

PAPER • OPEN ACCESS

Diversity of lactic acid bacteria isolated from fermented mare's milk products based on PCR-RFLP analysis

To cite this article: A I Mulyawati *et al* 2019 *IOP Conf. Ser.: Earth Environ. Sci.* **230** 012104

View the [article online](#) for updates and enhancements.

Diversity of lactic acid bacteria isolated from fermented mare's milk products based on PCR-RFLP analysis

A I Mulyawati, Y D Jatmiko, I Mustafa, T Ardyati and Suharjono

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia

E-mail: jatmiko_yd@ub.ac.id

Abstract. This study aimed to characterize the diversity of lactic acid bacteria (LAB) isolated from fermented mare's milk based on PCR-RFLP. The LAB was isolated from three fermented mare's milk products from three different regions i.e Dompu, Bima, and Sumbawa, Province of West Nusa Tenggara, Indonesia. Fermented milk samples (25 mL) was diluted into 225 mL of saline solution and spread plated onto MRS and M17 agar containing 1 % CaCO_3 , then incubated at 37 °C for 48 hours. Single colonies showing a clear zone were randomly selected and purified. The DNA was extracted by the heat-treatment method and amplified using an internal transcribed spacer (ITS) and 5.8S region spacer primers. The amplicons of the 5.8S region spacer were used for restriction fragment length polymorphism (RFLP) analysis using *Hae*III and *Hind*III enzymes. The DNA bands were characterized to obtain the LAB clusters. The representative of each LAB cluster was amplified using 16S rDNA primer then sequenced. Total of 41 isolates was grouped into 10 clusters based on the PCR-RFLP analysis. However, only three clusters were identified as LAB from *Lactobacillus* genus. The *Lactobacillus* species obtained were *L. plantarum* and two strains of *L. rhamnosus*. Four clusters were identified as *Staphylococcus* genus and three others were *Ochrobactrum* genus. Thus, the LAB isolates from fermented mare milk obtained were the member of *Lactobacillus* genus. However, the Gram-negative bacteria and *Staphylococcus* were still found in the fermented products indicating the poor quality of the products. Therefore, a good manufacturing practice should be implemented during the product preparation.

1. Introduction

The Fermented mare's milk is a popular traditional fermented milk product from Province of West Nusa Tenggara, Indonesia. It provides many benefits, especially on health aspects. The consumption of the traditional mare's milk could heal bronchitis, pneumonia, typhus, and decrease the cholesterol level and hypertension [1]. Fermentative microbes have played a pivotal role in preserving the fermented mare's milk product. Those microbes include lactic acid bacteria, acetic acid bacteria and yeasts. Lactic acid bacteria (LAB) are a Gram-positive bacteria, negative catalase, and produce lactic acid as the main product from carbohydrate fermentation [1]. It is a group of fermentation bacteria that provide health benefits such as the probiotic, anti-microbes, and biopreservative agent. In the previous study, *Lactobacillus brevis*, *Lactobacillus acidophilus*, and *Lactobacillus rhamnosus* that has a good potential as the probiotic agents were found in the fermented mare's milk [2]. It is clearly known that the LAB morphological, biochemical, and physiological characters (phenotypic) are difficult to be



distinguished. Thus, the study on LAB diversity from the fermented mare's milk based on its phenotypic characters is difficult to be done. However, the development of molecular characterization based on the genotypic characters can be used as the solution to do the LAB diversity analysis.

The diversity analysis method based on the genotypic characters using a molecular approach provides higher accuracy result and faster than the conventional method based on the phenotypic characters [3]. One of the methods used for the genotype-based characterization is polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis and then confirmed with 16S rDNA sequencing [4]. The PCR-RFLP analysis is a polymorphism characterization method by comparing the DNA bands from the amplicon digested using certain restriction enzymes [5]. Thus, this research aimed to evaluate the diversity of LAB isolated from fermented mare's milk products based on the PCR-RFLP analysis.

2. Materials and Methods

2.1. Bacterial isolation

Fermented mare's milk products were purchased from three different suppliers located at Bima, Dompu, and Sumbawa, Province of West Nusa Tenggara, Indonesia. Milk samples (25 mL) were diluted into sterile saline water (NaCl 0.85 %). Each sample was diluted into six dilutions (10^{-1} – 10^{-6}). The suspension (0.1 mL) was pour plated onto de Man Rogosa Sharpe (MRS) agar (Merck, Darmstadt, Germany) and M17 agar (Merck, Darmstadt, Germany) medium containing 1 % CaCO_3 then incubated on 37 °C for 48 hours [6]. Bacterial colonies that showed a clear zone around the colonies were assumed as the lactic acid bacteria. Colonies were morphologically characterized and enumerated. Colonies that showed different morphological characters were sub-cultured and purified to obtain a pure isolate. The LAB isolates were characterized based on its phenotypic characters such as cellular morphology using Gram staining and biochemistry (catalase test). The isolates that confirmed as Gram-positive and catalase negative were used for further analysis.

2.2. Genotypic characterisation

2.2.1. DNA extraction

The DNA extraction method used was the heat-treatment method [7]. One loop-full of the isolate was suspended into 200 μL of sterile ddH₂O in 1.5 mL of microcentrifuge tube. The bacterial suspension was homogenized using vortex (Scientific Industries, New York, USA) for one minute. It was then heated in the water bath at 95 °C for 20 minutes as the heat-treatment method to extract the DNA from the cell [7]. The suspension was centrifuged at $10,000\times g$ for 5 minutes at room temperature. The supernatant ($< 170 \mu\text{L}$) was transferred into a new microtube as the DNA template and stored at -20 °C.

2.2.2. Internal transcribed spacer (ITS) and spacer region 5.8S amplification

The amplification of ITS and 5.8S region was conducted by polymerase chain reaction (PCR) method. The primers used to amplify ITS and 5.8S region were designed by Rachman et al. [8]. The ITS primers used were R139-f (5'-TTG TAC ACA CCG CCC GTC-3') and 23S/p7-r (5'-GGT ACT TAG ATG TTT CAG TTC-3') while the 5.8S primers were tAla-f (5'- TAG CTC AGC TGG GAG AGC-3') and 23S/p10-r (5'-CCT TTC CCT CAC GGT ACT G-3') [8]. The PCR mixture reaction was containing 12.5 μL of GoTaq®Green Master mix (2x) (Promega, Wisconsin, USA), 1 μL of forward primer (10 pmol), 1 μL of reverse primer (10 pmol), 9.5 μL of nuclease-free water, and 1 μL of DNA template. The PCR procedure was started with initial denaturation at 94 °C for 5 minutes then continued with total 35 cycles of denaturation (94 °C; 30 s), annealing (53 °C for ITS primers and 56 °C for 5.8S primers; each for 60 s), and elongation (72 °C; 90 s) and finalized with final elongation at 72 °C for 5 minutes. The products were visualized using agarose gel electrophoresis. The agarose concentration used was 1.5 % using TBE buffer 1x and 1 μL of EtBr. The electrophoresis was run for 1 hour with the 100 V. The DNA bands were visualized under UV Transilluminator (Vilber Lourmat, Deutschland, Germany).

2.2.3. PCR-RFLP

The amplicons of 5.8S from each isolate was used as the samples for RFLP analysis. The PCR-RFLP mixture reaction was containing 9 µL of amplicons, 0.5 µL of enzyme buffer (Tango Buffer, Thermo Fisher Scientific, Walhatam, USA), and 0.5 µL of restriction enzymes. The restriction enzymes used in this study were *Hae*III and *Hind*III (Promega, Wisconsin, USA). The mixture was then incubated at 37 °C for 3 hours. Afterwards, the PCR-RFLP products were qualitatively analyzed using agarose gel electrophoresis with the same method as mentioned in the section 2.2.2. The all bacterial isolates were clustered based on the pattern of DNA bands resulted.

2.3. Bacterial identification

One bacterial isolate was selected represented each cluster. The DNA of selected isolates was extracted using DNA extraction mini kit (Geneaid, New Taipei City, Taiwan) according to manufacture's instruction. The DNA was amplified using 16S rDNA universal primers i.e 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-GGT TAC CTT CTT ACG ACT T-3') [9]. The PCR reaction conducted was similar to the method mentioned previously (section 2.2.2) except the annealing temperature was 55 °C. The 16S rDNA amplicon was confirmed using agarose gel electrophoresis (1.5 %). The amplicons were sent for sequencing at 1st BASE DNA Sequencing Service, Malaysia. Afterwards, the sequences were used to identify the bacteria using BLAST nucleotides from NCBI. The identified isolates were compared to the reference sequences from the GenBank database to construct the phylogeny tree using MEGA 6 software for windows based on Neighbour-Joining method and Tamura-Nei model.

3. Results and Discussion

3.1. Bacterial isolation

The total number of isolates obtained from this study was 41 isolates (18 isolates from Dompu; 10 isolates from Sumbawa; and 13 isolates from Bima). Each isolate was confirmed as Gram-positive bacteria and catalase negative (Table 1). The morphology of colonies were similar which was circular-shaped, entire-margin, convex-elevation, and creamy white. However, its size varied from 0.5-3.5 mm. The bacteria cell of group I, III, V, VII, VIII, and IX were rod-shape while group II, IV, VI, X were coccus.

Table 1. Phenotypic and genotypic characters of bacteria isolated from fermented mare's milk.

Group	Total Isolates	Sample	Phenotypic			Genotypic (PCR Analysis)		Genotypic (PCR-RFLP of 5.8S analysis)		Identity	Similarity (%)
			Gram	Cell shape	Catalase Activity	ITS (bp)	5.8S (bp)	HaeIII (bp)	HindIII (bp)		
I	1	Bima	+	Rod	-	750, 550	800	530, 270	390, 250, 200	<i>Lactobacillus rhamnosus</i>	97
II	2	Dompu	+	Coccus	-	780, 700, 600	800	800	800	<i>Staphylococcus epidermidis</i>	98
III	5	Dompu	+	Rod	-	800, 750, 590	780	780	780	<i>Lactobacillus rhamnosus</i>	98
IV	1	Dompu	+	Coccus	-	900, 880, 700	850	850	850	<i>Staphylococcus aureus</i>	98
V	4	Bima	+	Rod	-	1200, 750, 550	1000, 800	500, 550	950, 300, 250, 200	<i>Ochrobactrum</i> sp.	82
VI	1	Bima	+	Coccus	-	1200, 550	1000, 800	500, 550	1000, 300, 250, 200	<i>Staphylococcus aureus</i>	85
VII	10	Sumba wa	+	Rod	-	1200	1000	750, 250	1000	<i>Ochrobactrum oryzae</i>	98
VIII	10	Dompu	+	Rod	-	800, 750	700	700	700	<i>Lactobacillus plantarum</i>	75
IX	1	Bima	+	Rod	-	1200, 750, 550	1000, 800	550	800	<i>Ochrobactrum</i> sp.	91
X	6	Bima	+	Coccus	-	750, 550	790	590, 100	390, 250, 150	<i>Staphylococcus hominis</i>	97

3.2. Genotypic characterisation

PCR-RFLP analysis based on ITS and 5.8S spacer region has successfully grouped 41 isolates into 10 clusters (Table 1). The DNA bands pattern of ITS and 5.8S amplicon can be seen in Figure 1. The DNA bands of ITS region were varied from two or three bands of each group with the total base pairs can be seen in Table 1. The DNA bands result from ITS region and 5.8S region were clearly different. Rachman et al. [8] found that the primers coded for ITS region detection were resulted on more than one DNA bands can be produced. Some groups only have single band and the others have two bands. After the digestion reaction with *HaeIII* and *HindIII* enzymes, some isolates were shown different bands patterns (Figure 2). It can be clearly seen that the DNA bands pattern and their size of the 10 selected isolates were mostly different after digestion. It was already reported that PCR-RFLP analysis on LAB using *TaqI* and *HinfI* restriction enzymes can also be used to differentiate species by producing different DNA bands fragments or pattern [8].

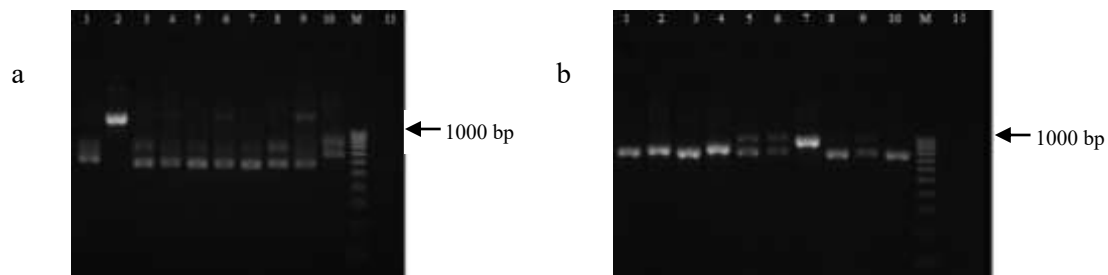


Figure 1. PCR analysis of ITS region (a) and 5.8S spacer region (b): 1-10 (Group I-X), 11 (sterile ddH₂O), M (DNA ladder 100 bp).

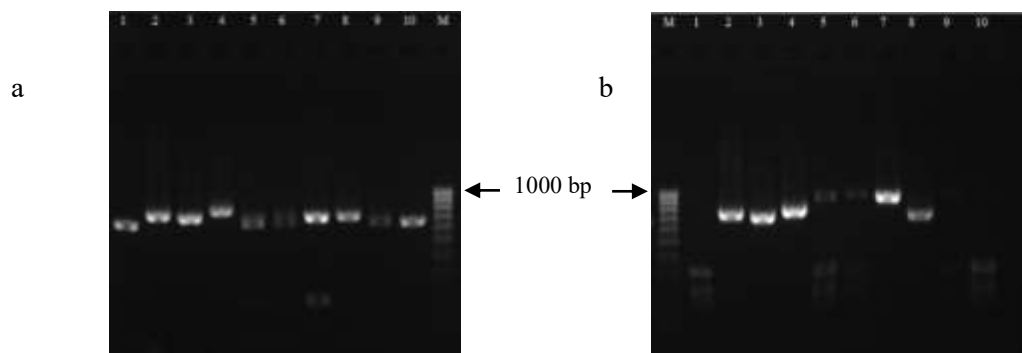


Figure 2. PCR-RFLP analysis of 5.8S spacer region: (a) *Hae*III and (b) *Hind*III: Lane 1-10 (Group I-X), M (DNA ladder 100 bp).

3.3. Bacterial identification

Among 10 groups, there were only three different genera identified as *Lactobacillus*, *Ochrobactrum*, and *Staphylococcus*. The lactic acid bacteria found in this study was *Lactobacillus* genus. Group VII was identified as *Ochrobactrum* genus (Table 1, Figure 3a) and Group II, IV, and X were identified as *Staphylococcus* genus (Table 1, Figure 3b). There were three groups identified as *Lactobacillus* genus i.e Group I, III, and VIII (Table 1, Figure 4). It can be seen that *Lactobacillus rhamnosus* found in this study was divided into two different strains based on its PCR-RFLP results. The phylogenetic tree of isolates coded BB3, BB4, and BB8 represented for group IX, V, and VI could not be constructed due to the low similarity (under 90 %) obtained when it was aligned with reference sequences. However, group IX and V were identified as *Ochrobactrum* genus with the similarity of 83 % and 82 %, respectively, while group XI was identified as *Staphylococcus* genus with the similarity of 75 %.

The fermented mare's milk products used as the samples in this study were derived from three different producer areas i.e Bima, Dompu, and Sumbawa. The diversity of LAB from three samples was different. This variation may be caused by the different nutrition contained in the samples especially the lactose. Mare's milk contains more lactose than cow's milk [10]. The lactose can be utilized by the LAB during the lactic acid fermentation to produce lactic acid [10]. The storage condition and packaging process during production can also influence the number of LAB that present. It can be noticed that the LAB species were only be found in Bima and Dompu samples. The different strains of *Lactobacillus rhamnosus* can be found on Bima and Dompu samples. However, the total isolates of *L. rhamnosus* isolated from Dompu sample were higher (5 isolates) than Bima sample (1 isolate). The *Staphylococcus epidermidis* and *S. aureus* can be found on Dompu sample while *S. pasteurii* and *S. hominis* can be found in Bima sample. The genus of *Ochrobactrum* bacteria was also can be found from Bima sample. Meanwhile, the *Ochrobactrum oryzae* was the only bacteria that can be found in Sumbawa sample. In this study, the Gram stain and catalase activity of each isolate was already tested and the result can be seen in Table 1. However, the *Ochrobactrum* species should be a

Gram-negative and catalase-positive [11], while the *Staphylococcus* species should be a Gram-positive and catalase-positive [12]. But, based on its 16S rDNA sequences, some groups were identified as *Ochrobactrum* and *Staphylococcus*.

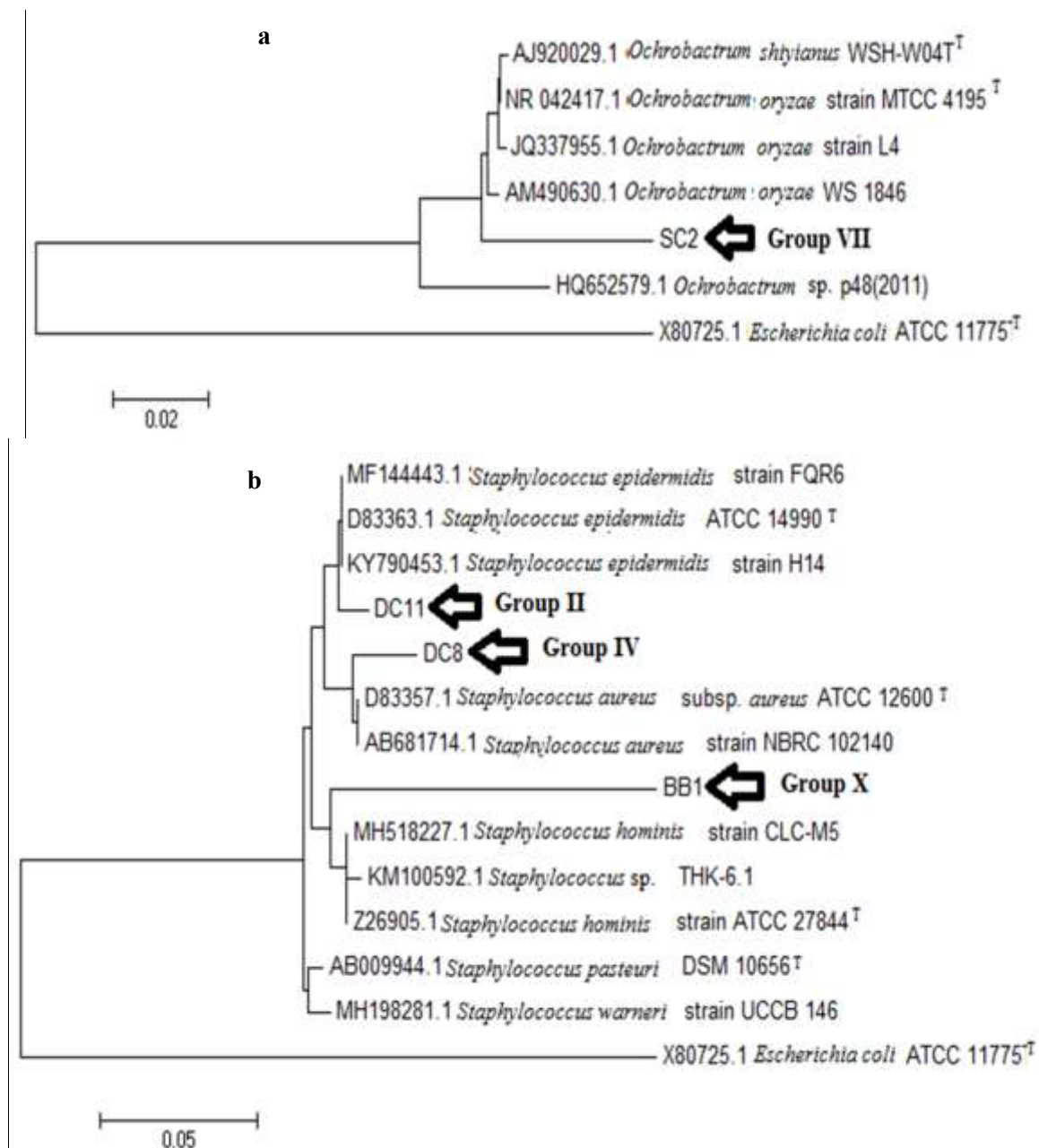


Figure 3. Phylogenetic tree of *Ochrobactrum* (a.) and *Staphylococcus* (b) and reference strains constructed by neighbour-joining method (1000x bootstraps) for 16S rDNA sequences.

The Gram-negative bacteria (*Ochrobactrum*) and pathogenic bacteria (*Staphylococcus*) were still can be found in the products. *Ochrobactrum oryzae* can cause the primary bloodstream infection related to a dialysis catheter [12], while *Staphylococcus* especially *Staphylococcus aureus* is a well-known as the human pathogen [13, 14]. The *Ochrobactrum oryzae* (MTCC 4195^T) was first isolated from surface-sterilized seeds and plant tissue of deep-water rice (*Oryza sativa*) cultivated in Suraha

Tal Lake in Northern India that can optimally growth at 37 °C and pH range from 4 to 9 [11]. Both genus of *Ochrobactrum* and *Staphylococcus* were the contaminants of milk products. It was already reported that during the milk processing line, the most contaminants was animal or feed origin such as *Ochrobactrum*, *Routltella*, *Rhodococcus*, *Brevibacterium*, *Alcaligenes*, and *Achromobacter* [15]. Thus, it can be assumed that the *Ochrobactrum* have contaminated the mare's milk during the production process i.e milking the mare's milk. The detection of *Staphylococcus* species in mare's milk products was related to the health of the mare. The presence of *Staphylococcus* was the contaminants derived from the animal itself due to the chronic mastitis disease [15].

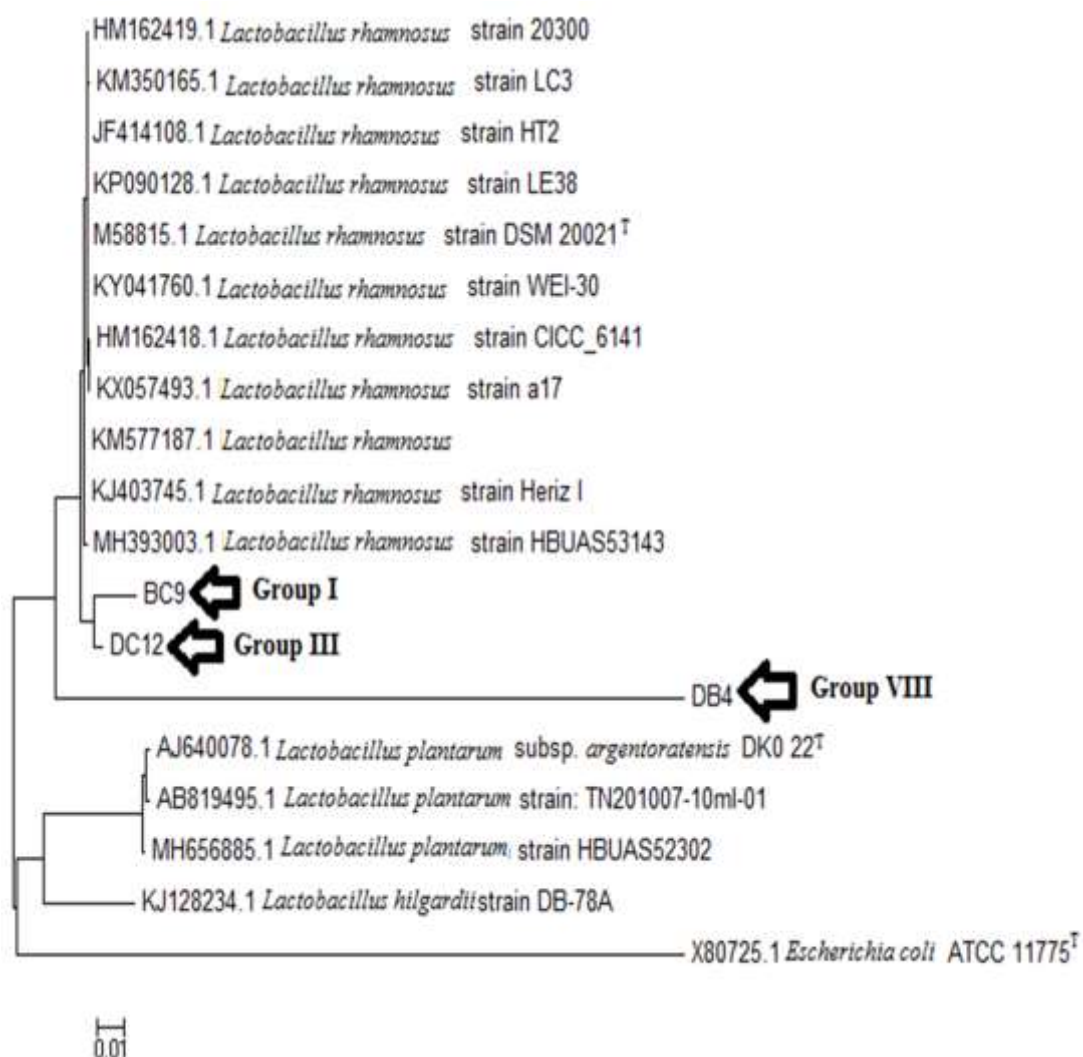


Figure 4. Phylogenetic tree of *Lactobacillus* and reference strains constructed by neighbour-joining method (1000x bootstraps) for 16S rDNA sequences.

4. Conclusion

Although the bacteria isolates showed a character of LAB based on phenotypic characterization, PCR-RFLP analysis has successfully clustered the bacterial isolates into 10 groups. The 16S rDNA sequencing results divided these groups into three genera i.e *Lactobacillus*, *Staphylococcus*, and *Ochrobactrum*. The dominant genus of bacteria isolated from Bima sample was *Staphylococcus*, Dompu sample was *Lactobacillus*, and Sumbawa sample was *Ochrobactrum*. The occurrence of

Gram-negative bacteria (*Ochrobactrum*) and pathogenic bacteria (*Staphylococcus*) must be a concern in term of food safety. The sanitation and preparation of the products should be further evaluated to prevent the contamination.

Acknowledgment

This work was financially supported by DPP/SPP Research Grant 2018, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya.

References

- [1] Sujaya N, Ramona Y, Widarini N P, Suariani N P, Dwipayanti N M U, Nocianitri K A 2008 Characterization of lactic acid bacteria isolated from Sumbawa mare milk *J. Vet.* **9** 2 52-59.
- [2] Shi T, Nishiyama K, Nakamata K, Aryantini N P D, Mikumo D, Oda Y 2012 Isolation of Potential Probiotic *Lactobacillus rhamnosus* Strains from Traditional Fermented Mare Milk Produced in Sumbawa Island of Indonesia *Biosci Biotechnol Biochem* **76** 10 1897–903.
- [3] Singh S, Goswami P, Singh R, Heller K.J 2009 Application of molecular identification tools for *Lactobacillus*, with a focus on discrimination between closely related species: A review *LWT - Food Sci. Technol.* **42** 2 448–57.
- [4] Rahman N, Xiaohong C, Meiqin F, Mingsheng D 2009 Characterization of the dominant microflora in naturally fermented camel milk *World J. Microbiol. Biotechnol.* **25** 11 1941–1946.
- [5] Temmerman R, Huys G, Swings J 2004 Identification of lactic acid bacteria: culture-dependent and culture-independent methods *Trends Food Sci. Technol.* **15** 7 348–359.
- [6] Perin L M, Miranda R O, Camargo A C, Colombo M, Carvalho A F, Nero L A 2013 Antimicrobial activity of the Nisin Z producer *Lactococcus lactis* subsp. *lactis* Lc08 against *Listeria monocytogenes* in skim milk *Arq Bras Med Veterinária E Zootec* **65** 5 1554–1560.
- [7] Bell J M, Paton J C, Turnidge J 1998 Emergence of vancomycin-resistant enterococci in Australia: phenotypic and genotypic characteristics of isolates *J. Clin. Microbiol.* **36** 8 2187–2190.
- [8] Rachman C N, Kabadjova P, Prévost H, Dousset X 2003 Identification of *Lactobacillus alimentarius* and *Lactobacillus farciminis* with 16S-23S rDNA intergenic spacer region polymorphism and PCR amplification using species-specific oligonucleotide *J. Appl. Microbiol.* **95** 6 1207–1216.
- [9] Chen Y-L, Lee C-C, Lin Y-L, Yin K-M, Ho C-L, Liu T 2015 Obtaining long 16S rDNA sequences using multiple primers and its application on dioxin-containing samples *BMC Bioinformatics* **16** 18 S13.
- [10] Abdel-Salam A M, Al-Dekheil A, Babkr A, Farahna M, Mousa H M 2010 High fiber probiotic fermented mare's milk reduces the toxic effects of mercury in rats *North Am. J. Med. Sci.* **2** 12 569–575.
- [11] Tripathi A K, Verma S C, Chowdhury S P, Lebuhn M, Gattinger A, Schlöter M 2006 *Ochrobactrum oryzae* sp. nov., an endophytic bacterial species isolated from deep-water rice in India *Int. J. Syst. Evol. Microbiol.* **56** 7 1677–1680.
- [12] Miller L G, Kaplan S L 2009 *Staphylococcus aureus*: a community pathogen. *Infect Dis. Clin. North Am.* **23** 1 35–52.
- [13] Borges L M S, Monteiro J, Miglioli L, Inoue F M, Coelho F M S L, Barbosa V L B, Cortez, D A, Vasconcelos E C Z, Souza E E, Abboud C S 2016 Identification of *Ochrobactrum oryzae* in primary bloodstream infection in a dialysis patient: can it be an emerging pathogen? *J. Microbiol. Infect. Dis.* **13** 6 128–131.
- [14] Pérez-Montarelo D, Viedma E, Murcia M, Muñoz-Gallego I, Larrosa N, Brañas P 2017 Pathogenic characteristics of *Staphylococcus aureus* endovascular infection isolates from different clonal complexes *Front Microbiol.* **8** 917 1-13.

- [15] Cleto S, Matos S, Kluskens L, Vieira M J 2012 Characterization of contaminants from a sanitized milk processing plant *PLoS ONE* 7 6 1-8.