Biodiversity of lactic acid bacteria of wild honey bee and Sumbawa horse milk by using RAPD-PCR

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Abstract. Wild honey bee and Sumbawa horse milk is the local product from West Nusa Tenggara, Indonesia, which has the characteristic of little sour in taste. The sour taste indicates lactic acid bacteria (LAB) existence that has a potential function as probiotics. The objective of this study is analysis of lactic acid bacteria biodiversity from wild honey bee and Sumbawa horse milk by using Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR). The genome of cultured samples from M17 and MRS medium were extracted, then identified by RAPD-PCR with primer (GTG)5 (5'- GTGGTGGTGGTGGTG -3). The scoring of DNA fragments was analyzed using the Numerical Taxonomy and Multivariate Analysis System (NTSYS) application. The result show five clusters from a common ancestor indicated the broad diversity of LAB in wild honey bee and Sumbawa horse milk.

1. Introduction

Honey is one of the nontimber forest natural resources used in Indonesia [1]. Wild honey bee and Sumbawa horse milk has become an icon in West Nusa Tenggara as it is one of the places producing forest honey and horse milk in Indonesia. Honey and milk has high nutrition facts and antioxidants. Several studies performed that honey has anti-inflammatory and antibacterial activity [2]. This antioxidant effect of honey is from enzymes, such as catalase, peroxidase, and glucose oxidase [3] and non-enzymes, e.g. phenolic constituents, organic acids, flavonoids, nitric oxide (NO) metabolites, carotenoid-derived compounds, proteins, and amino acids [4,5]. Honey also consisted of several vitamins, especially vitamin C and B complex with minerals, such as copper, calcium, magnesium, iron, phosphorus, manganese, phosphorus, zinc, and potassium [6]. In addition, honey has antibacterial capability from lactic acid bacteria which can suppress pathogen growth [7]. Antimicrobial substances produced by LAB are bacteriocins, diacetyl, hydrogen peroxide, lactic acid, and bacteriocin-like inhibitory substances [8].

Generally, the production of honey is from three kinds of honeybees. They are Apis cerana (local bee), Apis dorsata (wild bee), dan Apis mellifera (Europe bee) [9]. Honeybee species of Apis dorsata is utilized often in Sumbawa. The honey produced by this species is wild honey propagated in some
locations in Sumbawa, which are Kelungkung, Pelat, and Sempe village. This wild honey has a viscous texture, black colour, sweet but little sour in taste. LAB species of wild honey from Sumbawa are not widely known so that it requires a specific method to identify and characterize its LAB. Previously, detection of LAB used phenotype methods such as cell morphology, carbohydrate substrate differentiation, and growth average in various temperatures. Lack of reproducibility of those phenotype methods is the main issue because the result of one sample cannot be reproduced by others and biochemistry technique can not distinguish lactobacilli with similar morphology [10]. Recently, development in molecular was significant, especially identification technique and species characterization. RAPD-PCR is one of the methods to identify DNA polymorphism in genom sensitively and efficiently. The basis of this method is by using some primers to multiply the genome on the unidentified locus, which is a complement to primers used. Then, gel electrophoresis was used to detect and confirm the amplification band. The profiles of RAPD-PCR can be used to differentiate strains from Lactobacillus strains [11-13].

The objection of this research was to study lactic acid bacteria biodiversity from wild honey and Sumbawa horse milk using the RAPD-PCR method.

2. Materials and methods

2.1. Sample collection
Samples were collected from Kelungkung, Sempe, Pelat Village in Sumbawa, West Nusa Tenggara, Indonesia for 13 days, then were sent to laboratory in one day delivery on icebox to keep samples fresh and safe in good condition until observation. Horse milk samples were acquired from Penyaring Village and the isolates obtained from previous study [14] was used as positive control.

2.2. Isolation of Lactic acid bacteria
Isolation of LAB was using Awan’s method [15] with minor modification. Samples were cultured into M17 and the Mann Rogosa Sharp (MRS) liquid medium in the reaction tube. Plates were incubated at 30°C (M17) and 37°C (MRS) for 24 h, respectively. Next, the grown culture aliquot was objected to serial dilution (10⁻¹, 10⁻², 10⁻³) with a total volume of 100μL into the microtube. After that, the aliquot was spread onto a solid medium of M17 and MRS then incubated for 24 hours.

2.3. Isolation of genomic DNA
The isolation method of genomic DNA was done by Mustopa and Fatimah’s method with minor modification [16]. A single colony was pre-cultured into 5 mL of M17 and MRS liquid medium by resuspension then incubated for 24 hours. Then, cells were harvested by centrifugation at 6000 rpm at 4°C for 10 minutes. The supernatant was discarded, while the pellet was resuspended with TE buffer, added by 40μL lysozyme (60 mg/mL), and incubated at 37°C for an hour. Next, the mixture was added with 200μL SDS 10%, 100μL NaCl 5 M, 80 μL CTAB 10% and incubated at 68°C for 30 minutes. Chloroform was added at the same amount of the mixture and then centrifuged at 13000 rpm for 15 minutes at 4°C. Supernatant then was transferred into new tubes and was added with 1 mL ethanol, incubated at -20°C for 2 hours, and then centrifuged at 13000 rpm, 4°C for 10 minutes. The pellet then dried overnight. The DNA was dissolved by using ddH2O and ribonuclease free water.

2.4. RAPD-PCR
The RAPD-PCR was performed using a modified method of Chao et al. [17]. The solution used in PCR mixing were ddH2O 6.4μL, MyTaq 2μL, forward primer 0.2μL, reverse primer 0.2μL, DNA Taq Pol 0.2μL, and DNA template 1μL with 10μL in total volume. The primer used in PCR was (GTG)5 (5'-GTGTGGTGGTGGTG-3). Running PCR comprised of one cycle at 95°C for 7 minutes, 30 cycles at 95° for 1 minute, 55° for 1 minute, 65°C for 8 minutes, and one cycle at 65°C for 16 minutes. The PCR
products were run on 2% agarose gel electrophoresis with 1x TE buffer solution. The staining of the gel used ethidium bromide and DNA visualization used UV transluminator.

2.5. Similarity analysis
Electrophoresis gel photo resulted from RAPD-PCR was obtained to do table scoring by encoding “occurred DNA band” by number 1 and “non-occurred DNA band” by number 0. The scoring result was analyzed using the Numerical Taxonomy and Multivariate Analysis System (NTSYS) application with excel data to have dendogram and cluster results.

3. Results and discussion

3.1. Isolation of LAB
Honey samples were from three different locations in Sumbawa. They were Kelungkung, Pelat, and Sempe Village. The honey specimens have similar color, concentration, and texture. While horse milk samples were from Penyaring Village obtained by Kusdianawati et al. [14]. Those villages were chosen after collecting information about where the wild honey bee and horse milk were produced and utilized.

The samples of honey and horse milk were cultured in M17 and MRS medium both solid and liquid. The aim in this step was to get as much as LAB. Therefore, two mediums were used, de Mann Rogosa Sharp (MRS) and M17, because those mediums were specific mediums to culture LAB [18]. The M17 medium is recommended as the medium for the enumeration and growth and enumeration of lactic streptococci and their bacteriophages, while MRS Agar and Broth were used to grow the ‘lactic acid bacteria’ with species of Lactobacillus, Pediococcus, Streptococcus, and Leuconostoc.

Based on the growing results of cultured samples, only samples from Kelungkung village could grow at solid M17 and MRS medium with a total colony of 73. Samples from Pelat and Sempe Village could not grow in both M17 and MRS medium. It might because the concentration of LAB in the samples was too low or even zero level when collected and delivered. However, from these 73 colonies, only 14 could grow in a liquid medium. For this reason, only 14 colonies were taken for further analysis, the genomic isolation.

Table 1 disclosed total samples obtained from the medium in more detail. It was 12 colonies obtained from M17 medium and 2 colonies of MRS medium. For positive control, this study used LAB isolates from wild horse milk Sumbawa with a total sample of 10 and nuclease-free water as its negative control. Some LAB isolates used in this study were from laboratory collection.

The living cells of honey in various areas consisted of beneficial and pathogenic species. Honey had intrinsic condition that made harsh for microorganism to grow. Microorganism could grow in that condition were from microbial contamination sources either primary or secondary. Bees transferred some microorganism from the digestive tract into the nectar during the process to produce honey. The bacteria included: Bacillus spp., Lactobacillus rigidus, Clostridium spp., Streptococcus spp., and Gram-negative bacteria, Citrobacter spp., Flavobacterium spp., Enterobacter spp., Proteus spp., Achromobacter spp., Pseudomonas spp. and Klebsiella spp. [19-21]. Some other reports presented that living cells total number of aerobic bacteria in a gram honey varies from zero to tens per thousand [22-25]. These previous studies are in agreement with the results in this report.

3.2. Isolation of Genomic DNA
The cultures grown in M17 and MRS medium were isolated manually using the modified method of Mustopa and Fatimah [16] to obtain LAB genomic DNA. Isolation of genomic DNA was an important step to collect DNA samples from LAB in honey and in horse milk. This study has successfully isolated LAB genomic DNA from 14 single colonies in honey and 10 single colonies in horse milk. The isolated genomic DNA then stored at -20°C to prevent degradation.
Table 1. LAB isolates code of wild honey from Sumbawa and control.

| Number | Isolate code | Control/sample | Origin                  |
|--------|--------------|----------------|-------------------------|
| 1      | Lac 3 (L. lactis strain NM141-1) | Positive Control | Laboratory collection |
| 2      | Lactis InaCC | Positive Control | Laboratory collection |
| 3      | SKP K.3 (Weissella confusa) | Positive Control | Penyaring Village       |
| 4      | MRS SKP K.4 (L. fermentum) | Positive Control | Penyaring Village       |
| 5      | MRS SKP K.5 (L. garvieae) | Positive Control | Penyaring Village       |
| 6      | M17L 22 PR   | Positive Control | Laboratory collection |
| 7      | M17G 6       | Positive Control | Laboratory collection |
| 8      | KL M17 1     | Sample          | Kelungkung Village      |
| 9      | KL M17 2     | Sample          | Kelungkung Village      |
| 10     | KL M17 3     | Sample          | Kelungkung Village      |
| 11     | KL M17 4     | Sample          | Kelungkung Village      |
| 12     | KL M17 5     | Sample          | Kelungkung Village      |
| 13     | KL M17 6     | Sample          | Kelungkung Village      |
| 14     | KL M17 7     | Sample          | Kelungkung Village      |
| 15     | KL M17 9     | Sample          | Kelungkung Village      |
| 16     | KL M17 11    | Sample          | Kelungkung Village      |
| 17     | KL M17 12    | Sample          | Kelungkung Village      |
| 18     | KL M17 13    | Sample          | Kelungkung Village      |
| 19     | KL M17 16    | Sample          | Kelungkung Village      |
| 20     | KL MRS 10    | Sample          | Kelungkung Village      |
| 21     | KL MRS 11    | Sample          | Kelungkung Village      |
| 22     | M17G LG 1    | Positive Control | Laboratory collection |
| 23     | M17G LG 5    | Positive Control | Laboratory collection |
| 24     | M17G LG 7    | Positive Control | Laboratory collection |
| 25     | Nuclease Free Water | Negative Control | -                       |

3.3. RAPD-PCR

The isolated DNA genomic was identified later by seeing an amplification of primer (GTG)5 using random amplified polymorphic DNA. The aim was to recognize genotype diversity that expressed different phenotypes. Those genomic DNA from honey were then analyzed together with genomic DNA from horse milk and laboratory collection. The RAPD-PCR analysis indicated band profile of DNA with high variation. Figure 1 performed various band sizes from LAB of Sumbawa wild honey.
Visualization of RAPD-PCR by applying (GTG)\textsubscript{5} primer showed different genetic profiles which pointed the presence of DNA polymorphism from 25 isolates with codes 8 to 21 were from Sumbawa wild honey. Samples with code 8, 11, 13, 15, 16, 20, and 21 performed similar profile compared with code number 9, 10, 12, 14, 17, 18, 19 which showed different band size indicating not closely related. Isolates from Sumbawa horse milk previously acquired by Kusdianawati et al. [14]. Interestingly, the source of both wild honey and horse milk were very different. The horse milk originated from dairy product while the wild honey samples originated from non-timber forest product, but both of the products contained LAB.

3.4. Similarity Analysis

Amplification profile of RAPD-PCR was further analyzed to obtain dendogram. LAB diversity of dendogram was displayed on Figure 2, there were 5 clusters indicated different species. Cluster I and II consisted of 7 LAB isolates, 3 of them were from Kelungkung village encoded 10, 14, and 17. Cluster III contained 11 LAB isolates originated from Kelungkung village encoded 8, 9, 11, 12, 13, 15, 16, 18, and 19 of M17 medium and encoded 20 and 21 of MRS medium. Cluster IV and V consisted of 6 isolates were LAB isolated from Sumbawa horse milk as positive control. Based on dendogram, clusters 1 and 2 encoded 1, 5, 6, and 3 of positive control was closely related to bacteria isolates from wild honey bee encoded 17, 10, and 14. The similarity percentage between isolate number 17 from wild honey bee and number 6 from horse milk of Cluster 1 was 57%. Previously, it was known that the isolate number 3 was detected as Weissella confusa species [14]. Most of the bacterial isolates originated from Sumbawa wild honey were included in Cluster III. There was no positive control in this cluster indicated that bacteria in Cluster III had a distant relationship compared to the positive control. Cluster IV and IV were occupied with positive control isolates. It meant that isolates of Sumbawa wild honey bee with code number 2, 4, 7, 22, 23, and 24, were distantly related.
Figure 2. Dendrogram of 25 samples. Cluster I consisted of isolates number 1, 5, 6, and 17. Cluster II consisted of isolates number 3, 10, and 14. Cluster III consisted of isolates number 8, 9, 11, 13, 15, 16, 20, 21, 19, 18, and 12. Cluster IV consisted of isolates number 2 and 4. Cluster V consisted of isolates number 7, 22, and 23.

The dendogram indicated quite broad diversity based on the RAPD-PCR profile. There was a group of isolates from wild honey bees only, and there was also a group with positive control of horse milk. However, there was a cluster consist of LAB isolates from wild honey bee and horse milk that showed a close genetic relationship.

4. Conclusion
LAB biodiversity from wild honey bee and Sumbawa horse milk have been studied by using the RAPD-PCR method and continued by dendogram resulting in diverse amplification profiles and five Clusters from a common ancestor indicated a quite wide range of biodiversity.

Acknowledgments
The authors would like to thank to Research Center of Biotechnology, Indonesia Institute of Science for giving permission to do research activity in its laboratory and providing materials support. Also, for the valuable information from local community to finish this entire experiments.

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