Isolation and identification of lactic acid bacteria in hive of *Apis dorsata* from semi-arid tropical climate in Benu village, East Nusa Tenggara

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Abstract. Lactic Acid Bacteria (LAB) have been isolated from the hive of Giant Honey Bee (*Apis dorsata*) origin from tropical climate. Information about the diversity of LAB from the hive of *A. dorsata* from Benu Village has not been found yet. The information is important as a preliminary study of the potential of biological resources for food functional development in the future. Therefore, it is needed to research on diversity of LAB that isolated from the hive of *A. dorsata* from the village of Benu as an area of semi-arid tropical climate. The purpose of this study is determine the diversity of LAB isolated from the hive of *A. dorsata* from Benu Village. This research is important because it provides preliminary information about the diversity of LAB species from the hive of *A. dorsata* from Benu Village as a representation of semi-arid tropical climate. Profile of Denaturing Gradient Gel Electrophoresis showed the presence of seven LAB isolates from the hive of *A. dorsata* in Benu Village. This is an early indication that hive of *A. dorsata* from semi-arid tropic climate has the potential as a biological resources of LAB that important for food functional development in the future.

Keywords: lactic acid bacteria, *Apis dorsata*, semi-arid, tropical climate

1. Introduction
The Lactic acid bacteria (LAB) are Gram positive bacteria that produce lactic acid as the end product of carbohydrate fermentation [1]. The bacteria are also produce other compounds such as acetic acid, hydrogen peroxide, diacetyl and bacteriocins which have antagonistic power against pathogenic microbes. The benefits of LAB for human health include: increasing the body's immune system, overcoming lactose intolerance, reducing high blood pressure and improving the balance of abdominal microbiota [2]. These benefit makes honey as an important ingredient in the development of functional food products.
The benefits of honey are supported by the fact that honey is the result of natural processes that involve many components of the ecosystem, including microorganisms. The Lactic acid bacteria are group of microorganism that plays a role in the process of forming honey as an important functional food. The existence of these bacteria in the honeycomb is a description of the diversity of microorganisms that are involved in the process of honey formation. Therefore, knowing the diversity of bacteria is important to know its potential in the development of functional food products.

Timor Island has long been known as a producer of honey. *Apis dorsata* honey is the main honey product from this Island. Timor Island as a semi-arid area is thought to have an influence on the diversity of LAB in the hive bee of *A. dorsata*. Benu village is one of the villages in this area that produces honey. Information about the diversity of LAB from the hive of *A. dorsata* from Benu Village has not been found. Therefore, it is needed a research on diversity of LAB that isolated from the hive of *A. dorsata* from the village of Benu as an area of semi-arid tropical climate. The purpose of this study is determine the diversity of LAB isolated from the hive of *A. dorsata* in Benu Village.

2. Materials and methods

2.1 Sample collection

Hives of *A. dorsata* were collected from Benu Village in Kupang District, East Nusa Tenggara Province, Indonesia. The hives were collected randomly from three different trees and were subsequently mixed. The samples were stored in cool box and brought to laboratory.

2.2 Isolation of lactic acid bacteria

The samples (25 g) were suspended in 100 mL of 0.85% (w/v) NaCl (Merck, USA) and 100 µL aliquot of the suspension was spread on Mann, Rogosa and Sharpe agar (Merck, USA) supplemented with 1% of CaCO$_3$. The samples were incubated for 24 hours at 37°C under anaerobic conditions using Anaerobic Jars with Anaerocult A gas packs (Merck, Darmstadt, Germany). All colonies were scraped and dissolved in 500 µL nuclease free water and centrifuged at 12 000 g for 2 min. The supernatant was discarded and bacterial cells were collected for DNA extraction [3].

2.3 DNA extraction

Bacterial DNA was extracted with Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan). The DNA extraction procedure was carried out following the manual provided by the manufacturer. Gram positive buffer (200 µL) and lisozyme (200 µL) were added to the bacterial cells and incubated for 30 minutes at 37°C . Proteinase K (20 µL) were mixed to the cells suspension and incubated for 10 minutes at 60°C . After cooling, GB buffer (200 µL) were added to the cells suspension and incubated for 10 minutes at 70°C . Next step were DNA binding by absolute alcohol and washing by washing solution, then elution buffer were added to collect DNA. The purity of DNA were determined by Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA) [3].

2.4 Amplified 16S rRNA gene

The procedure was adapted from method as described by [3]. Bacterial DNA was amplified by PCR Applied Biosystem 2720 Thermal Cycler (Thermo Fisher Scientific, Massachusetts, USA). Amplification was performed with a universal primer for 16S rRNA gene that was hybridized with GC clamps [4]. DNA was amplified with primer 338F (ACTCCTACGGGAGGCAGCAG), 518R (ATTACCGCGCTGTGTCGAG) & 338F-GC (CGC CCG CCG CGC GGC GGG GGG GGG GGG GG GACG GGG GCG GCG GCG GCA GCA). PCR reaction contained 25 µL of final solution consisting of: 1.25 µL of 10 pmol of each primer, 12.5 µL Go Taq Green Mastermix 2x (Promega, Madison, USA), 2.5 µL DNA template and 7.5 µL nuclease free water (NFW). Amplification by PCR comprised 35 cycles of pre-denaturation at 94°C for 5 minutes, denaturation at 92°C for 30 seconds, annealing at 58°C for 30 seconds, elongation at 72°C for 45 seconds, and post-elongation at 72°C for 3 minutes. The PCR products were migrated on
1% agarose gel and stained with ethidium bromide 0.1% for visualization in G: BOX Gel Documentation (Syngene, Frederick, USA).

2.5 Denaturing gradient gel electrophoresis
The electrophoresis process was performed on D Universal Mutation Detection System (Bio-Rad, California, USA) at 60°C, 150 volt for 5 hours. A total of 20 µl of DNA and 5 µl of loading dye were migrated on 8 % polyacrylamide gel containing a denaturing gradient from 30-70%. The polyacrylamide gel was stained with ethidium bromide 0.1% for 15 minutes, then visualized in G: BOX Gel Documentation (Syngene, Frederick, USA). DNA bands on polyacrylamide gel were cut and eluted with 100 µl nuclease free water, which were then amplified with PCR TI-Thermocycler (Biometra, Goettingen, DE) using the same primers (338F and 518R) but without GC clamps [3, 5, 6].

3. Results
The hives of *Apis dorsata* was collected from Ceiba pentandra (L) Gaertn trees in Benu Village, sub-district of Takari, Kupang district, East Nusa Tenggara, Indonesia. Sample was split into B (fresh hives) and L (old hives) with different template (1 and 2). Code 1 was 0.5 µL of template. Code 2 was 1.0 µL of template. Result of electrophoresis on agarose gel showed DNA bands 180 base pairs (figure 1). This result indicated that the bacteria DNA has been amplified correctly by PCR method.

There were seven of DNA bands in DGGE profile (figure 2). Seven lines of DNA bands indicated seven species of lactic acid bacteria or seven OTU (Operational Taxonomy Unit). DNA bands of sample varies between hives. Sample B had more DNA bands than sample L. Sample B and sample L have DNA bands in lines 1, 2 and 7. The pattern of DNA bands in line 5 were only found in sample L but DNA bands in line 4 were only found in sample B. Sample L and sample B contained 6 lines of DNA bands with different kinds of lines.

![Figure 1](image.png)

**Figure 1.** Electrophoresis results after PCR with primers 338F-GC and 518R on agarose. All DNA bands have a size of 180 base pairs. Lad = Ladder (marker of DNA size). L = the old bee hive of *Apis dorsata*; B = the fresh bee hive of *Apis dorsata*; 1 and 2 state the difference in the number of DNA template (0.5 and 1 µL).
Figure 2. DGGE profile, there are 7 OTU of lactic acid bacteria in the hive bee of Apis dorsata from Benu Village. L = the hive bee of Apis dorsata from tree randu 1; B = the hive bee of Apis dorsata from Randu 2 tree.

4. Discussion
The hives of *A. dorsata* was collected from Ceiba pentandra (L) Gaertn trees in Benu Village, sub-district of Takari, Kupang district, East Nusa Tenggara, Indonesia. Trees of *C. pentandra*, grow high up to 70 m with horizontal branches, are compatible for *A. dorsata* to make the hives. Benu Village, a semi-arid tropical climate and Grassland is the dominant vegetation in Benu Village. Environment of the hives of *A. dorsata* were less support for honey bee (*A. dorsata*) to find flowers as sources of nectar and pollen for their feeding. This condition make honey bee working harder to collect nectar and pollen. Nectar and pollen are collected by honey bee from flowers that contain small amount of lactic acid bacteria. Lactic acid bacteria that isolated from the hives of *A. dorsata* have the unique character because they live in tropical savanna climate. Diversity of lactic acid bacteria from *A. dorsata* hives in Benu Village were different than others.

In this research found seven OTU of lactic acid bacteria from *A. dorsata* hives in Benu Village. This result was supported by Karyawati *et al.* [3] that found seven OTU of lactic acid bacteria from *A. dorsata* hives in sub-districts of Central Amfoang, South Amfoang and Central Fatuleu, Kupang District, East Nusa Tenggara (Indonesia). The seven OTU have closely related to *Lactobacillus kunkeei* YH-15 and *Lactococcus lactis* [3].

Molecular approaches can be used to analyse the genetic diversity of a complex microbial population. Analysis of microbial diversity was carried out using molecular biology techniques such as Denaturing Gradient Gel Electrophoresis (DGGE). The existence of lactic acid bacteria in the hives of *A. dorsata* have implications for future of health of honeybees colony and have relevance to human health as probiotics and functional food [7, 8]. The existence of LAB in bee hive from this area is an opportunity to obtain honey products that have benefit as probiotics. The opportunity is based on reports from previous studies that honeybees and their products are rich in sources of probiotics [9, 10]. It is very clear, this research is an initial activity that reveals bee hive in this area as a resource of functional food development.
5. Conclusion
Denaturing Gradient Gel Electrophoresis profile showed the presence of seven isolates of LAB from the hive of *A. dorsata* from the village of Benu. This indicated that hive of *A. dorsata* from semi arid tropical climate has potential as a biological resources of LAB.

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