.

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