Preliminary study on screening and selection of Lactic Acid Bacteria (LAB) from gastrointestinal tract of naleh fish (*Barbonymus* sp.)

Cut Yulvizar[1,4], *Dewi* Yunita[2], *Zainal* A Muchlizin[3], and *Suhartono* Suhartono[4*]

[1]Graduate School of Mathematics and Applied Science, Universitas Syiah Kuala, Banda Aceh 23111, Indonesia
[2]Department of Agricultural Product Technology, Faculty of Agriculture, Universitas Syiah Kuala, Banda Aceh 23111, Indonesia
[3]Department of Marine Sciences, Faculty of Marine and Fisheries, Universitas Syiah Kuala, Banda Aceh 23111, In
donesia
[4]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Syiah Kuala Banda Aceh 23111, Indonesia

Abstract. The naleh fish (*Barbonymus* sp.) is an indigenous species found in Nagan Raya Regency, Aceh Province. The presence of Lactic Acid Bacteria (LAB) in the gastrointestinal tract of the fish may potentially act as probiotics, thus promoting the growth of the naleh fish. The objective of this research is to isolate, characterise and select LAB through physiological testing. The research was conducted using a purposive sampling survey method and isolated the bacteria on selective media, namely Man, Rogosa, Sharpe (MRS) agar. The selection of physiological tests conducted included catalase tests, tests of the ability of the bacteria to survive at different temperatures, and tests of the effect of varying concentrations of NaCl. The research findings identified seven isolates with different colony characteristics of LAB. The morphology of the colonies was observed is punctiform (57.14%) and circular (42.85%). The colour is cream (14.28%) and white (85.71%). The edges were noted to be smooth (100%) and the elevation was observed to be flat (100%). The cell morphology is coccus - shaped (100%) and the bacteria are classified as Gram staining positive (100%). The growth of all LAB isolates from the gastrointestinal tract of the naleh fish is can survive by differences in temperature, NaCl concentration and catalase negative test.

1 Introduction

Extensive research has demonstrated the significant role of probiotic microorganisms in improving the health of both fish and humans. The prevention or treatment of bacterial diseases in fish usually involves the administration of antibiotics. Continuous administration of antibiotics can lead to bacterial resistance to the type of antibiotic [1]. An alternative to address this issue is the use of probiotics. Probiotics are live microorganisms that can have

* Corresponding author: suhartono@usk.ac.id

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beneficial effects on an organism by improving and maintaining a good microbial balance in the gastrointestinal tract. In general, several types of bacteria from the gastrointestinal system (intestine and stomach) of fish belong to the group of lactic acid bacteria (LAB) that can act as probiotics. Lactic acid bacteria are bacteria with potential as probiotics in aquaculture and are widely used to enhance the growth of aquatic organisms [3]. A number of lactic acid bacteria (LAB) species, including Lactobacillus, Lactococcus, Leuconostoc, Enterococcus, Streptococcus, Carnobacterium, Weissella, and Pediococcus, have been identified as potential probiotic candidates for use in fish aquaculture [3].

There are several commercial probiotics for fish aquaculture that may be purchased, including EM4, Bio-Sak, Pro-w, Bactazyme, Biowish AquaFarm, and Aqua Probiotik. Nevertheless, the performance of probiotics is considerably augmented when they integrate indigenous strains of microorganisms, which are obtained from the gastrointestinal tract and the relevant environment of the host organism [4]. It should be noted that not all fish species can be treated with the same probiotic [5].

A number of studies have been conducted to isolate and identify probiotics for use in aquaculture. Some previous studies showed that probiotic and isolat origin varied such as: *Micrococcus, staphylococcus, Bacillus* from mackerel(Rastrelliger sp.) [6]; *Staphylococcus* spp., *Micrococcus sadentarius*, *Lactobacillus acidophillus* and *Micrococcus lylae* from milkfish (*Chanos chanos*) [7]); *Bacillus cereus*, *Strenotrophomonas maltophilia* from duck grouper(*Cromileptes altivelis*) [8]; *Lactococcus lactis* ; *Enterococcus faecalis*, *Lysinibacillus* sp. and *Citrobacter freundii* from ikan Tilapia (*Oreochromis niloticus*) [9] then *Lactobacillus plantarum*, *Pseudomonas aeruginosa*, dan *Bacillus subtilis* from Rohu fish(*Labeo rohita*) [10] ; *Lactobacillus* sp. from goldfish (*Cyprinus carpio*) [11] ; *Vibrio* sp, *Staphylococcus* sp, dan *Pseudoalteromonas* from vannamei shrimp (*Lithopenaeus vannamei* [12].

A review of the existing literature indicates that no studies have been conducted to isolate Naleh fish. Naleh fish (Barbonymus sp.) is native fish distributed in Nagan Raya District, Aceh Province [13]. The naleh fish is regarded as a consumable fish with considerable economic value and has a potential ornamental fish [14]. This fish is a cryptic species and has the potential to become a new species with a limited distribution (endemic) in the South West Coast of Aceh Province [15]. Naleh fish has started to be cultivated in the last 10 yea.

Based on research recommendations from previous research, further research is needed to meet the nutritional requirements of naleh fish [16]; [17]. Therefore, this problem can be overcome through efforts to improve feed management with the addition of probiotic bacteria local isolates (indigenous) derived from the gastrointestinal tract of naleh fish. Probiotics applied can also prevent and overcome disease attacks in fish, maintain water quality in ponds, and increase the productivity of naleh fish. The purpose of this study was to isolate and phenotypically characterize LAB isolates with probiotic potential from the gastrointestinal tract of naleh fish.

2 Materials and methods

2.1 Sample preparation and isolation bacteria Lactic Acid Bacteria (LAB)

Lactic acid bacteria (LAB) were isolated from the Naleh fish, which was obtained from the Nagan Raya District. The naleh fish samples were carefully contained within a dual-layer plastic bag that was saturated with oxygen and aquatic medium. Ice cube were methodically positioned around the plastic enclosures to maintain a reduced temperature during the transportation from the Nagan Raya District to the Microbiology Laboratory of the

Department of Biology at Universitas Syiah Kuala, Banda Aceh. The naleh fish selected for the study are healthy and exhibit no visible lésions on their body (Figure 1.) A dissection was performed under aseptic conditions to isolate the probiotic bacteria.

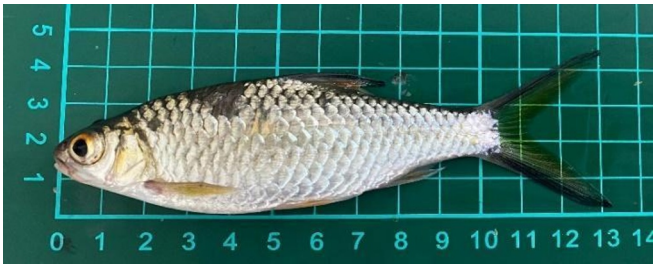

Fig. 1. Naleh fish (*Barbonymus sp*.).

One gram of the sample was placed into a sterile tube containing physiological NaCl, homogenized, and vortexed. The inoculum source was taken in an amount of 1 mL and diluted in a 10-1 serial dilution tube. The dilution was performed by taking 0.1 mL of bacterial suspension from the 10-1 dilution with a micropipette and placing it into a test tube containing 9 mL of physiological NaCl to obtain a 10-2 dilution. The same procedure was repeated until a series of dilutions up to 10-8 was achieved. Each dilution was taken 0.1 mL of bacterial suspension using a micropipette, inoculated into each petri dish containing TSA media using the spread plate method, and incubated for 24 hours in an incubator at 37 °C. After that, the bacterial colonies that grew on the TSA media were inoculated onto MRSA and MRSA+$CaCO_3$ media. The bacterial colony isolate was streaked in four quadrants on each medium with repeated purification until a pure isolate was obtained. Then incubate for 48 hours in an incubator at 37°C [18].

2.2 Characterisation of bacteria from the gastrointestinal tract of naleh fish

The characterisation of the LAB was conducted through macroscopic and microscopic. The macroscopic characterisation involved the observation of the morphology of the bacterial colony, encompassing aspects such as shape, edges, colour and elevation of the colony. The microscopic characterisation of bacterial cells was conducted using the Gram staining technique.

2.3 Screening potential probiotic LAB for in vitro assays

The selection of LAB into probiotic potential bacteria in vitro includes resistance to temperature, gastric acid, pH and bile salts.

a. Catalase test
This test was conducted by dripping 2 drops of Hydrogen Peroxide (H_2O_2) onto a glass slide containing LAB isolates. A positive test of this reaction is characterised by the formation of gas bubbles, indicating that the LAB isolate produces a catalase enzyme that can convert Hydrogen Peroxide (H_2O_2) into water (H_2O) and Oxygen (O_2) [19].

b. Growth at different temperature concentrations
The heat resistance test of LAB bacterial isolates was conducted using 10 mL of MRSB media. Then, the isolates were incubated at 15°C, 37°C, and 45°C for 7 days. A positive

result will be marked by the presence of bacterial growth within that temperature range. The negative control consists of sterile media [20].

c. Growth at different Sodium chloride (NaCl) concentration
Sodium chloride (NaCl) tolerance test using NaCl concentrations of 2%, 5%, and 10%. Sterile MRSB media is placed in test tubes with bile salt concentrations according to the treatment. A total of 9.9 mL of treated MRSB solution is then added with 0.1 mL of 24-hour-old LAB culture. The cultures in the test tubes are then incubated at 37°C for 24 hours. A positive result is indicated by the formation of a precipitate at the bottom of the tube and a change in color to a more turbid state compared to before incubation. Negative control consists only of sterile media [21].

3 Results and discussion

3.1 Isolation and characterization Lactid Acid Bacteria (LAB)

A total seven isolates LAB were obtained from the gastrointestinal tract in the form of the stomach and the intestines of naleh fish (*Barbonymus* sp.). Seven bacterial isolates are the result of initial screening using de man rogosa sharpe agar (MRSA) media. Isolates that grow are given different codes.

Table 1. Colony characteristics and cell morphology of LAB.

Isolate Code	Colony				Cell	
	Form	Pigmentation	Elevation	Edge	Form	Gram Stain
J2103	Punctiform	Cream	Flat	Smooth	Coccus	Positive
J232	Circular	White	Flat	Smooth	Coccus	Positive
J234	Punctiform	White	Flat	Smooth	Coccus	Positive
J185	Punctiform	White	Flat	Smooth	Coccus	Positive
J141	Punctiform	White	Flat	Smooth	Coccus	Positive
B144	Circular	White	Flat	Smooth	Coccus	Positive
B283	Circular	White	Flat	Smooth	Coccus	Positive

This study used de man rogosa sharpe agar (MRSA) media as a selective media in LAB growth. Lactic acid bacteria (LAB) can live on MRSA media because it contains several components that can support the growth of these bacteria. MRSA media contains dextrose, meat extract, yeast extract, Ammonium Citrate, Magnesium Sulfate, Peptone, Sodium Acetate, and Dicalcium Phosphate. Dextrose is a fermented carbohydrate that serves as carbon and energy source [22]. Ammonium Citrate serves to support LAB growth at low pH conditions. Potassium Phosphate and Sodium Acetate serve as buffer solutions to keep the pH low. Magnesium Sulfate serves as a source of ions and sulfate. Peptone, meat extract and yeast extract are sources of nutrients for growth because they contain Nitrogen, Vitamins, Minerals and Amino Acids

The results of the study obtained seven pure culture isolates from the gastrointestinal tract of naleh fish. Colony morphology observed in bacterial isolates included the shape, edges, elevation and colour of the colonies (Table 1). The overall morphology of LAB colonies had punctiform (57.14%) and circular (42.85%) colony shapes; cream (14.28%) and white

(85.71%) colours; smooth edges (100%) and flat elevations (100%). Cell morphology with a coccus shape (100%) and classified as Gram positive bacteria (100%).

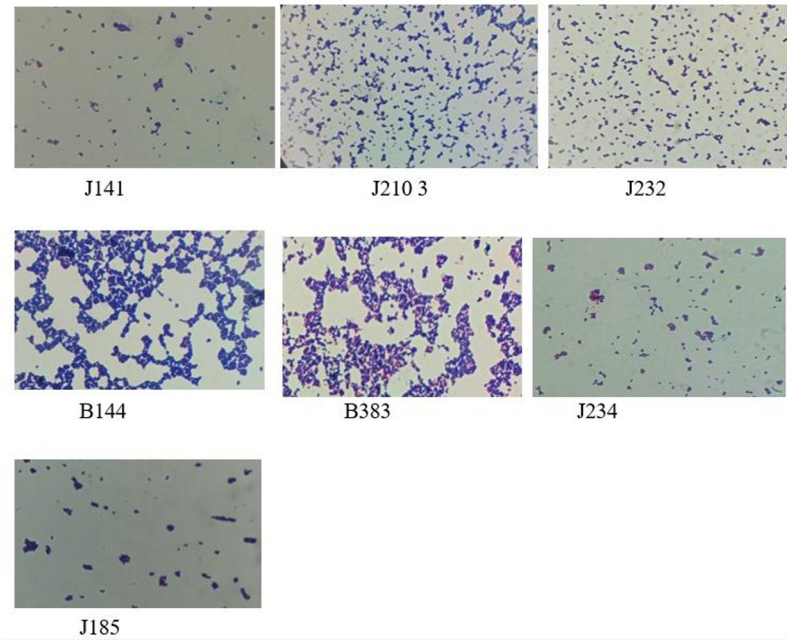

Fig. 2. Gram staining results on each isolate.

The cell shape in this study can be seen in Figure 1. The results showed that the bacterial colonies that grew on MRSA media were white and milky white in colour. This indicates that the bacteria contain carotenoid pigments. The colour of growing colonies looks different because it is influenced by pigments produced by bacteria [23]. Some pigments found in bacteria include melanin, anthocyanins, carotenoids, phenazine and tripyrilmethene. These pigments will then give different colours to each bacterial colony. Melanin pigments will give brown, orange, red and black colours. Antasionin pigments will give red and blue colours. Carotenoid pigments will give red, orange, yellow, beige and milky white colours. Phenazine gives deep orange, yellowish orange and orange red colours. Tripyrilmethene which gives red pigment.

LAB isolation research has been conducted by several researchers with a variety of different numbers of isolates. A total six LAB isolates isolated from the gastrointestinal tract of gourami fish (*Osphronemus goramy*) [24]. Eight LAB isolates isolated from the gastrointestinal tract of star pomfret (*Trachinotus blochii*) [25]. However, there are 96 LAB isolates isolated from the gastrointestinal tract of mangrove crabs (*Scylla* sp.) [26].

The results of Gram staining demonstrated that seven isolates were identified as belonging to the group of Gram-positive bacteria (100%) and exhibited the morphology of cocci cells (100%). One of the characteristics of LAB is Gram positive, forming either coccus or bacilli isolates. Previous research show that 21 LAB isolates exhibited gram-positive characteristics with purple cell walls, as evidenced by the retention of the purple colouration observed in Crystal Violet staining [8].

The stomach and intestines were the sources of the LAB isolates used in this investigation. The ileum and cecum contain a large number of LAB that are Gram-positive bacteria. Gram staining reaction causes all colonies that are gram positive to appear purple [26]. Peptidoglycan in the cell walls of Gram positive bacteria is thicker than that of Gram negative bacteria, and Gram positive bacteria maintain their crystal violet color without changing it.

The majority of the lipids layer that makes up the cell walls of gram-negative bacteria [27]. Therefore, in the Gram staining process, the lipid layer can inhibit the adherence of the primary stain to the bacterial cell, especially when washed with alcohol, which can damage the lipid layer, causing Gram-negative bacteria to appear red in the Gram staining results [28].

3.2 LAB selection by physiological test

The results of the research indicated that the seven LAB isolates were categorized as catalase negative, which is distinguished by the absence of bubbles (Table 2). LAB traits typically have a negative reaction to catalase. The purpose of this test is to identify the species that produce the catalase enzyme. This enzyme detoxifies Hydrogen Peroxide by breaking it down into water and Oxygen gas [29]. The Oxygen gas produced in the form of bubbles clearly confirms a catalase positive result. LAB do not produce the enzyme catalase so they cannot convert Hydrogen Peroxide (H_2O_2) into water (H_2O) and Oxygen (O_2). Bacteria that cannot produce the enzyme catalase are classified as catalase negative.

Table 2. Physiological characterization of isolates LAB.

No.	Isolat	Catalase Activity	Growth at different temperature			Growth at different NaCl concentrations	
			15˚C	30˚C	45˚C	5%	10%
1.	B1 4 4	-	+	+	+	+	+
2.	J1 4 1	-	+	+	+	+	+
3.	B2 8 3	-	+	+	+	+	+
4.	J2 10 3	-	+	+	+	+	+
5.	J2 3 2	-	+	+	+	+	+
6.	J1 8 5	-	+	+	+	+	+
7.	J2 3 4	-	+	+	+	+	+

+ : Positive reaction; - : Negative reaction

Based on the research results, it was obtained that the seven LAB isolates could grow at 15˚C, 30˚C, and 45˚C (Table 2). This test was conducted to determine the ability of LAB growth at different temperatures. The best growth was obtained at 30˚C with turbidity and sediment at the bottom of the tube. All isolates showed more turbidity at temperature 30 ˚C compared between temperatures 15˚C and 45˚C. The growth of bacteria in a liquid medium is observed in the form of sediment or turbidity [30]. All isolates were identified as belonging to the mesophilic. LAB are mesophilic and thermophilic, with some able to grow at 5˚C and the highest at 45˚C. A number of studies have demonstrated that LAB can grow at different temperatures. For instance, LAB isolates from ale-ale Pekasam have been shown to grow between 15°C and 45°C [1].

Table 2 shows that all BAL isolates can grow at salt (NaCl) concentrations of 5% and 10%. BAL isolates grew in 5% salt, indicated by the medium changing to a cloudy color and the presence of sediment at the bottom of the tube [31]. Tolerance to NaCl is an indicator of the osmotic tolerance level of bacteria. NaCl is an active osmotic agent that can inhibit certain bacteria. To survive and reproduce in the gastrointestinal tract, probiotics must tolerate several environmental condition barriers in the gastrointestinal tract, including increased osmolarity in the small intestine [32]. Resistance to acidic conditions is an important criterion for probiotics to survive in the host's gastrointernal tract [33]. The LAB obtained were able to survive in acidic environment because the isolates were isolated from the gastrointestinal tract of fish, so they have adapted at pH fluctuations. This is related to the ability of LAB to

live and thrive in the gastrointestinal tract of fish in probiotic applications given the acidic conditions in the fish stomach.

4 Conclusion

The conclusion of this study was to obtain seven LAB isolates with different colony and cell morphology. Seven LAB isolates with probiotic potential were selected based on in vitro testing, namely catalase test, media tolerance test at different temperatures and NaCl concentrations. Further investigation is required in order to develop a set of selection criteria for LAB probiotics as defined by the World Health Organisation (WHO).

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