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Molecular identification, characterization, and antimicrobial activity of isolated lactic acid bacteria from *dali ni horbo*

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Abstract. Various dairy products come from Indonesia and are fermented that leading to the growth of lactic acid bacteria. According toataknese culture, water buffalo milk can be fermented to form Dali ni Horbo. This study was aimed to isolate, characterize, molecular identification, and evaluate the antimicrobial effect from the lactic acid bacteria in Dali ni horbo. Dali ni horbo as the sample was encoded D1, D2, and so on. The lactic acid bacteria were isolated from Dali ni Horbo by MRSA. Moreover, the colonies of bacteria were enriched into some isolates. After that, the isolates were characterized by gram staining, catalase, and type of fermentation. Meanwhile, to evaluate the antimicrobial activity, this study used the disc diffusion test against two different pathogens. At last, the selected isolate was molecularly identified from the 16S rRNA marker. Dali ni horbo contain 4.3×10^8 CFU/ml. of *Lactobacillus fermentum strain A1753* The isolated bacteria were bacilli-shaped, positive gram, homofermentative, and heterofermentative bacteria without catalase activity. The isolated bacteria have potent antimicrobial activity against the negative pathogen (ranged from 9.8 mm – 12.15 mm) than positive gram pathogen (ranged from 7.15 mm – 11.05 mm). Hence, Dali ni horbo contain lactic acid bacteria that can be natural preservative and potential probiotic.

1. Introduction

Lactic Acid Bacteria (LAB) is a positive gram bacteria that has coccus or basil shape, non-forming spore, motile, facultatively anaerobic, and none catalase activity. There are several genera' of these bacteria include *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weisella* [1].

Lactic Acid Bacteria has a significant role in the food serving industry. This bacteria can act as a natural preservative due to its metabolites that have antimicrobial activity. These were organic acid and Bacteriocin. Lactic acid bacteria also affect the texture and taste of the food [2, 3].

The metabolite of the bacteria was the result of fermentation activity. Based on their fermentation activity, this bacteria was classified into homofermentative and heterofermentative. Homofermentative is the group of lactic acid bacteria that only form lactic acid as the metabolite. Meanwhile, the heterofermentative bacteria form lactic acid and various metabolites like acetate, ethanol, and carbon dioxide [4].



Milk is a favorable medium for the growth of bacteria. Therefore, the milk may contain the lactic acid bacteria that can act as the preservative and affect its texture and taste. Due to the taste, this product is preferred by most consumers. Furthermore, it shifts the diet of the community to consume healthy food like the fermented products that increase the demand for the fermented product [5].

There are various dairy products in Indonesia. These can come from cow's milk or water buffalo's milk. Based on their animal, the water buffalo was more profitable than the cow. Its due to water buffalo can survive in the troublesome environment, especially when the good feed was not enough. Several dairy products from water buffalo's milk in Indonesia are Dangke (South Sulawesi), Dadih (West Sumatera), and Dali (North Sumatera) [4].

The water buffalo's milk has been contributed to increasing the income for cattle farming. This product also improves community nutrition. Dali ni horbo is a fermented water buffalo's milk that comes from bataknese culture in North Sumatera. The type of water buffalo used to ferment was swamp-type of water buffalo [4].

Several studies of various dairy products had been performed to explore their probiotic potential. Fachrial et al. (2014) isolated and explore the antimicrobial activity of Dadih. They reported that there was 8.4×10^9 CFU/g of Lactic Acid bacteria in Dadih, and it had a bacteriocin that weighted lower than 10 kDa. Due to its molecule weight, the Bacteriocin is Class II Bacteriocin that has stability on the high temperature [4].

The previous study of Water Buffalo's milk from Enrekang district was performed by Andiani (2012) reported that the water buffalo's milk contain *Lactobacillus* as the lactic acid bacteria. The isolates were non-motile, gram-positive, and bacilli-shape lactic acid bacteria. Meanwhile, based on physiologic properties of bacteria, there were no catalase and oxidase activity, unable to reduce nitrate, unable to form Hydrogen sulfide, and able to ferment glucose, lactose, or sucrose into acid without gas [6]. Based on the previous study, this study was aimed to isolate, characterize, molecular identify and evaluate antimicrobial effect from the lactic acid bacteria in Dali ni horbo as the fermented product of water buffalo's milk that was a bataknese food.

2. Materials and Method

2.1. Reagents

Dali ni horbo as the samples, Isolates of *Escherichia coli* and *Staphylococcus aureus*, MRS Broth (Oxoid) media, MRS agar, Nutrien Agar Merck, Nutrient Broth Merck, aqua dest, solution of 3% Hydrogen Peroxide, immersion oil, crystal violet, iodine, and 70% alcohol.

2.2. Sample collection

This study was performed between June-August 2019 in The Biochemistry Laboratory of Medicine Faculty at the University of Prima Indonesia. The sample was collected from a bataknese restaurant in Medan. Moreover, the sample was isolated into some isolates and was encoded D1, D2, and so on. The following figure showed the sample that was used in this study.

2.3. Preparation of medium

The media used in this study were MRSA (*DeMan Rogosa Sharpe* Agar) and MRSB (*DeMan Rogosa Sharpe* Broth). These media was specific for lactic acid bacteria due to the contains of nutrient were specific for the lactic acid bacteria included beef extract, yeast extract, di-potassium hydrogen phosphate, di-ammonium hydrogen citrate, sodium acetate, magnesium sulfate, and manganese sulfate [7]. The MRSB was made by mixing the MRSB powder into the amount of aquadest. The ratio of powder (gram) and aquadest (ml) was made based on the manufacture instructions.



Figure 1. *Dali ni horbo*

2.4. Estimation of bacterial population, enrichment and isolation of bacteria

The estimation of bacteria numbers used Total Plate Count (TPC) methods. The amount of 1 gram sample was added into the MRS Broth, and then it was homogenized and incubated for 24 hours at 37°C. After that, these MRS Broth were dissolved into several dilution serials. The serial dilution of MRS Broth was cultured into MRS Agar from 10^{-4} to 10^{-6} dilution serial and incubated for 48 hours [8]. The amount of 0.1 ml of MRS Broth was cultured into MRS Agar by spread methods at the sterile condition in laminar airflow. These media were incubated for 48 hours at 37°C. After 48 hours, the colonies that were formed in MRS Agar were estimated the number of bacteria by the following formula [9]:

$$\text{CFU} = \text{Number of Colony} \times \frac{1}{\text{Dilution}} \times \frac{1}{\text{Volume of Samples}} \quad (1)$$

Moreover, five colonies that were circular, mucoid, convex, and smooth-edge were enriched into MRS Agar by strike methods. These enrichment media was incubated for 48 hours at 37°C, and then these were identified and characterized.

2.5. Characterization of bacteria

The bacteria that have been isolated were characterized according to their gram staining, catalase, and fermentation activity. The gram staining was performed by fixing the isolated bacteria into the object-glass. Then, these were dropped by crystal violet and incubated at room temperature for 30 seconds. After that, it was rinsed by aqua dest. Then, these were drops by iodine, and these were rinsed again by the 70% alcohol and aquadest. At last, these were dropped by safranin, incubated for 30 seconds, and rinsed by the aquadest.

Before the microscopic view, the object was dropped by the immerse oil and view at 100x magnitude. The gram-positive would show the violet color by crystal violet. Meanwhile, the gram-negative would show a red color by safranin. The catalase activity was evaluated by dropping the isolated bacteria by 3% Hydrogen peroxide in the object-glass. The formation of air bubbles in the sample indicates a positive result (Catalase-Positive bacteria). It is due to the formation of oxygen by the degradation of hydrogen peroxide molecule [10]. The fermentation activity was evaluated by culture the bacteria into the Durham tube. The culture was incubated for 48 hours at 37°C. The result was evaluated by observation of the bubble formed in the tube [10].

2.6. Antimicrobial assay

Disc diffusion methods were used to evaluate the antimicrobial assay against *Escherichia coli* and *Staphylococcus aureus* as the pathogen. The isolates of pathogens were obtained from the microbiology laboratory of Medicine Faculty at Universitas Prima Indonesia. Initially, the isolates of the bacteria were cultured into 10 ml sterile Nutrient broth and incubated anaerobically. After that, the pathogen bacteria were cultured spreadly by cotton swab and incubated at 37°C for 18-24 Hours.

Furthermore, the discs were soaked into the isolate of Lactic Acid Bacteria that had been isolated from the samples. After that, these discs were placed in the nutrient agar that had been cultured by pathogens. Then, plates were incubated for 48 hours. At last, the zone of inhibition was measured using sterile Vernier calipers.

2.7. Molecular identification of bacteria

Several steps performed to identify molecularly of bacteria. These steps included isolation of DNA, amplification of DNA, electrophoresis gel, and sequencing the nucleotide. The isolate of Lactic Acid Bacteria in MRSB was placed into 2 ml Eppendorf tube, centrifuged at 14,000 rpm for 3 minutes. It formed a supernatant and pellet cell. The supernatant was removed, and remain pellet cell was added by the 2 ml isolate of lactic acid bacteria in MRSB. It was repeated for three times of centrifuge. Remain pellet cell was added 500 μ L 1 x TE, 40 mL SDS, and 5 μ L Proteinase K. After that, it was homogenized by vortex and incubated by water bath at 37°C for an hour. Moreover, it was added by 500 μ L PC and centrifuged at 14,000 rpm for 3 minutes. Then, take the top of the layer, and it was added by 600 μ L PC, flipped it three-time, and centrifuged at 14,000 rpm for 5 minutes. After that, take the top of the layer, move it into the Eppendorf tube, and then add 1/10 part of sodium acetate. Then add 6/100 part of cold isopropanol, flipped it, and centrifuged at 14,000 rpm for a minute, then remove the supernatant and washed it with 1ml cold ethanol. Dried it and added 25 μ L TE to dissolve the pellet.

The gene that was amplified in this study was the gene that encoded 16s rRNA. The PCR performed the amplification in 35 cycles. The cycle was adjusted as pre-denaturation for 5 minutes at 95°C, denaturation for a minute at 94°C, annealing for a minute at 56°C, extension for 1.5 minutes at 72°C, final extension for 5 seconds at 72°C. The universal primer used in this study were 27f (5'-AGA GTT TGA TCC TGG CTC AG - 3') and 1492r (5'-GGT TAC CTT GTT ACG ACT T - 3'). This procedure was performed using 1% agarose (b/v) that contains ethidium bromide. The amount of 2 μ L isolate was mixed with 1 μ L loading buffer Bromophenol Blue (BPB) and 7 μ L 1x TE. The electrophoresis was performed under 100 volt for 30 minutes. Meanwhile, a 1 kb DNA ladder (Gene ruler) was used as the marker [9]. The purified samples were determined by their nucleotide sequences by forward-reverse DNA. The sequence that was obtained was analyzed by the Basic Local Alignment Search Tool (BLAST) then compared to the NCBI (<http://www.ncbi.nlm.nih.gov>) database. Meanwhile, the Sequence Alignment was performed by ClustalW2.

2.8. Data analysis

All data obtained in this study include the number of the colony, the characteristics, the zone of inhibition, and molecular identification. All data were analyzed descriptively that was shown as the table and bar chart.

3. Results and Discussion

3.1. Estimation of bacterial population

Dali ni Horbo as the sample estimated contain 4.3×10^8 CFU/ mL. The following figure showed the colony that was formed in the MRSA for the estimation of the bacteria. The fermentation of water buffalo's milk looks like an increase in the number of bacteria in the water buffalo's milk. Damayanthi (2014) reported that the water buffalo's milk swamp type and river-type were 3.79×10^8 and 5.08×10^5 CFU. Meanwhile, based on the recent study results, the Dali ni horbo had a higher number of bacteria than the raw water buffalo's milk [4].



Figure 2. The colony of lactic acid from sample at 10^{-6} dilution serial

As a comparison, the previous study reported that yogurt was contain lactic acid bacteria. They grouped the sample into three groups of samples include A1 (10 grams sucrose and 200 ml skim milk), A2 (20 grams sucrose and 200 ml skim milk), and A3 (30 grams sucrose and 200 ml skim milk). The estimation of bacteria from sample A1, A2, and A3 were 6.924 log CFU/ml, 7.006 log CFU/ml, and 7.054 log CF/ml, respectively [11]. Fachrial et al. (2018) reported that there are lactic acid bacteria in the fermented palm oil sap. It had 1.4×10^7 CFU/ml. The number of active and viable lactic acid bacteria up to 10^6 CFU/g indicates it is probiotic. Based on the estimation of the number of bacteria in the Dali ni horbo, it can be probiotic due to the number of lactic acid bacteria higher than 10^6 CFU/g [12].

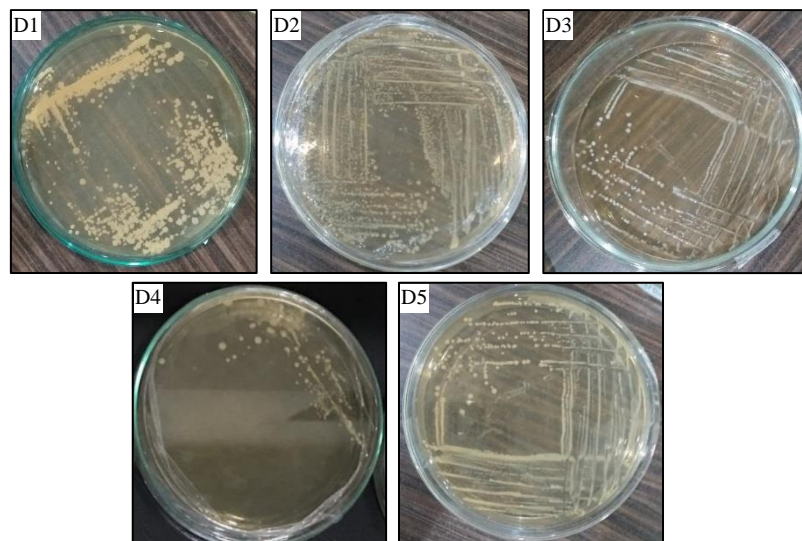


Figure 1. Enrichment of lactic acid bacteria from sample A and B

Furthermore, the colonies from each sample were enriched into some plates of MRSA. Then, the following figure showed the result of the enrichment. Based on the figure above, most of the colonies appeared white to white-yellowish colored. The edge of the colonies was smooth and wave. Some colonies were elevated and convex, and other colonies were flat. The following table showed the summary of the morphology of the samples.

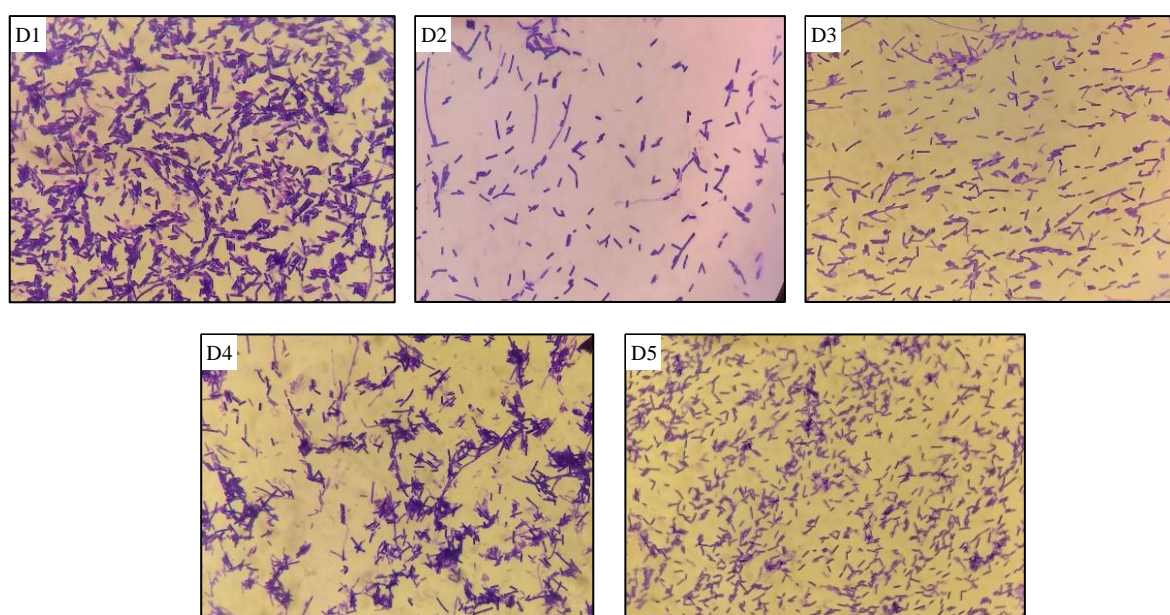
Table 1. The morphology of colonies from sample A and B

Isolate	Morphology of Colony		
	Shape	Elevation	Colour
D1	Large circular	Flat	Cream
D2	Small circular	Convex	White
D3	Small circular	Convex	White
D4	Large circular	Convex	Creamy White
D5	Small circular	Convex	White

Based on the table above, the colonies from Sample A and B have the morphology of lactic acid bacteria, especially *Lactobacillus*. This bacteria's properties were white to yellow color, circular, wave, thin, and wide surface with the greasy edge in the slants agar [13].

3.2. Characterization of bacteria

The isolated bacteria that had been enriched were staining by gram staining, and the following figure showed the staining results.

**Figure 2.** Gram staining from purified lactic acid bacteria (D1-D5)

Based on the table above, all isolates were positive gram lactic acid bacteria. They showed the violet-color bacilli-shaped bacteria. Moreover, the colonies were evaluated their catalase and fermentation activity. The following table showed the summary of the colonies' characteristics.

Based on the table above, most of the isolates were bacilli-shape, positive gram, and homofermentative lactic acid bacteria without catalase activity. However, there was an exception that the D3 isolate had heterofermentative activity. This finding indicates that the isolates were lactic acid bacteria. Moreover, the characteristic of lactic acid bacteria indicated the characteristic of *Lactobacillus*. *Lactobacillus* was positive gram bacteria without catalase activity. $0.5\text{--}1.2 \times 0.5\text{--}1.5 \mu\text{m}$ [10,14].

Table 2. The characteristics of isolates

Isolate	Characteristics			
	Shape	Gram	Catalase	Fermentation
D1	Bacilli	+	-	Homofermentative
D2	Bacilli	+	-	Homofermentative
D3	Bacilli	+	-	Heterofermentative
D4	Bacilli	+	-	Homofermentative
D5	Bacilli	+	-	Homofermentative

3.3. Antimicrobial assay

Furthermore, each isolate was evaluated their antimicrobial activity, and the following table showed the result of the antimicrobial assay.

Table 3. Diameter of inhibition zone from the isolates

Isolates	<i>E. coli</i> (mm)	<i>S. aureus</i> (mm)
D1	-	-
D2	-	-
D3	9.8	7.15
D4	11.75	10.65
D5	12.15	11.05

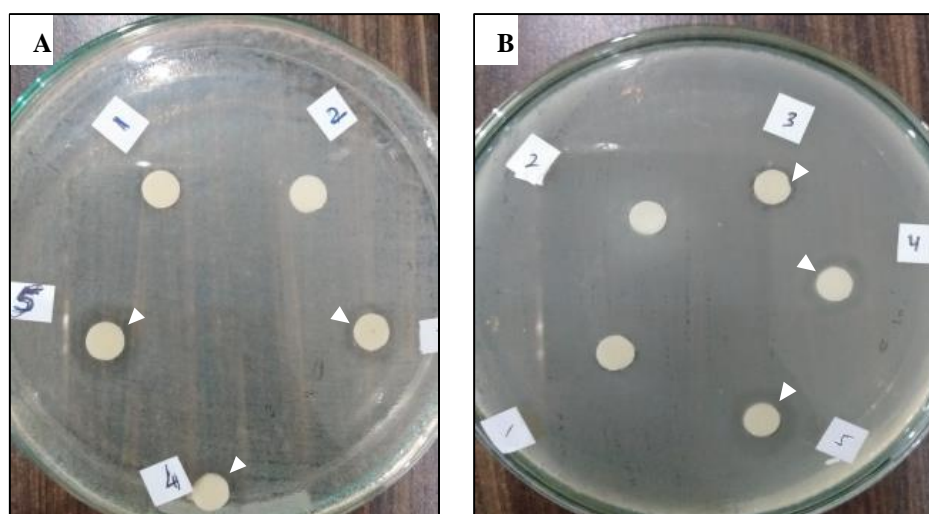


Figure 3. The inhibition zone (white-arrowhead) from the isolates against *E. coli* (A) and *S. aureus* (B)

Based on the table and figure above, The D1 and D2 isolates did not show any antimicrobial activity. However, remain isolates had antimicrobial activity against *E. coli* as negative gram bacteria and *S. aureus* as positive gram bacteria. The antimicrobial activity of the isolates was shown by the clear zone that forms around the disc. This clear zone is also known as the inhibition zone. The diameter of the inhibition zone from D3, D4, and D5 isolates against *E. coli* was 9.8 mm, 11.75 mm, and 12.15 mm, respectively. Meanwhile, the inhibition zone diameter from D3, D4, and D5 isolates against *S. aureus* was 7.15 mm, 10.65 mm, and 11.05 mm, respectively. According to the inhibition zone, the antimicrobial activity of isolates against gram-positive was greater than gram-negative bacteria.

The antimicrobial activity of the isolates is due to the presence of their metabolite. The metabolites were lactic acid, ethanol, carbon dioxide, and other secondary metabolites like bacteriocin and hydrogen peroxide. The lactic acid can diffuse into the environment of the pathogen. It will impact the stability of the membrane cell. The unstable membrane cell will disturb the absorption of nutrition by the pathogen. Moreover, the metabolism and growth of the bacteria will be impaired. On the other hand, the presence of Bacteriocin that was formed by the lactic acid bacteria also take a role in the antimicrobial activity, and it acts as the single hit inactivation that means a molecule of bacteriocin attack a cell of bacteria [10, 15].

This study's result was similar to Fachrial et al. (2014) study. They reported that the lactic acid bacteria isolated from dadih had antimicrobial activity against *S. typhii* and *E. coli* as negative gram pathogen and *S. aureus* as negative gram bacteria. The inhibition zone's diameter from their isolates against *S. typhii*, *E. coli*, and *S. aureus* were 10.55 mm-14 mm, 12.5 mm-14 mm, and 10.5 mm – 12mm respectively. Meanwhile, The widest inhibition zone was against *S. typhii*, *E. coli*, and *S. aureus* was shown by E1 (14 mm), E4 (14 mm), and E1 (12 mm), respectively. Hence, their isolates from dadih showed potent antimicrobial activity against negative and positive gram pathogens [9].

Another study that was performed by Melia and Juliyasari (2011) also showed a similar result. They reported that Dadih, which contain 2% *Lactococcus lactis* had greater antimicrobial activity against *S. aureus* than *E. coli* and *S. typhii*. It meant that *Lactococcus lactis* that formed Bacteriocin more effective against *S. aureus* as positive gram bacteria than gram-negative bacteria [16].

3.4. Molecular identification

Based on the result of DNA genome sequencing, it was continued to trimming and assembling by BioEdit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). It was compared to data from BLAST that had been registered in NCBI/ National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>) for determination of taxonomy from the isolate.

```
AATTGATTGATGGTGCTTGACCTGATTGATTTTGGTCGCCAACGAGTGGCGGACGGGTGAGTAACAC
GTAGGTAACCTGCCCAGAAGCGGGGACAACATTTGGAAACAGATGCTAATACCGCATAACAACGTTG
TTCGCATGAACAACGCTTAAAGATGGCTTCTCGCTATCACTTCTGGATGGACCTGCGGTGCATTAGC
TTGTTGGTGGGGTAACGGCCTACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACAA
TGGGACTGAGACACGGCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGCAAG
CCTGATGGAGCAACACCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAA
CACGTATGAGAGTAACTGTTTACATACGTTGACGGTATTTAACCAGAAAGTCACGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGAGAGTGCAGGCGG
TTTTCTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGGAGAAGTGCATCGGAACTGGATAACTTGAG
TGCAGAAGAGGGTAGTGGAACCTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACCAGTG
GCGAAGGCGGTACCTGGTCTGCAACTGACGCTGAGACTCGAAAGCATGGGTAGCGAACAGGATTAGA
TACCCTGGTAGTCCATGCCGTAAACGATGAGTGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCGG
AGCTAACGCATTAAGCACTCCGCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGG
GGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCCTTACCAGGTCTTGAC
ATCTTGCGCCAACCTAGAGATAGGGCGTTTCTTCGGGAACGCAATGACAGGTGGTGCATGGTTCGTC
GTCAGCTCGTGTGCTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTTACTAGTTGCCAG
CATTAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAGATC
ATCATGCCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGTCGCGAACTCGCG
AGGGCAAGCAAATCTCTTAAACCGTTCTCAGTTCGGACTGCAGGCTGCAACTCGCCTGCACGAAGTC
GGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC
GTCACACCATGAGAGTTTGTAAACCCAAAGTCGGT
```

Figure 6. Sequencing result of 16S rDNA

Based on the figure above, the most highly similar or homology molecular microorganism against the D5 isolates was *Lactobacillus fermentum strain A1753*. The analysis of the similarity gene from the isolates against *Lactobacillus fermentum strain A1753* was evaluated by Max Score, Total Score, Query coverage, E- Value, and Max Identities. Max Score and T Score were the same, Query coverage around 100%, E-value around 0 with max identities around 100%. It means the *Lactobacillus fermentum strain A1753* highly similar to the D5 Isolates.

Lactobacillus fermentum was the group of lactic acid bacteria. It has growth and pH resistance, similar to other types of probiotic. It can be isolated from lactic acid food (like cheese, juice, and fermented milk) and gastrointestinal tract of humans and animals. Some probiotic strains of *Lactobacillus fermentum* are commercially available. One of the products was *L. fermentum* ME-3. Moreover, *Lactobacillus fermentum* possesses antimicrobial activities against some species of intestinal pathogens like *Salmonella spp.* and *Staphylococcus aureus*, anti-inflammatory, as well as high antioxidative activity. The antimicrobial effect of *L. fermentum* due to the presence of LysM domain protein, it causes lysis of the pathogen's cell wall but does not affect lactobacilli [17–20].

4. Conclusions

Dali ni horbo contain bacteria ranged from 2.2×10^8 to 4.3×10^8 CFU/ mL of Lactic Acid Bacteria. The lactic acid bacteria are bacilli-shaped, positive gram, homofermentative, and heterofermentative bacteria without catalase activity. Based on their DNA genomic, the lactic acid bacteria is *Lactobacillus fermentum strain A1753* that has potent antimicrobial activity against negative pathogen than positive gram pathogen.

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