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Selection of Proteolytic Bacteria from the Digestive Tract of Vanname Shrimp (*Litopenaeus vannamei*)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The feed consumed by shrimp is not all digestible but some are excreted in the form of waste in the form of feces and other metabolic waste such as urine and ammonia. The amount of feed released into feces depends on the suitability of feed components with enzymatic capabilities in the shrimp digestive tract or digestibility. One of the efforts to maintain aquaculture water quality is by using probiotics that can be mixed with feed and applied directly to aquaculture medium. The study aimed to obtain proteolytic and probiotic candidate bacteria from the digestive tract of vanname shrimp. Bacterial screening was carried out by isolating bacteria from the digestive tract of shrimp obtained from pond farmers in the Suppa area of Pinrang Regency. The results of the isolation of probiotic candidate bacteria from the digestive tract of shrimp obtained a total of 20 isolates of proteolytic candidates for further selection, then five isolates with different morphological characteristics were selected. The five isolates were then tested for proteolytic activity. The results

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showed the greatest proteolytic activity was observed in the UM5 isolate with an activity value of 17 mm; followed by the UM1 isolate at 10 mm; UM2 at 9 mm, UM4 at 7 mm; and UM3 at 5 mm. Then tested for resistance to acidic and alkaline conditions (pH 2.5) and (pH 7.5), bacterial attachment test, and antagonistic test against *Vibrio harveyi* bacteria. The five isolates can survive in acidic conditions (pH 2.5 and alkaline (pH 7.5)) for eight hours. Antagonistic tests showed that the five selected isolates (UM1, UM2, UM3, UM4, and UM5) can inhibit the growth of *Vibrio harveyi* bacteria by forming an inhibition zone around the isolate. The highest inhibition zone was found in the UM5 isolate at 19 mm, followed by the UM2 isolate at 16 mm, UM4 at 15.5 mm, and UM3 at 15 mm. While the smallest antagonistic was obtained in isolate UM1 at 12 mm. The bacterial population in the swab shows the ability to colonize or attach a bacterium so the greater the number of bacteria in the swab, the better the bacteria are in sticking. The largest bacterial population was found in isolate UM5 which amounted to 2.53 x 107 Cfu/ml. isolate UM 4 amounted to 7.27 x 106 cfu/ml. Furthermore, UM3 amounted to 6.78 x 106 Cfu/ml. Then UM 2 amounted to 3.32 x 106 cfu/ml. the smallest population was shown by isolate UM1, which amounted to 4.5 x 105 cfu.ml.

Keywords: Shrimp; digestive tract; probiotics; Vibrio harveyi bacteria.

1. INTRODUCTION

Feed quality determines the growth rate of shrimp. Not all feed consumed by shrimp can be digested, but some are excreted in the form of waste in the form of feces and other metabolic waste such as urine and ammonia. The amount of feed released into feces depends on the suitability of feed components with enzymatic capabilities in the shrimp digestive tract or digestibility. Intensive ponds that rely on excessive artificial feed cause the quality to be less good thus it can cause pollution of pond water. One of the efforts to maintain aquaculture water quality is to use probiotics that can be mixed with feed and applied directly to the water in the pond [1]. According to Ding et al. [2] probiotics are supplemental nutrients consisting of live microbes that are beneficial to the host animal. Probiotics can increase appetite, improve metabolism, inhibit pathogenic bacteria, increase enzyme activities, and increase growth [3,4,5]. The use of probiotics can have a good effect if it is done under several criteria including the probiotic species utilized, the method used, and the dose and time of administration [1].

The addition of probiotics to feed has been widely applied in aquaculture activities and shown to improve growth, feed efficiency, feed digestibility, digestive enzyme activities, survival rate, protein retention, carbohydrate utilization, and the immune system of fish or shrimp both in the larval, fry and enlargement phases [6-14].

The presence of protease-producing proteolytic bacteria in the digestive tract of the shrimp is needed to help digest complex substances in the form of protein in a shorter time [15]. Protease enzymes play a role in hydrolyzing pro-protein into simple compounds, so it will be easily absorbed by the body and will increase the growth rate [16]. Efforts to increase growth in shrimp by utilizing proteolytic bacteria-producing protease enzymes that can be obtained from the digestive tract. This study was conducted to obtain proteolytic bacteria and probiotic candidate bacteria from the digestive tract of vanname shrimp (*Litopenaeus vannamei*).

2. MATERIALS AND METHODS

This research was conducted in April - June 2021. Samples of vannamei shrimp were obtained from vannamei shrimp ponds in Suppa, Pinrang Regency, South Sulawesi Province, Indonesia. Bacterial isolation, screening, and testing were carried out in the Fish Health Laboratory, Pangkep State Polytechnic of Agriculture**.**

2.1 Bacterial Isolation

The source of inoculum was obtained from the digestive tract of adult vanname shrimp, with an average size of 10-15 g, by removing the digestive tract of the shrimp. The digestive tract was then weighed and measured in length then crushed and diluted. Each 1g of the digestive tract was diluted with 9 ml of sterile physiological solution (NaCl 0.85%). The diluted samples were then grown in seawater complete (SWC) media with 2% skim milk added as an energy source. Cultures were made in duplicate and

then incubated at 290C for 24 hours. Microbial growth is indicated by the turbidity of the culture media.

Serial dilutions were carried out from 10-2 to 10-10 by taking 0.1 ml of bacterial suspension derived from nile tilapia (*Oreochromis niloticus*) intestine and inserted into the first dilution medium containing 0.9 ml of physiological solution (NaCl 0.85%). Of the first dilution medium, 0.1 ml was taken and inserted into the second dilution medium until the last dilution medium. To obtain pure isolates, from each dilution series, 0.1 ml was taken and then spread into selective media. The selected media used is SWC Agar +2% casein media. Each media was then incubated for 24 h to 48 h at 29^0C. Bacterial colonies that produce protease will produce a clear zone around the isolate. The purification method was repeated with the same technique and media until a single and uniform bacterial colony was obtained.

2.2 Proteolytic Activity Testing

The test was aimed to measure the amount of Proteolytic activity of each isolate. The proteolytic test was performed according to Benmebarek et al. [17].

2.3 Bacterial Growth Phase

The attainment of the bacterial exponential phase can be determined by the bacterial growth phase. The preparation of the culture was done by inoculating 0.1 ml of bacterial isolate into 10 ml of liquid culture medium and incubating for 24 h at 29°C. This prepared fresh culture was then taken 1% and inoculated into 90 ml sterile culture medium and incubated again at 29°C. Bacterial growth was observed every 2 h by measuring the optical density (OD) value, using a spectrophotometer with a wavelength of 620 nm [18,19].

2.4 Antagonistic Activity Test

This test was conducted to determine the ability of probiotic candidate bacteria to inhibit pathogenic bacteria. The pathogenic bacteria used was *V. harveyi*. 0.1 ml of liquid culture of pathogenic bacteria was taken and 0.9 ml of sterile physiological solution was added. For pathogenic bacteria, 0.1ml was then taken and spread on a plate. The sterile filter paper was dipped in the probiotic candidate suspension and placed on top of SWC solid media that had been spread with *V. harveyi*. The bacterial culture was incubated at 29°C for 24 h and a clear zone was observed as a result that the probiotic candidate could inhibit *V. harveyi.*

2.5 Stomach Acid and Bile Salt Resistance Test

The ability of bacteria to survive in the low pH stomach and the alkaline digestive tract was tested by gastric acid and bile salt resistance. This method refers to Ngatirah et al. (2000) and Menconi et al [20] by inoculating 1 ml of bacterial isolate into a series of tubes containing 9 ml of sterile media solution with pH 2.5 (adjusted by the addition of HCL) and pH 7.5 (adjusted by the addition of NaOH) and then incubated at 29 °C. Furthermore, the growing bacterial cells were counted by the cup count method every 2 h for 8 h. Resistance to gastric acid and bile salts were determined by the difference in colony counts between control and treatment. The smaller the difference, the more resistant to gastric acid and bile salts.

2.6 Attachment Test

This test refers to the method based on Dewanti & Wong [21] which used steel plates. The steel plates were first sterilized by soaking in a detergent solution heated to a temperature of 40-45°C for 24 h, then the steel plates were rinsed with hot water at 40-50°C until clean and then dried, then autoclaved at 121°C for 20 min. The test was carried out by placing the steel plate in a 1 L Erlenmeyer in a standing position. The Erlenmeyer was previously filled with 250 ml of sterile SWC and inoculated with 1 ml of fresh bacterial culture. The Erlenmeyer was covered with aluminum foil and placed in a shaker for 24 h at 29°C. After 24 h, the steel plates were rinsed with phosphate buffer (BF) solution. Then the surface of the plate was wiped evenly using a swab. The swab was put into a test tube containing 10 ml of BF and vortexed for 1 min. Serial dilutions were then made and the bacterial population was counted using the cup count method.

The number of bacteria growing on the media in the Erlenmeyer was also calculated by taking 1 ml of liquid from the growth media and diluting it with 9mL of Phosphate buffer. Furthermore, the calculation of the growing bacterial population was carried out using the total plate count

method. Bacteria that can form biofilms well will be able to attach to the substrate such as the intestine.

2.7 Data Analysis

This study used an experimental design in the form of a completely randomized design with three replications. The data obtained were analyzed using analysis of variance with a confidence level of 95%. To see the treatment differences, further tests were carried out by Duncan's Multiple Range test using the SPSS 14 computer program.

3. RESULTS AND DISCUSSION

3.1 Isolation of Probiotic Candidate Bacteria

The 8 vanname shrimp that were used for bacterial isolation were obtained from the farmer of a shrimp pond in the Suppa area of Pinrang Regency. Before isolating, each fish's digestive tract was crushed and homogenized. The isolation of probiotic candidate bacteria resulted in 20 proteolytic probiotic candidates being used for further selection. These bacterial isolates were taken based on different colonies. Colony morphology of bacterial isolates obtained is a large round, medium round, shiny white, thinly serrated, small round, transparent white, shiny cream color, smooth edges, slightly convex elevation, convex, irregular, cream color; large round thinning to the edge, serrated edges, cream color; round dilated, filamentous edges, clear color; round, thinning to the edge, clear color.

3.2 Proteolytic Activity

The results of the proteolytic activity test of isolated bacteria are presented in Fig. 1.

Fig. 1 shows the 5 bacterial isolates with the largest proteolytic index from the 20 isolates obtained from the bacterial isolation stage. The area of the casein hydrolysis zone is used as the first reference basis in the selection of proteolytic bacteria, which indicates their ability to utilize proteins for their survival, by first breaking down proteins into amino acids. Although according to Zhang et al. [22] in some cases, the area of the casein hydrolysis zone is not automatically directly correlated with its productivity in producing protease enzymes, this method is still quite effective for initial selection. Gupta and Khare [23] and Tang (2008) also performed this clear zone method to select proteolytic bacteria, which in the last stage obtained *Pseudomonas aeruginosa* capable of producing large amounts of protease.

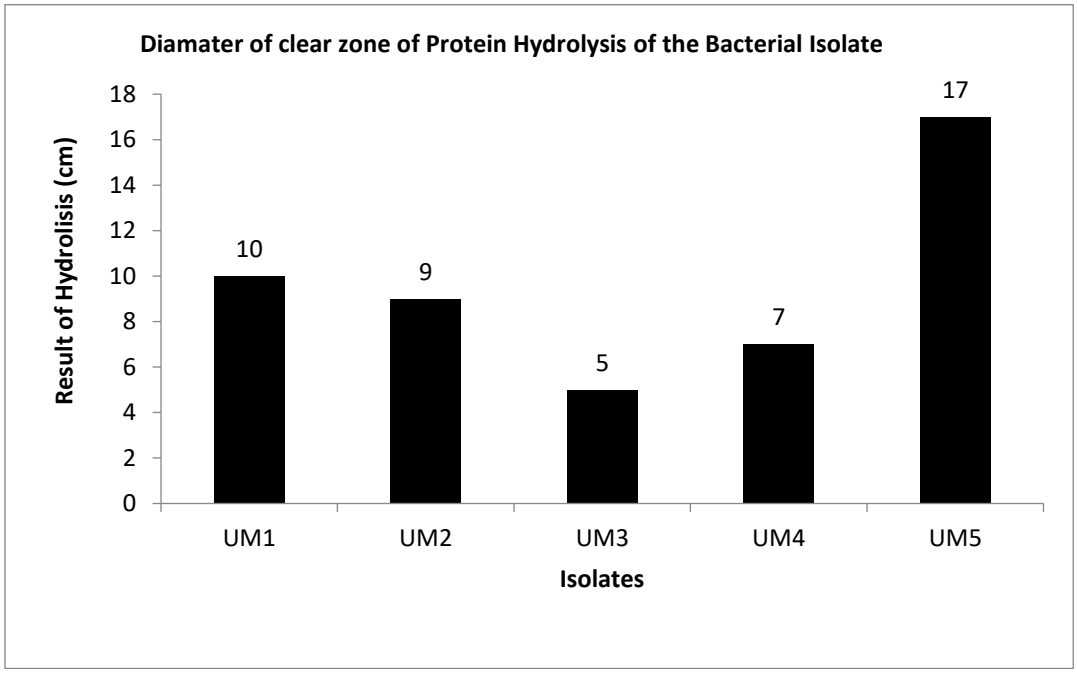

Fig. 1. Diameter of clear zone resulting from caffeine hydrolysis by proteolytic bacterial isolates

3.3 Bacterial Growth Phase

Observation of the growth phase of bacteria was conducted by observing changes in population and optical density (OD) values. This is related to the right cell harvest to produce a product or metabolite compound, including enzymes, antibacterials, vitamins, organic acids, fatty acids, amino acids, and peptides. According to Irianto [24] the fastest exponential phase is quite good bacteria used as probiotics. The results showed that isolates UM1, UM2, UM3, UM4, and UM5 had the fastest growth phase, each reaching the end of the exponential phase at the 12th h and beginning to decline at the 14th h (Fig. 2).

It can be seen from the growth curve that each isolate has varying growth. The observation of optical density values obtained the average end of exponential with the highest number of cells occurring at 16 h and 18 h. This time is then used as the basis for harvesting bacterial cells.

Bacterial growth is a complex process involving numerous anabolic (the synthesis of cell constituents and metabolites) and catabolic (the breakdown of cell constituents and metabolites) reactions [25]. Ultimately, these biosynthetic reactions result in cell division. During exponential growth, the rate of increase of cells in the culture is proportional to the number of cells present at any particular time. The exponential growth requires a number of factors to be present in excess in the growth medium, including sources of carbon, nitrogen, phosphate, and certain trace elements [26].

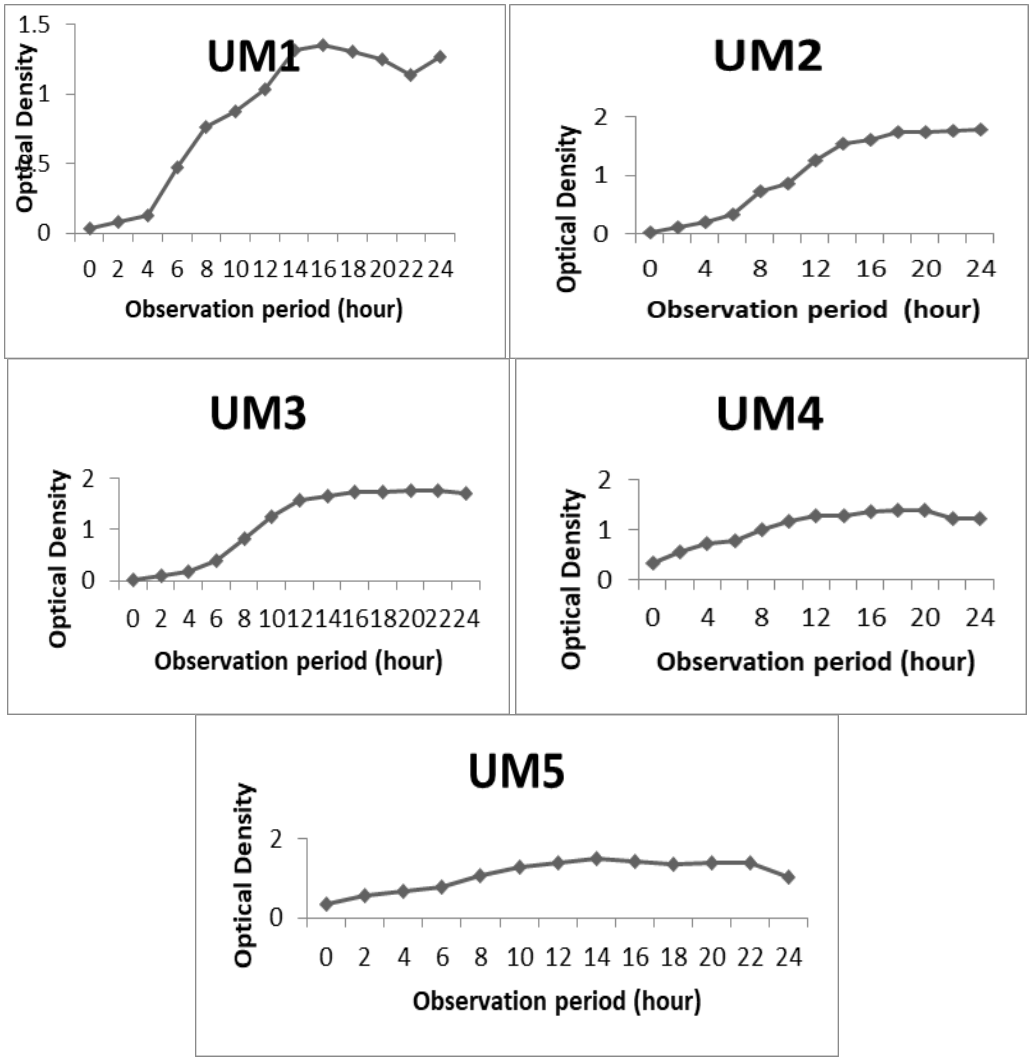

Fig. 2. Curve of optical density values

3.4 Antagonistic Activity Testing

One of the important criteria in the selected candidate of probiotics is that the bacteria can produce antimicrobial substances to suppress the growth of pathogenic bacteria in the digestive tract of vanname shrimp. In this study, the antagonistic activity of probiotic candidate bacteria used *V. harveyi* (Fig. 3).

Based on Fig. 3, the largest inhibition zone was shown in isolate UM5 at 19 mm, followed by isolate UM2 at 16 mm, then UM4 at 15.5 mm, UM3 at 15 mm. While the smallest antagonistic ability was obtained in isolate UM1 at 12 mm.

The ability of beneficial bacteria to inhibit the development of pathogenic bacteria indicates their ability to maintain the balance of microflora in the digestive tract of vanname shrimp. This potential is related to its ability to produce antimicrobial compounds; peptides that are synthesized from ribosomes. Surono (2004) stated that antimicrobials produced by microflora are lactic acid, peroxide, and bacteriocins. Normal flora in the digestive tract has an important protective function to suppress pathogenic bacteria and viruses, stimulate local and systemic resistance, and alter intestinal metabolic activity. In addition, normal flora also suppresses pathogenic bacteria due to competition for nutrients and attachment sites in the gut (Verschuree et al. [27] Irianto [24].

In the bacterial community, in order to survive adversity and coexist with other microorganisms, bacteria are constantly fighting for nutrients and niche space [28]. In terms of bacterial antagonism, it is important to define producer strains as bacterial strains that are capable of producing toxic compounds that inhibit the growth of other bacteria, whereas Non-producer strains, however, are generally sensitive to such substances [29]. Competition between bacteria can be influenced by the production of these toxic substances and the producer strains are advantaged compared to non-producer strains or sensitive strains by dominating a niche or sensitive strains by dominating the niche they reside and live [30].

3.5 Resistance to Stomach Acid and Bile Salts

The resistance of isolates to gastric acid and bile salts illustrates the ability of probiotic bacterial candidates to survive in acidic and alkaline conditions, which is expressed in the difference in the log number of bacterial isolates in the control and treatment media during the observation period. The difference in the log number of isolates for each period is presented in Fig. 4.

The next criterion for bacterial isolates to consider as probiotics is their ability to withstand acidic and alkaline conditions. Tolerance to gastric acid and bile salts is the most important requirement for probiotic candidates. This is because when the bacteria enter the fish body, they will pass through the stomach in an acidic atmosphere and will then pass through bile salts with an alkaline pH in the intestine. The resistance of bacterial isolates to gastric acid and bile salts is reflected by their resistance to acidic and alkaline media, which is expressed in a log decrease in the number of isolates in the treatment media during the observation period. The smaller the log decrease, the greater the resistance of bacterial isolates at low pH and high pH. Bacteria that successfully survive at low pH conditions are declared to be resistant or resistant to stomach acid. While bacteria that survive at alkaline pH are declared to be resistant or resistant to bile salts.

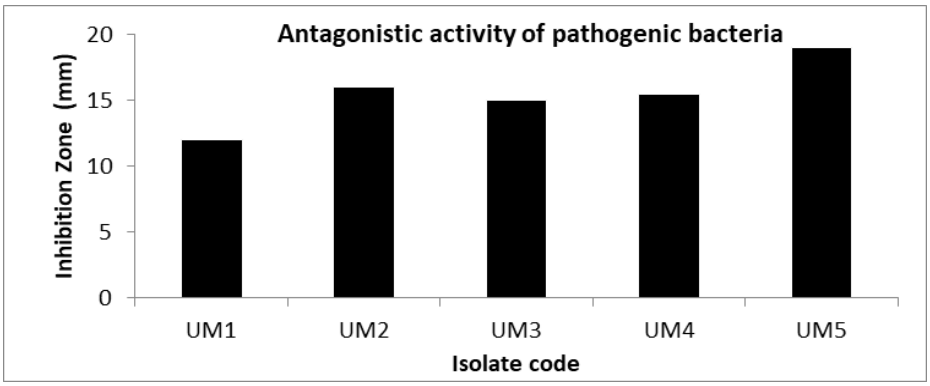

Fig. 3. Antagonistic activity of probiotic candidate bacteria against *V. harveyi*.

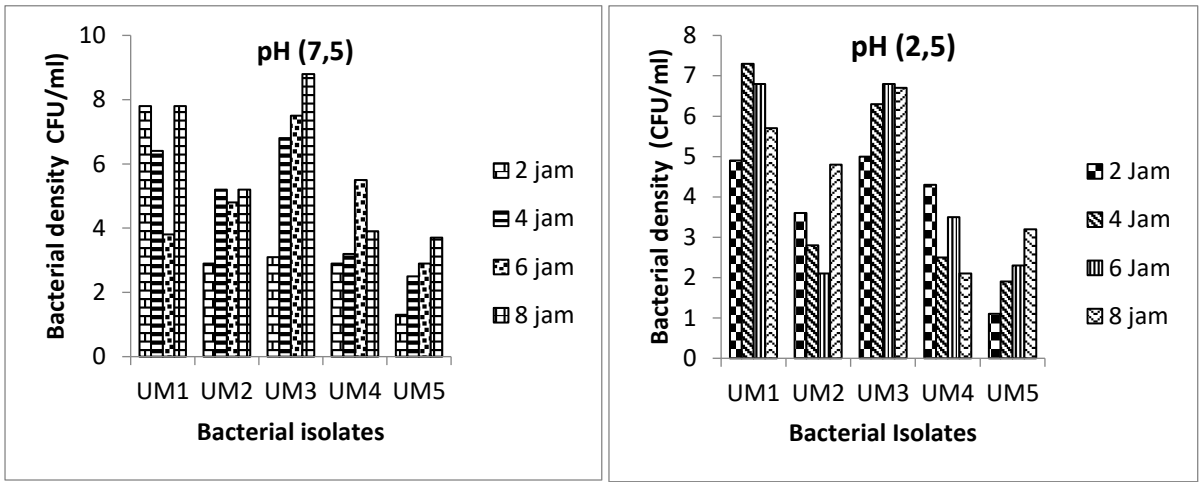

Fig. 4. Difference in log (cfu/ml) bacterial counts at pH 2.5 and pH 7.5 with normal pH

The results showed that all probiotic candidate bacteria isolated from the digestive tract of vanname shrimp could survive in acidic and alkaline media. This indicates that the bacteria can survive in low-pH stomachs due to gastric acid secretion and are also able to survive with high-pH bile salts. Bacterial isolates were still able to live until the end of the 8 h observation. This ability is thought to be because the isolate is a normal microflora of the digestive tract that has adapted to the conditions of gastric acid and bile salts in the digestive tract. The same results were also obtained in the research of Putra (2010), all bacterial isolates successfully isolated from the digestive tract of tilapia were able to survive in acidic and alkaline media. Andriani et al. [31] stated that in order to thrive and grow within the digestive tract, probiotic bacterial candidates must be able to pass through various environmental threats. One such threat is when bacteria enter the upper intestinal tract where bile is secreted into the gut. Bile secretion is a mixture of bile acids, cholesterol, fatty acids, phospholipids, bile pigments, and some detoxifying xenobiotics, the combination of which is bactericidal to commensal microorganisms in the body.

The results showed that isolate UM5 had the smallest log difference when compared to the other four isolates in the observation period of 4 to 8 h both under acidic conditions (pH 2.5) and under alkaline conditions (pH 7.5). This indicates that the UM5 bacterial isolate can survive better in acidic and alkaline conditions than other bacterial isolates. The tolerance ability of a bacterium to changes in media acidity is due to the ability of bacteria to regulate the pH of the cytoplasm compared to the extracellular pH (Aslamyah [6] in Putra 2010).

3.6 Adherence Test

The adherence factor is a factor possessed by bacteria to attach and form biofilms on solid surfaces [32]. Things that affect the nature of bacterial attachment to solid surfaces are the hydrophobicity between bacterial cells, the distance between cells, and the presence of receptors on host cells [33]. The attachment test of probiotic candidates gave different results for each probiotic candidate as shown in Table 1. The selected probiotic candidates showed the ability to attach to the substrate. Isolate UM 5 has a colony count of 2.53 x 107 Cfu/ml which means this isolate can stick well.

Table 1. Plating test results of proteolytic bacterial isolates on stainless steel plates

Isolates	Bacterial Population CFU/ml
UM1	$4{,}55 \times 10^5$
UM2	$3{,}32 \times 10^6$
UM3	$6{,}78 \times 10^6$
UM4	$7{,}27 \times 10^6$
UM5	$2{,}53 \times 10^7$

In addition to having high antagonistic activity against pathogenic bacteria, one of the criteria for probiotic bacteria is the ability to colonize the intestinal surface. This is because bacteria that are unable to colonize will be released by intestinal contractions. To colonize well in the digestive tract, probiotics must have the ability of adhesion or sticking. According to Aslamyah [6]

adhesion can be considered the first stage of colonization and is proportional to viability and metabolic activity.

The bacterial population in the swab shows the ability to colonize or attach to a bacterium thus the greater the number of bacteria in the swab, the better the bacteria is doing the attachment. The largest bacterial population was found in isolate UM5 which amounted to 2.53 x 107 Cfu/ml the greater number of bacteria was 7.27 x 106 Cfu/ml. Then followed by UM 3 at 6.78 x 106 Cfu/ml. Then followed by UM2 at 3.32 x 106 Cfu/ml. the smallest population size was shown in isolate UM1, which amounted to 4.5 x 105 Cfu/ml.

To date, no one has reported the number of ideal bacterial populations attached to solid substrates. However, similar results where the increase in population on a solid substrate, followed by a decrease in planktonic population were obtained by Wirawati and Aslamyah [6] in their research. However, all bacterial isolates tested in this study had adhesion ability as indicated by the presence of bacterial colonies that were able to adhere to stainless steel plates that were identified with intestinal solid substrates [34].

4. CONCLUSIONS

Based on the growth curve, the ability of bacterial isolates to produce antimicrobial compounds; peptides synthesized from ribosomes, which include lactic acid, peroxides, and bacteriocins, the resistance of bacterial isolates to gastric acid and bile salts is reflected by their resistance to acidic and alkaline media and the ability of bacterial attachment and biofilm formation on solid substrates. The study found five isolates; UM1, UM2, UM3, UM4, and UM5 have the potential proteolytic probiotic bacteria.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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