

## **Molecular Analysis of Lactic Acid Bacteria Isolate Sr2 from Bali Cattle Rumen**

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### **Abstract**

Bali cattle with its distinctively genetic characteristics, namely simple life or easy to adapt on unfavorable environmental condition was known as cattle pioneer. These characteristics were due to specific type of lactic acid bacteria (LAB). These facts indicate that the LAB identification with specific advantages was absolutely necessary for further research in order to prevent incorrect interpretation. Generally, molecular approach is the accurate method of identifying microorganism, and in this case, we used the analysis of the 16S rRNA gene. Research activities began with cultivation of SR2 isolates on specific media deMannRogosa Sharpe (MRS) followed by Gram stain, catalase test, and formation of CO<sub>2</sub> gas. Molecular analysis was performed by PCR of the 16S rRNA gene using primers 27F and 1492R. The PCR products were sequenced and nucleotides data were analyzed by Mega 5.2 Software tool. The results showed that phenotypical isolates of SR2 showed characteristics as LAB isolates, and the results of the 16S rRNA gene analysis showed that isolates SR2 included into *Lactococcus lactis* strain with a bootstrap value of 100.

**Keywords:** *Bali cattle, Lactic acid bacteria, Molecular analysis, Rumen, SR2 isolate*

### **Introduction**

Bali cattle are known as cattle pioneer as one of the local cattle with its genetic characteristic that can live by utilizing forage that are less nutritious, not selective for the food and have a higher digestibility against fiber foods [1-3]. Besides, it is theoretically known that dietary factors contributes greatly to the changes in the gut flora [4]. Flora in the digestive tract are increasing in number and complexity along the canal and is estimated to be abundance up to 10<sup>12</sup> bacteria per gram of content of the gastrointestinal tract with an estimate not less than 500 species [4,5].

The results of conventional isolation and identification of the 15 samples of LAB from rumen fluid of Bali cattle managed to identify 20 strains of LAB. All 20 strains

were identified by the new limited phenotypic ally plastic test that is based on biochemical tests (test lollies) and Gram staining. Generally, the identification of a bacterium is based on phenotypically plastic testing, for more accurate identification that should be based on molecular testing. According to Hill and Jinn man, the detection of microorganisms preferably using molecular methods considering the high sensitivity and specificity [5].

The discovery of polymerase chain reaction (PCR) and DNA sequencing, bring major changes to do complete bacterial genome sequencing. Comparison of the genomes of bacterial species indicates that the 16S rRNA gene had a high similarity between species within the same genus, so it is used

as the gold standard in bacteria specification [6]. The use of molecular methods by analyzing the 16S rRNA gene has been widely and commonly used by researchers. With this method, it is possible to determine the genus and species of bacteria, even allows in determine the phylogenetic relationships and the discovery and classification of new bacterial species [6]. Based on the above background, the molecular study of SR2 LAB in the Bale cattle rumen as one of the 20 strains were identified to be interesting to be served.

## Materials and methods

### Isolate Cultivation

Cultivation of SR2 isolate is done by growing isolates in MRS broth media, followed by Gram staining and catalase test. Confirmed isolates were further identified molecularly.

### DNA isolation

SR2 LAB isolates, it is DNA genome isolated by culture centrifugation at 16,000 x g for 1 minute, the supernatant is discarded, and the cell pellet was added with 200  $\mu$ L of positive Gram buffer that has previously been filled with lysozyme enzymes (0.8 mg / 200  $\mu$ L). Once evenly mixed, was then incubated at 30 ° C for 30 minutes. To the sample was added 20  $\mu$ L of K proteins, plainly vortexes and incubated at 60°C for 10 minutes.

The next stage is addition of 200  $\mu$ L of GB buffer before returning and incubated at 70°C for 10 minutes. After this incubation stage, the sample is added to 200  $\mu$ L of absolute ethanol. After being vortexed, the sample was transferred to GD column that was placed on a new 2  $\mu$ L tube and centrifuged at 16,000 x g for 2 minutes. Container tube was replaced, and the column was added with 400  $\mu$ L of W1 buffer then centrifuged at 16,000 x g for 30 seconds.

The intercepted fluid was removed and the column was placed back on a new container tube, add 600  $\mu$ L of wash buffer that was previously added by ethanol and then centrifuged at 16,000 x g for 30 seconds. Centrifuging back for 3 minutes at 16,000 x g to dry the column matrix. Stages of dissolving DNA is done by addition of 100  $\mu$ L

Elution buffer (TE buffer) followed by centrifugation at 16,000 x g for 30 seconds. The intercepted DNA is stored at 4°C and aliquoted for storage, for a prolonged period should be stored in a -20°C freezer.

### 16s rRNA Gene Analysis

16S rRNA gene from SR2 isolates were amplified using B27F universal primers with the arrangement of 5-AGAGTTTGATCCTGGCTCAG-3 and U1492R with 5-GGTTACCTTGTTACGACTT-3 arrangement such as that are used by Khunajakr et al (2008) and Lim et al (2009) [7,8].

A total of 1  $\mu$ L product of genomic DNA was added to the PCR tube, then 25  $\mu$ L of Dream Tag Green added, 5  $\mu$ L of forward and reverse primer were added with a concentration of 10 pmol, and sterile water (demineralized water / deionized water) was added until the final volume was 40  $\mu$ L. The PCR reaction is programmed with step of single denaturation at a temperature of 94°C for 7 minutes followed by 35 cycles, each consisting of a stage of denaturation at a temperature of 94°C for 1 minutes, stage of attachment (annealing) at a temperature of 56°C for 1 minutes, and elongation stage (extension) at a temperature of 72°C for 1.5 minutes. An additional extension phase at the end of the cycle was carried out at a temperature of 72°C for 5 minutes.

### Electrophoresis with Agarose 1.5%

For the determination of PCR products, DNA separation (electrophoresis) is done with agarose gel stained by ethium bromide. A total of 1.5 grams of agarose is added with 100  $\mu$ L of TAE IX buffer, next step is to be heated to dissolve completely. After the gel is formed, the comb is removed and DNA product from PCR is inserted in the wells that have been available.

The gel is then transferred to the electrophoresis tank containing 1x TAE buffer. A total of 3  $\mu$ L PCR product and 1  $\mu$ L of loading buffer were mixed and added to existing gel wells. One of the wells is loaded with 2  $\mu$ L of marker as the standard measure of amplified DNA product, and then the electrophoresis is carried out at a voltage of 100 volts for 30 minutes. The bands thus

formed were viewed under UV light (trasilluminator) and then picture was taken.

### Sequencing

The PCR products were first purified using Micro Spin™ S-300 HR Columns (Amersham Pharmacia Biotech Inc.). Then was sequenced using the Big Dye Terminator Cycle Sequencing Kit, FS (Perkin-Elmer) with PRISM™ ABI 1330 DNA Sequencing System (PE Applied Biosystems).

### Alignment and Phylogenetic Analysis

The arrangement of 16S rRNA nucleotide homology was being aligned at NCBI (<http://www.ncbi.nlm.nih.gov/>) and analysis

of kinship (phylogenetic position) is made using the "Clustal W program package" with a 5.2 Mega program.

### Result and Discussion

#### Isolate cultivation

The results of cultivation of SR2 LAB isolates were conducted by growing isolates in MRS broth media in an anaerobic atmosphere, and showed the lush growth of bacteria which indicate that the optimal bacteria grow in anaerobic atmosphere as well as the characteristics of LAB bacteria. SR2 isolate staining results showed the shape like a short trunk (cocci) in purple that can be seen in Fig. 1.



Fig 1: Gram staining LAB SR2 isolates

Fig. 1 shows that the SR2 isolates with purple reflecting characteristic of Gram-positive bacteria. Hadioetomo (1985) mentions that the Gram-positive bacterial cell walls was thicker, when the shrinkage occurs during alcohol treatment for dehydration, causing the pores of the cell wall of Gram-positive bacteria that be shutdown so as to prevent the dissolution of the violet-iodine complex crystal in the bleaching step [9]. Other form of confirmation test such as catalase test showed SR2 isolates is negative. This result classifies SR2 isolates is included in the group of homofermentative LAB. SR2 isolates did not show any gas bubbles during catalase test. Bacteria that belong to the homofermentative is not able to form gas,

along with lactic acid among the fermentation products, the homofermentative group also produces carbon dioxide [10, 11].

#### 16S rRNA gene analysis

The results of analysis of the 16S rRNA gene of the SR2 isolates show that successfully amplification of 16S rRNA gene with a

length of 1502 bp PCR product using B27F and U1492R primers. Further analysis of the sequencing results showed genetic distance of isolates of SR2 with other strains in Genbank as table 1, as well as phylogenetic picture that can be seen in Fig. 2.

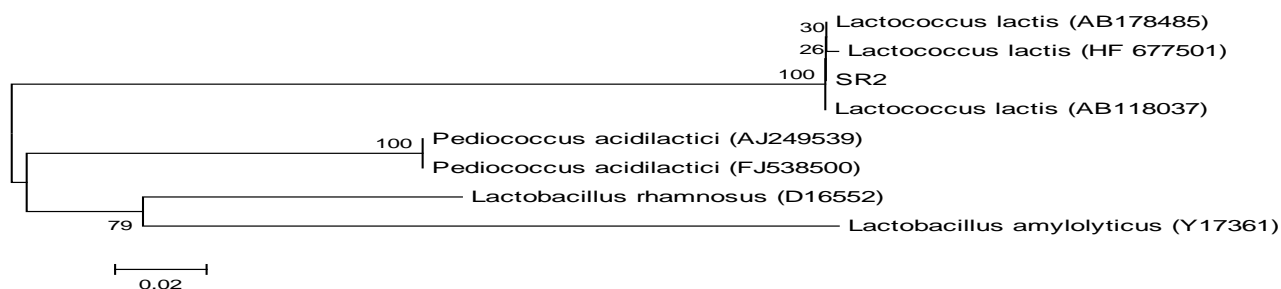


Fig 2: Phylogram constructed based on Neighbor Joining algorithm from 16S rRNA gene nucleotide of SR2 LAB to others isolates registered in Genbank.

**Table1: Gene distance and nucleotide number that is different between SR2 isolates and LAB strains deposited in Genbank.**

SR2	SR2	<i>Lactococcus lactis</i> (AB178485)	<i>Lactococcus lactis</i> (AB118037)	<i>Lactococcus lactis</i> (HF677501)	<i>Pediococcus acidilactici</i> (AJ249539)	<i>Pediococcus acidilactici</i> (FJ538500)	<i>Lactobacillus rhamnosus</i> (D16552)	<i>Lactobacillus amylolyticus</i> (Y17361)
<i>Lactococcus lactis</i> (AB178485)	0.000							
<i>Lactococcus lactis</i> (AB118037)	0.000	0.000						
<i>Lactococcus lactis</i> (HF677501)	0.002	0.002	0.002					
<i>Pediococcus acidilactici</i> (AJ249539)	0.268	0.268	0.268	0.271				
<i>Pediococcus acidilactici</i> (FJ538500)	0.268	0.268	0.268	0.271	0.000			
<i>Lactobacillus rhamnosus</i> (D16552)	0.278	0.278	0.278	0.282	0.175	0.175		
<i>Lactobacillus amylolyticus</i> (Y17361)	0.357	0.357	0.357	0.361	0.271	0.271	0.222	

The data in Fig. 2 shows that the SR2 strain LAB isolates showed a genetic similarity with the placement in one cluster with strain of *Lactococcus lactis* (AB 178 485), *Lactococcus lactis* (AB118037) and *Lactococcus lactis* (HF 677 501). Clustering is confirmed by the data in Table 1, which shows that there is no genetic distance between SR2 strains with the strain of *Lactococcus lactis* (AB 178 485), and *Lactococcus lactis* (AB118037) and there are only two nucleotides of 1000 nucleotides compared and different from the *Lactococcus lactis* strain (HF 677 501). If it is compared as similarity strain, the data in Table 1 can be interpreted that the SR2 strain has similarities about 100%, 100%, and 99.8% with the strain of *Lactococcus lactis* (AB 178 485), *Lactococcus lactis* (AB118037) and *Lactococcus lactis* (HF 677 501) respectively.

Referring to the concept of similarity of nucleotides or nucleotide differences between the nucleotides compared, it is known that if the similarity of nucleotides was more than 90% or has a nucleotide differences between 1 and 1.5% (14–22 bp), the nucleotides compared are categorized in same species [12]. Based on the concept of the SR2 strains of local isolates of LAB is referred to as *Lactococcus lactis*. Kim et al (1999) revealed that the *Lactococcus lactis* is a group of very important commercial

bacterium and has been used extensively in the production of milk-derived products, and as a food preservative such as nisin [13]. Application of these bacteria also recently expanded into the areas of health such as application as probiotics.

*L. lactis* as a probiotic can also be used as an additive for animal feed such as cattle. Santiananda et al found that administration of probiotics in animal feed can boost milk production in cattle. The addition of probiotics is to give a stimulatory effect on rumen bacteria that affect the increase in overhaul of lactic acid, resulting in the stabilization of ruminal pH, increased use of ammonia that is used to improve protein synthesis by microbes, increase microbial populations influence on increasing the digestibility of the fiber material so that the impact on the increase in feed consumption and substrate supply to the intestines that effect on increasing production response [14,15].

LAB from Bali cattle was different with LAB from cattle in other areas. The bacterial species difference may be caused by cattle diet and the type of cattle that affects the differences in the intestinal environment. According to research by Han et al, five types of LAB are found in cattle in Japan; that is *L. acetolerans*, *L. Pontis*, *L. casei*, *L.*

*Suebicus*, and *L. plantarum*. The research sample taken from silage, rumen fluid, and feces of cattle from there [16]. While the research conducted by Bolanle et al found five types of LAB in 30 cattles in Nigeria, namely: *Enterococcus hirae*, *E. Durans*, *E. faecium*, *E. faecalis*, *Weissella confuse* are types of bacteria that have antibacterial agen [17].

Bolanle et al found that *Enterococcus hirae*, *E. Durans*, *E. faecium*, *E. faecalis*, *Weissella confuse* can inhibit the growth of potentially pathogenic bacteria such as *E. coli* and *Klasiella Sp.* LAB can produce antimicrobial agents that use as strong antagonistic activity against various microorganisms, including pathogenic microorganisms and parasites. Metabolites such as organic acids, hydrogen peroxide, ethanol, diacetyl, acetaldehyde, acetone, carbon dioxide, reuterin, reutericyclin and bacteriocins are examples of microbacterial agent produced by LAB. Organic acids that produced by LAB is to reduce the pH levels and increase the production of hydrogen peroxide. This product is evidences of antibacterial activity against a variety of

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pathogenic microorganisms including gram-positive and gram-negative bacteria. But there is no evidence that the SR2 LAB isolates can manufacture the same products as *Enterococcus hirae*, *E. durans*, *E. faecium*, *E. Faecalis*, and *Weissella confuse* that can be used to inhibit the activity of pathogenic bacterias [17].

## Conclusion

Based on the results of molecular identification showed the strain of SR2 LAB isolates from Bali cattle rumen fluid belongs to the strain of *Lactococcus lactis*. These strains are predicted to have a commercial value that refers to a type of strain which has been first discovered by researchers.

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