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Low pH resistant lactic acid bacteria from cow rumen waste

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ABSTRACT This study aims to obtain a new strain of lactic acid bacteria (LAB) with the ability to survive at low pH, resulting from isolation, selection, and identification from rumen waste. The research included four stages: isolation of bacteria from rumen waste, selection, and identification. The selection procedure involved growth of LAB in a low pH medium. Molecular identification procedure was conducted using the 16S rRNA gene sequence amplification method with universal primers 27F (AGAGTTTGATCCTGGCT CAG) and 1429R (TAGGGTTACCTTGTTACGACTT). The results of the isolation and identification were analyzed descriptively, revealing that five petri dishes (5a, 10a, 10b, 16a, and 18b) contained lactic acid bacteria which produced clear zones. Among these, isolate 18b was the only LAB strain that survived in a pH 3.5 medium. The results of the molecular identification using the 16s rRNA gene showed that isolate 18b belonged to *Limosilactobacillus fermentum*.

KEYWORDS Identification; Isolation; Lactic acid bacteria; Low pH

1. Introduction

Lactic acid bacteria (LAB) are Gram-positive, non-sporing bacteria, with either bacillus or cocci shaped. They are facultative anaerobes and capable of fermenting lactose, with lactic acid as the main product. LAB play an important of breaking down proteins into amino acid monopeptides available to the body and produce bacteriocins (Widyadnyana et al. 2015). Bacteriocins help in tackling the incidence of infection, are safe and do not cause resistance (Marshall and Arenas 2003).

The digestive process of ruminants passes through 4 compartments of the stomach, namely, the rumen, reticulum, omasum, and abomasum. The first and main process of digestion in ruminants is in the rumen, where the feed undergoes fermentation process. The fermentation process cannot be separated from the role of microbes, which often called rumen microorganisms (MOR).

The types of bacteria found in the rumen contents are acid-forming, lipolytic, amylolytic, proteolytic, and cellulolytic bacteria (Soetrisno et al. 1994). Cahyaningtyas et al. (2019) stated that the total LAB in the rumen content was influenced by the feed given (ratio of forage and concentrate), bacterial composition, and fermentation conditions in the rumen.

The results of Suardana and Suardana (2007) research, showed that the total yield of LAB living in the rumen contents of fistulous Ongole Peranakan cattle after spray drying was 9.1 log CFU/mL in fresh rumen content and 8.9 log CFU/mL. Research conducted by Winurdana (2016) reported that the addition of fresh rumen contents and carbohydrate source additives (bran and cassava flour) resulted in a very rapid decrease in pH and resulted in high lactic acid production.

Silage production is growing rapidly in countries with cold and sub-tropical climates. The principle of making silage is forage fermentation by microbes that produce a lot of lactic acid. The most dominant microbes are from the lactic acid bacteria group which is capable of fermenting under anaerobic conditions. Lactic acid produced during the fermentation process will act as a preservative to prevent the growth of spoilage microorganisms.

Leguminous forage is highly preferred by livestock and breeders because it has high palatability and high crude protein content. However, there are several drawbacks that make legumes less suitable for use as silage, such as their high anti-nutrient content (saponins and tannins), high buffer capacity (BC), and increased risk of silage damage. Given these challenges, it is necessary to study the presence of bacteria in rumen waste that can be used as an agent for legume fermentation.

The identification of lactic acid bacteria species can be done through examination of morphological, metabolite, phenotypic, and genotypic characteristics. The phenotypic

method is considered less efficient and less accurate, as it relies on phenotypic expression under laboratory conditions, which may lead to misidentification (Widyadnyana et al. 2015; Chenoll et al. 2003). The genotypic method that is widely applied in microbial identification studies is 16S and 23S rRNA gene sequence analysis (Widodo et al. 2013).

2. Materials and Methods

The material used is rumen waste. The flow of this research involves the isolation of bacteria from the waste contents of the rumen, followed by the determination of lactic acid bacteria from pure cultures. Next, low pH-resistant lactic acid bacteria are selected, and finally, the identified bacteria resistant to low pH are analyzed.

2.1. Isolation of bacteria from rumen waste

The isolation of bacteria in this study involved extracting bacteria from the waste medium of the rumen contents, enriched with tamarind leaves as a legume material. This process is based on Singleton and Sainsbury (1988) method to obtain pure cultures. Pure cultures were obtained by the scratch plate method which is based on the principle of dilution with a view to obtaining individual species. It is assumed that each colony can be separated from one type of cell that can be observed (Afrianto 2004).

2.2. Determination of lactic acid bacteria from pure cultures obtained

The determination of lactic acid bacteria was carried out by growing pure bacterial cultures obtained using the pour plate method. Colonies that grew and produced clear zones were identified as lactic acid bacteria. Lactic acid bacteria obtained from this process were then measured for lactic acid production and growth rate.

2.3. Selection of low pH resistant lactic acid bacteria

The lactic acid bacteria obtained were selected based on isolates with lactic acid production and high growth rates. The isolates were then grown in low pH media, at pH 3.5, pH 4, and pH 4.5.

2.4. Identification of selected lactic acid bacteria resistant to low pH

Isolates of lactic acid bacteria that survived at the lowest pH were identified molecularly using 16S rRNA gene sequence analysis. Bacterial isolates identification was conducted molecularly through analysis of the 16S rRNA gene. One single colony for each isolate was inserted into the PCR tube using a sterile toothpick. Bacterial cells were then added with 50µL of PCR reaction mix containing 25 µL taq RM (KAPA), 0.4 µL 100 pmol primer 9F (5′-AAGGAGGTGATCCAGCC-3′), 0.4 µL 100 pmol primer 1541R (5′-AAGGAGGTGATCCAGCC-3′) and 24.2 L H_2O. The PCR conditions used were as follows: predenaturation 96 °C for 5 min, denaturation 96 °C for 30 s, anneling 55 °C for 30 s, pre-elongation 72 °C for 1 min, elongation 72 °C for 7 min for 30 cycles (Tamura et al. 2007). The results of the PCR were visualized by electrophoresis using 1% agarose in 1× TAE buffer solution. Electrophoresis was conducted at 100 V, 400 A for 30 min. The gel from the electrophoresis was then immersed in an EtBr solution for 30 min and then washed in distilled water. The results of the electrophoresis were then observed on the gel isolate under UV light. The PCR products were then sequenced at PT Macrogen, South Korea to determine the base sequence. The sequencing results were then aligned with GenBank data using BLAST-N (Basic Local Alignment Search Tool – Nucleotide) from the NCBI (National Center for Biotechnology Information) website (https://blast.ncbi.nlm.nih.gov/).

3. Results and Discussion

The isolation of bacteria from rumen waste contents began with preparing a source of bacteria by sieving trembesi (*Sammanea saman*) leaves. The source of bacteria was then diluted 10 times in 10 tubes. After that, it was then grown in MRS media using the pour plate method in 20 petri dishes, with two replications per sample. After growth, lactic acid bacteria were screened through the formation of a clear zone. It was then found that 5 petri dishes had formed a clear zone.

Of the 5 petri dishes that formed a clear zone, lactic acid bacteria were grown into 25 petri dishes with MRS media. After being incubated for 42 hours, the bacteria were purified into 25 petri dishes and incubated for 36 hours. After incubation, those that formed the most colonies were selected, namely petri dish numbers 4, 5, 8, 10, 16, 17, and 18. Then the colonies were transferred to MRS media with 3 replications. Then, the bacteria that grew a lot were seen again so that petri dish 5a, 10a, 10b, 16a, and 18b were selected. After that, the bacteria were transferred to a liquid medium. Then measured the production of lactic acid and the growth rate of lactic acid bacteria. The data on lactic acid production and growth rate can be seen in Table 1 and Figure 1.

Figure 1 shows that the five isolates had an increasing growth trend. Because the five isolates of lactic acid bacteria obtained had relatively similar lactic acid production and growth rate, a selection process was conducted to assess their resistance in low pH media. This selection is needed to ensure their compatibility as the agents for legume fermentation. Legumes have high buffer capacity, which can hinder the success of the ensilage process in the legume fermentation because the acidity levels needed were not achieved. Therefore, a selection of lac-

TABLE 1 Concentration of lactic acid (mg/mL).

Code Isolate	blanko	5a	10a	10b	16a	18b
Lactic acid	0.01	0.119	0.117	0.105	0.082	0.079

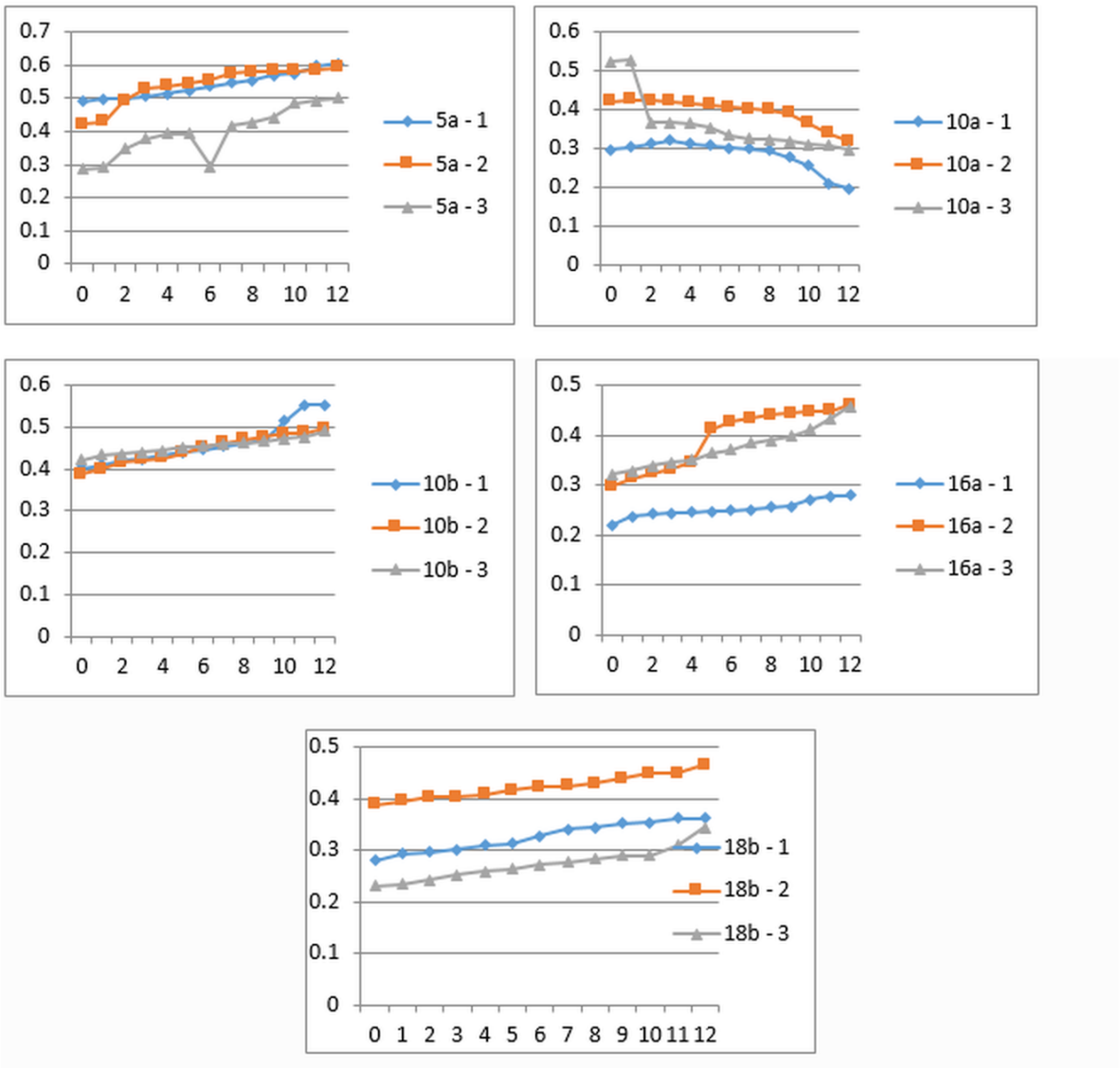

FIGURE 1 Increasing growth trend of the five isolates.

tic acid bacteria was conducted by growing it on several acidic media, specifically MRS media at pH of 3.5, pH 4, and pH 4.5. The process was conducted because bacteria that can live at low pH can inhibit the buffer capacity of legumes. The viability of the five isolates is presented in Table 2. Table 2 shows that the lactic acid bacteria isolate 18b was able to survive in media with a pH of 3.5. Therefore, isolate 18b was selected to be identified further.

TABLE 2 Viability of lactic acid bacteria in low pH media.

Code of lactic acid bacteria isolate	pH 3.5	pH 4	pH 4.5
5a	-	+	+
10a	-	+	+
10b	-	+	+
16a	-	+	+
18b	+	+	+

Notes: - = lethal; + = existence

3.1. Molecular result

The identification of isolate number 18b was conducted molecularly using 16S rRNA gene sequence analysis. The results of 16S rRNA gene amplification can be seen by the appearance of PCR product fragments with a size of 2,500 base pairs (bp) which is the expected size when using a combination of primers B27F and U1492R (Figure 2).

Figure 2 shows the amplification of the 16S rRNA gene LAB isolate 18b, isolated from rumen waste with primers B27F and U1492R on 0.8% agarose. The results of the Basic Local Alignment Search Tool (BLAST) showed that isolate 18a had similarity with *Limosilactobacillus fermentum* strain AR3, *Limosilactobacillus fermentum* strain HBUAS517 and *Limosilactobacillus fermentum* strain HBUAS51683. This high similarity was indicated from the clustering of LAB isolate 18b in one cluster with the reference strain, holding a bootstrap value of 100%. The bootstrap value shows close kinship if it has a high value, which is more than 70% (Mount 2001).

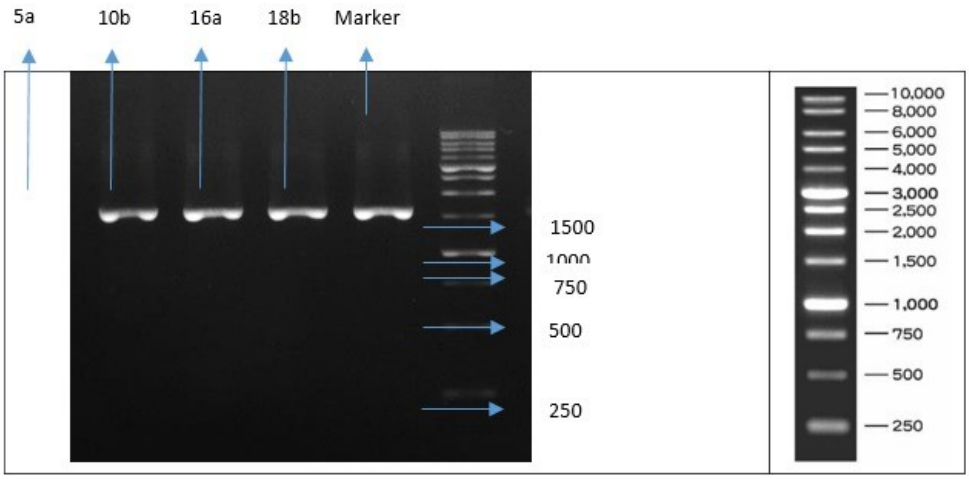

FIGURE 2 Amplification of the 16S rRNA gene LAB isolate 18b.

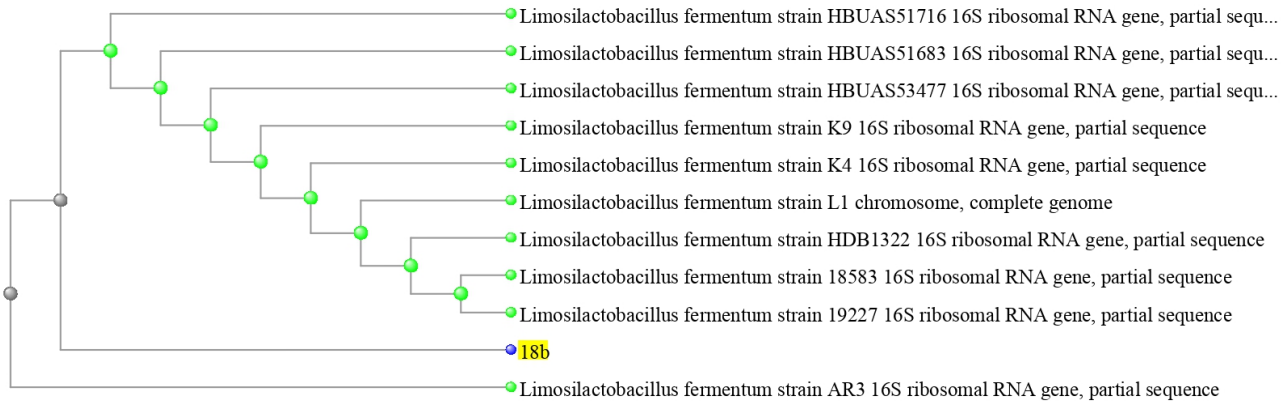

FIGURE 3 Phylogenetic tree of 18b isolate based on the 16S rRNA gene sequence.

Reller et al. (2007) stated that the results of bacterial identification based on the 16S rRNA gene sequence can be accepted as a genus if it has a homology of more than 97% and as a species if the homology is more than 99%. These results are then constructed to define a phylogenetic tree as shown in Figure 3.

Limosilactobacillus fermentum belongs to the genus *Limosilactobacillus*. Species in this genus are heterofermentative and adapted to the intestinal tract of vertebrates but are also used for a variety of applications including the fermentation of food and feed. *L. fermentum* differs from most or all other species in the genus in that it has a nomadic lifestyle and is not a stable member of the gut microbiota of humans or animals. It has been found that some strains of *L. fermentum* have natural resistance to certain antibiotics and chemotherapy. They are considered as potential vectors of antibiotic resistance genes from the environment to humans or animals to humans (Cahyaningtyas et al. 2019).

Testing of *L. fermentum* against solutions of different pH concentrations revealed that it has a strong pH tolerance with its ability to grow and survive several hours after incubation in 3 pH-level solutions (Agussalim 2020). The *L. fermentum* strain has also been tested in different bile concentrations and was shown to have good bile tolerance when incubated with 3 g L^{-1} bile salts.

4. Conclusions

This research can be concluded that lactic acid bacteria isolate 18b selected lactic acid bacteria are resistant to low pH and identified as *Limosilactobacillus fermentum*.

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Authors' contributions

AKS coordinates the entire research process, report writing, and article writing. K responsible for collecting rumen content waste samples and handling them prior to lactic acid bacteria isolation. ZB responsible for screening lactic acid bacteria from rumen content waste, isolating lactic

acid bacteria, and their rejuvenation. CH responsible for cultivating lactic acid bacteria in low-pH media, identifying lactic acid bacteria, and assists in writing the scientific article.

Competing interests

The authors declare that there are no competing interests that could influence the results, interpretation, or presentation of this study.

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