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Genetic diversity of lactic acid bacteria isolated from Sumbawa horse milk, Indonesia

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Abstract. *Kusdianawati, Mustopa AZ, Fatimah, Budiarto BR. 2020. Genetic diversity of lactic acid bacteria isolated from Sumbawa horse milk, Indonesia. Biodiversitas 21: 3225-3233.* LAB from Sumbawa horse milk has good potential antimicrobial and probiotic agents. It is known, the study on LAB diversity based on its phenotypic characters is difficult to be distinguished. However, the development of molecular characterization based on the genotypic characteristic could be done for LAB diversity analysis. The aim of this study is to obtain the genetic diversity of LAB from Sumbawa horse milk collected from Penyaring Village and Lennanguar Village, Sumbawa, West Nusa Tenggara Indonesia. LAB strains were identified based on their genotypic characteristics, including their randomly amplified polymorphic DNA (RAPD) primers profiles and 16S ribosomal RNA (rRNA) sequences. The result of RAPD-PCR analysis showed 5 clusters of dendrograms resulted from GTG5 and LB2 primer amplification. Based on 16 rRNA sequences result, the phylogenetic tree was constructed and revealed 7 species of LAB i.e: SK 1.5, SKP K.3, SKP K.5, SKP K.9/SKP K.7/M.SKP K.3, SKL K.4, M.SKL K.1/ M.SKL K.5, and SKP K.4 belonging to the species of *Enterococcus faecium*, *Weissella confusa*, *Lactococcus garvieae*, *Enterococcus thailandicus*, *Lactobacillus fermentum*, *Enterococcus faecalis*, and *Lactococcus petauri*. In this study, the bacteria from *Enterococcus* sp., *Lactococcus garvieae*, and *Lactococcus petauri* existed as a novel of bacteria which means they have not been isolated and identified in Sumbawa horse milk compared to the previous findings.

Keywords: 16S rRNA analysis, LAB, RAPD-PCR analysis, Sumbawa horse milk

INTRODUCTION

Sumbawa is one of the districts in Nusa Tenggara Barat (NTB). Sumbawa horse milk is a local food product in Sumbawa, which has unique characteristics. The milk is not clumped when heated and acidic due to the presence of lactic acid bacteria (LAB) (Hermawati et al. 2004). The results extraction of active compounds from Sumbawa horse milk produced an organic compound as protein (galactoequin or galactoferrin) that has strong antimicrobial activity with bacteria pathogen (Hermawati 2005). Also, Sumbawa horse milk has a LAB that can ferment milk into acids and produce antimicrobial compounds such as bacteriocin, lactic acid, organic acids (acetate and hydrogen peroxide) (Hakim et al. 2013; Manguntungi et al. 2018; Sujaya et al. 2008; Zacharof and Lovitt 2012). LAB isolated from Sumbawa horse milk has antimicrobial activity to *Staphlococcus aureus, Escherichia coli, Salmonella thypimurium*, and *Shigella flexneri* (Suyaja et al. 2008)

Sumbawa horse milk contains the highest lactose (6.2 g / 100 g) compared to other dairy milk. The high lactose content is produced by horse milk fermentation (pH 3.1) from the excessive organic acids of LAB (Sujaya et al. 2008). Some of LAB bacteria species contained in Sumbawa horse milk consist of *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus salivarius*, *Lactobacillus delbrueckii* subsp. *delbrueckii* and *Lactococcus lactis* subsp. *Lactic*, *Lactobacillus bulgaricus*, *Lactobacillus rhamnosus*, *Streptococcus lactis*, *Tarula* sp., *Weissella* sp., *Leuconostoc* sp., or *Ochrobactrum oryza* and others LAB group which have not been identified (Mulyawati 2019; Sujaya et al. 2008; Widiada 2006). Yeasts group are also existed causing low pH of milk ranged in 3.1 (Sujaya et al. 2008). However, until now, there is still less of research addressing the genetic diversity of LAB found in Sumbawa horse milk. In the previous study, the LAB from Sumbawa horse milk has good potential antimicrobial and probiotic agents (Shi et al. 2012; Sujaya et al. 2008). It is known, the study on LAB diversity based on its phenotypic characters (morphological, biochemical, and physiological characters) is difficult to be distinguished. However, the development of molecular characterization based on the genotypic characteristic could be done for LAB diversity analysis. The results of this study expected to provide information on polymorphism patterns and obtained a phylogenetic tree from Sumbawa horse milk.

The genetic diversity of LAB is seen from amplification LB2 (Ben Omar 2000) and GTG5 primer (Gevers et al. 2001) on the LAB genomic DNA using Random amplified polymorphic DNA (RAPD). RAPD is a molecular technique to apply certain markers to study genetic diversity. RAPD is a useful approach to assessing genetic variation for the conservation of wild microorganisms; It is

based on PCR amplification of genomic DNA with arbitrary nucleotide sequence primers. The RAPD marker can detect high levels of DNA polymorphism and can produce fine genetic markers (Welsh and McClelland 1990; Williams et al. 1990). The analysis of RAPD result can be computerized that allow rapid identification of the unknown isolates. Therefore, there are several recent studies conducted on identifying genetic diversity of LAB using RAPD method (Chao et al. 2008; Chao et al. 2012; Delavenne et al. 2012; Mustopa and Fatimah 2014). This study aims to obtain LAB genetic diversity based on their genotypic characteristics through isolation, molecular marker screening using RAPD primers, and identify 16S rRNA sequences in Sumbawa horse milk.

MATERIALS AND METHODS

Study area

Samples were collected at horse farms in Penyaring Village and Lennanguar Village, Sumbawa, West Nusa Tenggara (NTB), Indonesia. The time to take a Sumbawa horse milk sample from two different villages were done on the same day. Condition of milk samples obtained from horses in fresh and good condition. Milk was stored at 4 °C using iceboxes to keep milk safe in good condition until observation in the laboratory. The volume of the collected fresh milk was 500 mL. The research was conducted from May to August 2019 in the Laboratory for Applied Genetic Engineering and Protein Design, Research Center for Biotechnology, Indonesian Institute of Sciences and Molecular Biology Laboratory and Genetic Resources Research and Development. Bogor, West Java, Indonesia.

Procedures

Isolation and identification of Lactic Acid Bacteria

LAB isolation method has been modified from Mustopa (2013). The LAB isolation of Sumbawa horse milk was carried out using a serial dilution method (10^{-6}) using 0.85% NaCl. Aliquot of 100 µL Sumbawa horse milk resulting from multilevel dilution (10^{-6}) is, then, spread into the solid medium of de Mann Rogosa Sharp (MRS) and M17. The LAB isolates grown in MRS media were incubated at 28 °C and 37°C for 24 hours, while the isolates of LAB grown in M17 media were incubated at 30 °C for 24 hours. A single bacterial colony produced from the isolates then grown in 5 ml of MRS and M17 liquid media.

Identification of genetic diversity LAB bacteria was conducted by isolation of LAB genomic DNA, PCR-RAPD, and PCR 16S rRNA as follows:

Isolation of genomic DNA

LAB genomic DNA isolation has been modified from Mustopa and Fatimah (2014) method. LAB colonies were cultured in 5 mL MRS (at 28 °C and 37 °C for 24 hours) and M17 (at 30 °C for 24 hours) media. Cell harvesting was performed by centrifugation at 11000 x g for 10 minutes. The pellet was resuspended with TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA), 40 µL of lysozyme (60 mg/mL) at 37 °C for 60 min. The solution was added with isolation buffer (200 µL SDS 10%, 100 µL NaCl 5 M, 80 µL 10% CTAB) and incubated at 68°C for 30 minutes. Then, chloroform was added 1:1 (v/v), and centrifuged at 23000 x g for 10 minutes. The top phase solution was transferred to a new microtube, then isopropanol was added with a volume ratio of 1:1 and centrifuged. The DNA pellets were added by 1 mL 70% cold ethanol and inverted. Then, centrifuged at 10000 x g for 2 minutes at 4°C. The DNA pellets were aired overnight. Dried DNA was solubilized in 27 µL of sterile water (ddH_2O) and 3 µL RNAse. The DNA solution incubated at 37°C for 30 minutes, then stored at 4°C.

RAPD-PCR

RAPD-PCR was performed using methods and amplification conditions as described by Chao et al (2008). The primers used were LB2 (5'-GGTGACGC-3 ') (Ben Omar 2000) and GTG5 (5'-GTGGTGGTGGTGGTG-3') (Gevers et al. 2001). The solutions used in the PCR mix were ddH_2O 3 µL, MyTaq master mix (Bioline) 7.5 µL, primers 1.5 µL, DNA template 3 µL with total volume was 15 µL.

Single primer was used in two different PCR conditions. The LB2 primer was used in the first PCR condition, which consists of 1 cycle of 94 °C for 2 min; 6 cycles of 94 °C for 30 s, 36 °C for 1 min, and 72 °C for 90 s; 30 cycles of 94 °C for 20 s, 36 °C for 30 s, and 72 °C for 90 s; and finally 1 cycle of 72 °C for 3 min. The GTG5 primer was used in the second PCR condition, which consists of 1 cycle of 95°C for 7 min; 30 cycles of 95°C for 1 min, 55°C for 1 min, and 65°C for 8 min; and finally 1 cycle of 65 °C for 16 min. PCR products were separated on 2% (w/v) agarose gel electrophoresis using 1x TAE buffer. The gels were stained in ethidium bromide solution and photographed under UV transilluminator.

The result of the amplification band profile obtained from RAPD-PCR was analyzed using NTSYS 2.02 in the form of binary data (0 and 1) to obtain a cluster analysis and produce dendrogram. The polymorphic information content (PIC) was computed as :

$$PIC_i = 2f_i\,(1\text{-}f_i)$$

Where PIC_i is the polymorphic information content 'i', f_i is the frequency of the amplified allele (band present), and $(1\text{-}f_i)$ is the frequency of the null allele (band absent) (Roldán-Ruiz et al. 2000; Soengas et al. 2006).

PCR amplification for 16S rRNA

Primary sequences of 16S rRNA primer 8F (5'-AGAGTTTGATCATGGCTCAG-3') and 16R (5'-AAGGAGGTGATCCAACCGCA-3') (Chao et al. 2008). The solutions used in the PCR mix were ddH_2O 38.5 µL, 5 × MyTaq Green 7.5 µL, primers 8F 1 µL, primers 16R 1 µL, DNA template 2 µL with total volume was 50 µL. The PCR conditions were 96 °C for 5 min; 35 cycles consisting of 96 °C for 1 min, 55 °C for 3 min, and 72 °C for 1 min; and 72 °C for 7 min. The PCR products were subjected to gel electrophoresis on 1% agarose gel, followed by ethidium bromide staining and photographed under UV transilluminator.

Sequencing and phylogenetic relationships of the lactic acid bacteria

The DNA sequencing was performed in the 1st BASE, Singapore. Similarity analysis was performed by online Basic Local Alignment Search Tool (BLAST) analysis in NCBI. The phylogenetic tree was constructed by Treeview software.

RESULTS AND DISCUSSION

Isolation of Lactic Acid Bacteria

Sumbawa horse milk was obtained from two different locations, Penyaring Village (SKP) and Lenangguar Village (SKL), Sumbawa Regency, NTB. Based on the physical appearance of horse milk there were differences in color, the thickness of milk, and strong acid odor as illustrated in (Figure 1). Therefore, the result of organoleptic tests shown from Sumbawa horse milk was white, sour taste, distinctive odor and having pH with a range of 2.73-4.25 (Hermawati 2005). The sour taste of Sumbawa horse milk has been correlated with the number of LAB which depends on variations in storage time (Hakim et al. 2013).

The result of isolation LAB from Sumbawa horse milk has produced 41 single colonies (Table 1). Then their genetic diversity identified was elucidated using PCR-RAPD and PCR 16S rRNA.

A **B**

Figure 1. Sumbawa horse milk samples. A. Horse milk from Lenangguar Village (color: yellowish-white, thickness of milk: very thick, acidic odor: very stinging). B. Horse milk from Penyaring Village (color: white, thickness of milk: more dilute, smell acid: sting)

Identification of Lactic Acid Bacteria

RAPD-PCR

Identification of LAB genetic diversity in this study used the RAPD-PCR method by applying primer GTG5 and LB2 (Mustopa and Fatimah 2014). As pointed in Figure 2, the RAPD-PCR profile showed various sizes of DNA amplification bands from each LAB specimen of two different horse milk sources. Interestingly, two different primers used in RAPD showed a different pattern of DNA bands where GTG5 primer (PIC 0,38) more likely gave variation in terms of LAB identified compared to LB2 primer (PIC 0,32). Furthermore, dendrogram analysis of LAB based on amplification bands of each isolate showed the diversity of LAB (Figures 3 and 4).

Table 1. LAB isolate from Sumbawa horse milk

Isolate code	Medium	Temp.	Origin
LAB 1.1	MRS	37 °C	Penyaring Village
LAB 1.2	MRS	37 °C	Penyaring Village
LAB 1.4	MRS	37 °C	Penyaring Village
LAB 1.5	MRS	37 °C	Penyaring Village
LAB 1.6	MRS	37 °C	Penyaring Village
LAB 1.7	MRS	37 °C	Penyaring Village
LAB 1.8	MRS	37 °C	Penyaring Village
LAB SK 1.5	MRS	37 °C	Penyaring Village
LAB SK 2.1	MRS	37 °C	Penyaring Village
LAB SK 2.3	MRS	37 °C	Penyaring Village
SKP K.1	MRS	37 °C	Penyaring Village
SKP K.2	MRS	37 °C	Penyaring Village
SKP K.3	MRS	28 °C	Penyaring Village
SKP K.4	MRS	28 °C	Penyaring Village
SKP K.5	MRS	28 °C	Penyaring Village
SKP K.6	MRS	28 °C	Penyaring Village
SKP K.7	MRS	28 °C	Penyaring Village
SKP K.8	MRS	37 °C	Penyaring Village
SKP K.9	MRS	37 °C	Penyaring Village
SKP K.10	MRS	37 °C	Penyaring Village
SKP K.11	MRS	37 °C	Penyaring Village
M.SKP K.1	MRS	37 °C	Penyaring Village
M.SKP K.2	M17	30 °C	Penyaring Village
M.SKP K.3	M17	30 °C	Penyaring Village
M.SKP K.4	M17	30 °C	Penyaring Village
M.SKP K.5	M17	30 °C	Penyaring Village
SKL K.1	M17	30 °C	Lenangguar Village
SKL K.2	MRS	37 °C	Lenangguar Village
SKL K.3	MRS	37 °C	Lenangguar Village
SKL K.4	MRS	37 °C	Lenangguar Village
SKL K.5	MRS	37 °C	Lenangguar Village
SKL K.6	MRS	37 °C	Lenangguar Village
SKL K.7	MRS	28 °C	Lenangguar Village
SKL K.8	MRS	28 °C	Lenangguar Village
SKL K.9	MRS	28 °C	Lenangguar Village
SKL K.10	MRS	28 °C	Lenangguar Village
M.SKL K.1	MRS	28 °C	Lenangguar Village
M.SKL K.2	M17	30 °C	Lenangguar Village
M.SKL K.3	M17	30 °C	Lenangguar Village
M.SKL K.4	M17	30 °C	Lenangguar Village
M.SKL K.5	M17	30 °C	Lenangguar Village

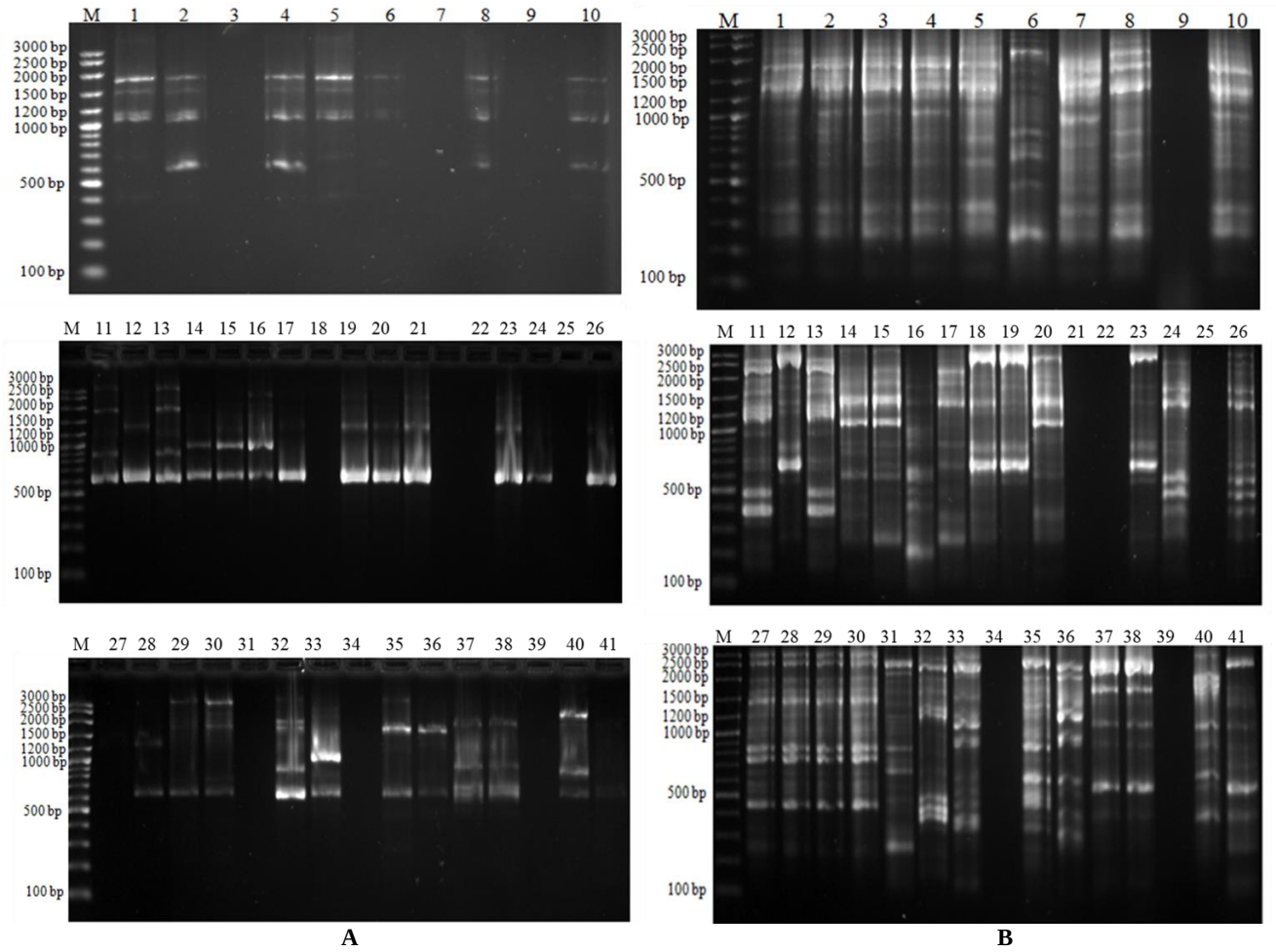

Figure 2. RAPD profiles of LAB DNA amplification using primers A. LB2 and B. GTG5 in 2% agarose gel electrophoresis. From left to right: First lane: 1 kb DNA ladder, second lane up to end: LAB isolates from Sumbawa milk horse

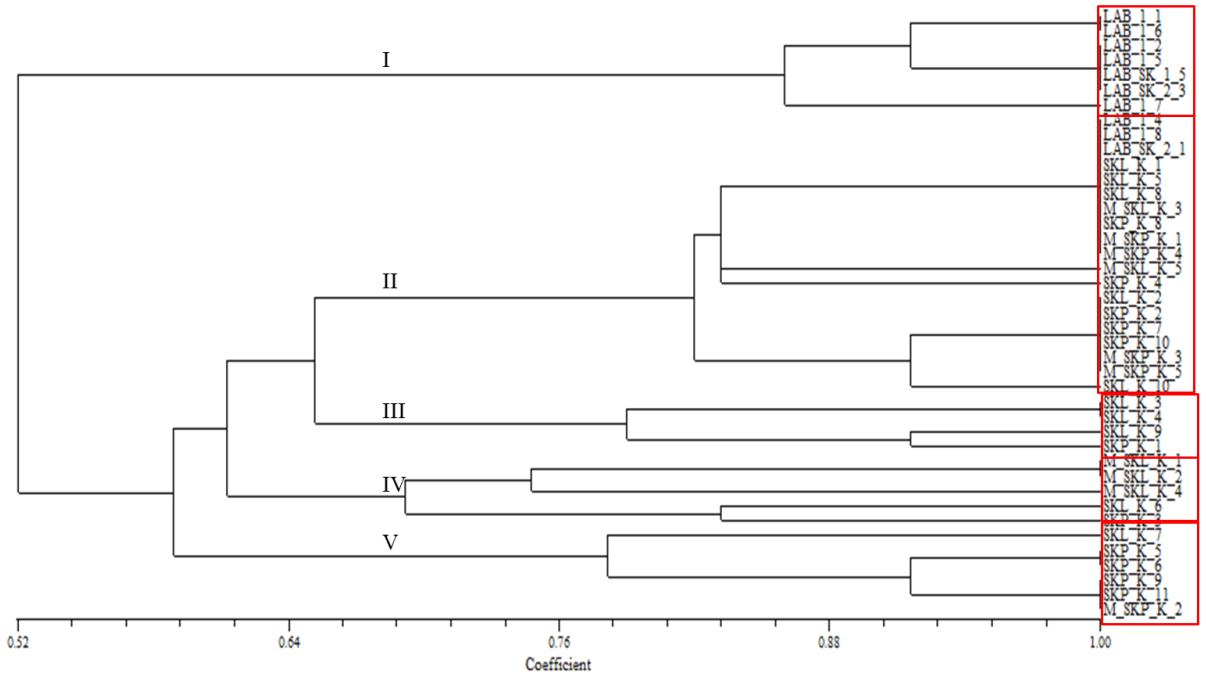

Figure 3. Dendrogram of LAB isolates from Sumbawa horse milk using LB2 primer has separated 5 clusters

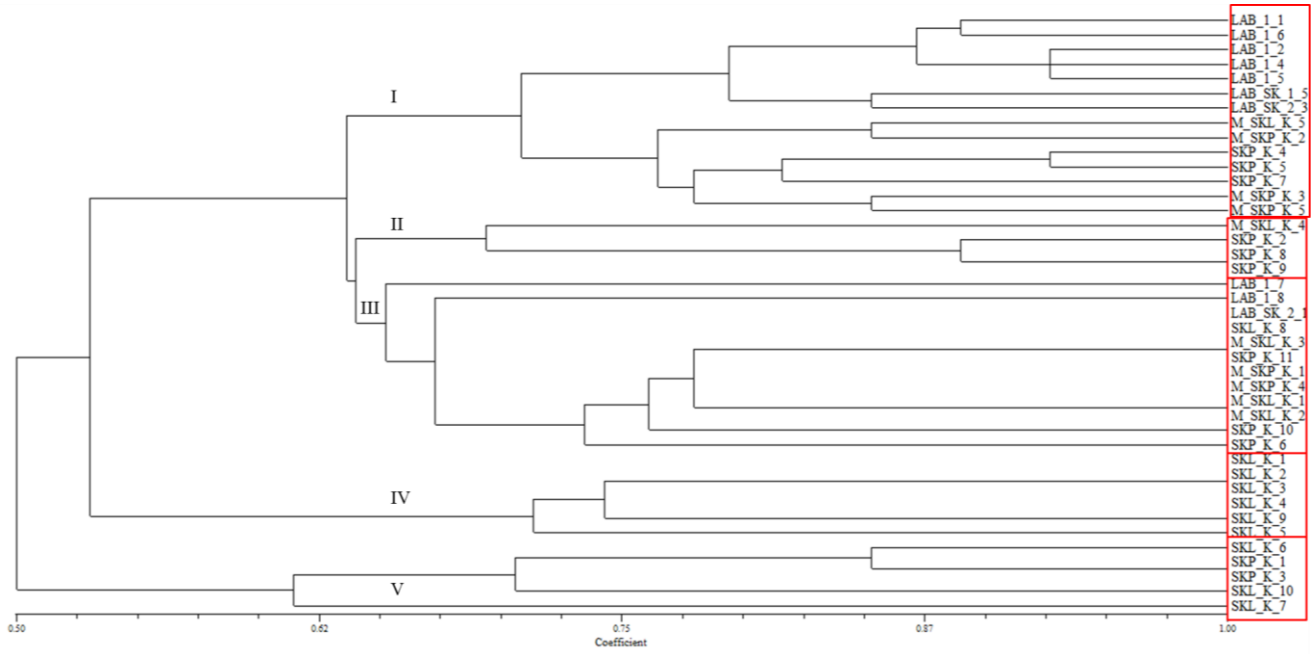

Figure 4. Dendrogram of LAB isolates from Sumbawa horse milk using GTG5 primer has separated 5 clusters

Based on the dendrogram analysis of LAB genetic diversity from Sumbawa horse milk using LB2 primers at 68% similarity, 5 clusters were found (Figure 3). The cluster I consisted of 7 LAB isolates from horse milk in the Penyaring Village. Cluster II consisted of 12 LAB isolates from horse milk in Penyaring Village and 7 LAB isolates from horse milk in Lenangguar Village. Cluster III consisted of 1 LAB isolate from horse milk in Penyaring Village and 3 LAB isolates from horse milk in Lenangguar Village. Clusters IV consisted of 1 LAB isolate from horse milk in Penyaring Village and 4 LAB isolates from horse milk in Lenangguar Village. Clusters V consisted of 5 LAB isolates from horse milk in the Penyaring Village and 1 LAB isolate from horse milk in Lenangguar Village. This indicates that LAB isolates in clusters II, III, IV, and V obtained from horse milk in the Penyaring Village have genetic similarities with LAB obtained from horse milk in Lenangguar Village. Whereas LAB isolates from horse milk of Penyaring Village in cluster I have different genetic diversity from LAB isolates obtained from horse milk in Lenangguar Village.

Based on the dendrogram analysis of the diversity of LAB from Sumbawa horse milk using GTG5 primers at 67% similarity, 5 clusters were found(Figure 4). Cluster I consisted of 13 LAB isolates from horse milk in the Penyaring Village and 1 LAB isolate from horse milk in Lenangguar Village. Cluster II consisted of 3 LAB isolates from horse milk of the Penyaring Village and 1 LAB isolate from horse milk in Lenangguar Village. Cluster III consisted of 8 LAB isolates from wild horse milk in the Penyaring Village and 4 LAB isolates from horse milk in Lenangguar Village. Cluster IV only consisted of 6 LAB isolates from horse milk in Lenangguar Village. Cluster V consisted of 2 LAB isolates from horse milk in Penyaring Village and 3 LAB isolates from horse milk in Lenangguar Village. The level of LAB genetic diversity obtained from the RAPD-PCR amplification result using GTG5 primers was more diverse (PIC 0,38). In some clusters such as cluster IV which only consisted of LAB isolates from horse milk Lenangguar Village had different genetic diversity from LAB isolates from horse milk in Penyaring Village. Cluster I, II, III, and V indicate that LAB isolates obtained from horse milk in the Penyaring Village have genetic similarities to LAB isolates obtained from horse milk in Lenangguar Village.

PCR Amplification for 16S rRNA of the Lactic Acid Bacteria

Based on the dendrogram result, the similarity percentage of GTG5 primer of LAB isolates from Sumbawa horse milk were more diverse compared to LB2 primers. Hence, the 5 clusters resulted from GTG5 primer at similarity percentage of 67% were used as representatives to determine the LAB strain for amplification of 16S rRNA (Figure 5).

Sequencing and phylogenetic relationships of the lactic acid bacteria

Based on PCR amplification result using 16S rRNA primers, a band of 1500 bp was obtained. Then only ten LAB isolates as representative from each cluster of GTG5 primer were selected i.e.: SKP K.3, SKP K.5, SKP K.9, SK 1.5, SKL K.4, M.SKL K.1, M.SKL K.5, SKP. K4, SKP K.7, and M.SKP K.3 to proceed to a sequence (Table 3).

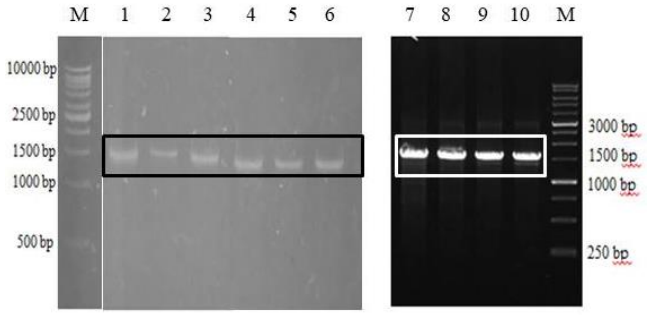

Figure 5. 16S rRNA profile of Sumbawa horse milk LAB isolates as representative from each cluster of GTG5 primer. From *left to right*: Lane 1: 1 kb marker (DNA ladder Vivantis), lane 2: SK 1.5, lane 3: SKP K.3, lane 4: SKP K.5, lane 5: SKP K.9, lane 6: SKL K.4, lane 7: M.SKL K.1, lane 8: M.SKL K.5, lane 9: M.SKP K.4, lane 10: SKP K.7, lane 11: M.SKP K.3, lane 12: 1 kb DNA ladder

Based on Table 3, seven species of LAB that were successfully identified, *E. faecium* sp., *W. confusa* sp., *L. garvieae* sp., *E. thailandicus* sp., *L. fermentum* sp., *E. faecalis* sp., and *L. petauri* sp. The phylogenetic tree of the 16S rRNA sequence in Figure 6 contained sequences of LAB strains from Sumbawa horse milk (this study), sequences of several LAB from Gram-positive bacteria such as *Lactobacillus plantarum*, *Pediococcus* obtained from GenBank and sequences of some Gram-negative bacteria such as *Escherichia coli, Pseudomonas aeruginosa, Chlamydia trachomatis, Helicobacter typhlonius, Legionella anisa* from GenBank (Figure 6).

Discussion

The genetic diversity result from 41 single LAB colonies (Table 1) with PCR-RAPD using GTG5 primers was more diverse than the LB2 primers (Figure 2 and Table 2). Based on the result of DNA amplification on LAB genome (Figure 2), the GTG5 primer produced a more diverse band when compared to the LB2 primer. Analysis of LAB genetic diversity based on bands of the same size and bands of different sizes of each isolate showed the diversity of LAB (Figures 3 and 4). Based on the dendrogram analysis, the percentage of similarity of LAB isolates from Sumbawa horse milk using LB2 primers was 68% and GTG5 primers were 67%. PCR amplification using the GTG5 primer was proven to be useful for the differentiation of a wide range of *lactobacilli* at the species, subspecies, and potentially up to the strain level (Gevers et al. 2001). Abdollahniya et al. (2018), the results of the RAPD marker from LAB traditional dairy products indicated that intra-species diversity was greater than inter-species diversity. Diversity of lactic acid bacteria isolated from fermented mare's milk products based on PCR-RFLP analysis had a total of 41 isolates that were grouped into 10 clusters. However, only three clusters were identified as LAB from *Lactobacillus* genus (group I with 97%, group III with 98%, and group VIII 75%) (Mulyawati et al. 2019).

The identification of LAB from Sumbawa horse milk using 16 rRNA sequence was successfully identified three closely related type strains bacteria from *Enterococcus* sp. (M.SKP.K3), *Lactococcus garvieae* (SKP K.5), and *Lactococcus petauri* (SKP K.4) as a novel bacteria in Sumbawa horse milk compared to the previous findings reported by Mulyawati 2019, Sujaya et al. 2008 and Widiada 2006.

Based on 16S rRNA sequence obtained that M.SKP K3 isolates closely related type *E. thailandicus* which is showing 96,91% similarity. This bacteria can be a new strain type or unknown isolate. In a previous study, about the similarity of 16S rRNA of the unknown isolate with reference species or strain below 97% represented the new type strain of the unknown isolate. But, for determining an isolate as a new species certain analysis should be done along with 16S rRNA analysis, DNA hybridization, % G+C content, morphological, biochemical, and physiological characterization of the isolate determines the novelty of the bacterial isolate as a new species or type strain (Baltz et al. 2010; Mignard and Flandrois 2006).

Table 2. Results of polymorphic information content (PIC) LB2 and GTG5 primers in RAPD analysis

Primer	PIC
LB2	0.32
GTG5	0.38

Table 3. LAB isolate Sumbawa horse milk species

Isolate	Finished sequences totals (bp)	Closely related type strains Based on 16S rRNA gene	Percentage similarity (%)	Accession number
SK 1.5	1397	*Enterococcus faecium*	99.86	CP032308.1
SKP K.3	1397	*Weissella confusa*	99.39	MK818759.1
SKP K.5	1397	*Lactococcus garvieae*	99.51	LC376029.1
SKP K.9	1397	*Enterococcus thailandicus*	100	AA00296
SKL K.4	1397	*Lactobacillus fermentum*	99.93	KY249642.1
M.SKL K.1	1397	*Enterococcus faecalis*	100	CP030045.1
M.SKL K.5	1464	*Enterococcus faecalis*	100	CP045045.1
SKP K.4	1446	*Lactococcus petauri*	100	CP045924.1
SKP K.7	1446	*Enterococcus thailandicus*	98.62	LT223669.1
M.SKP K.3	1002	*Enterococcus thailandicus*	96.91	CP023074.1

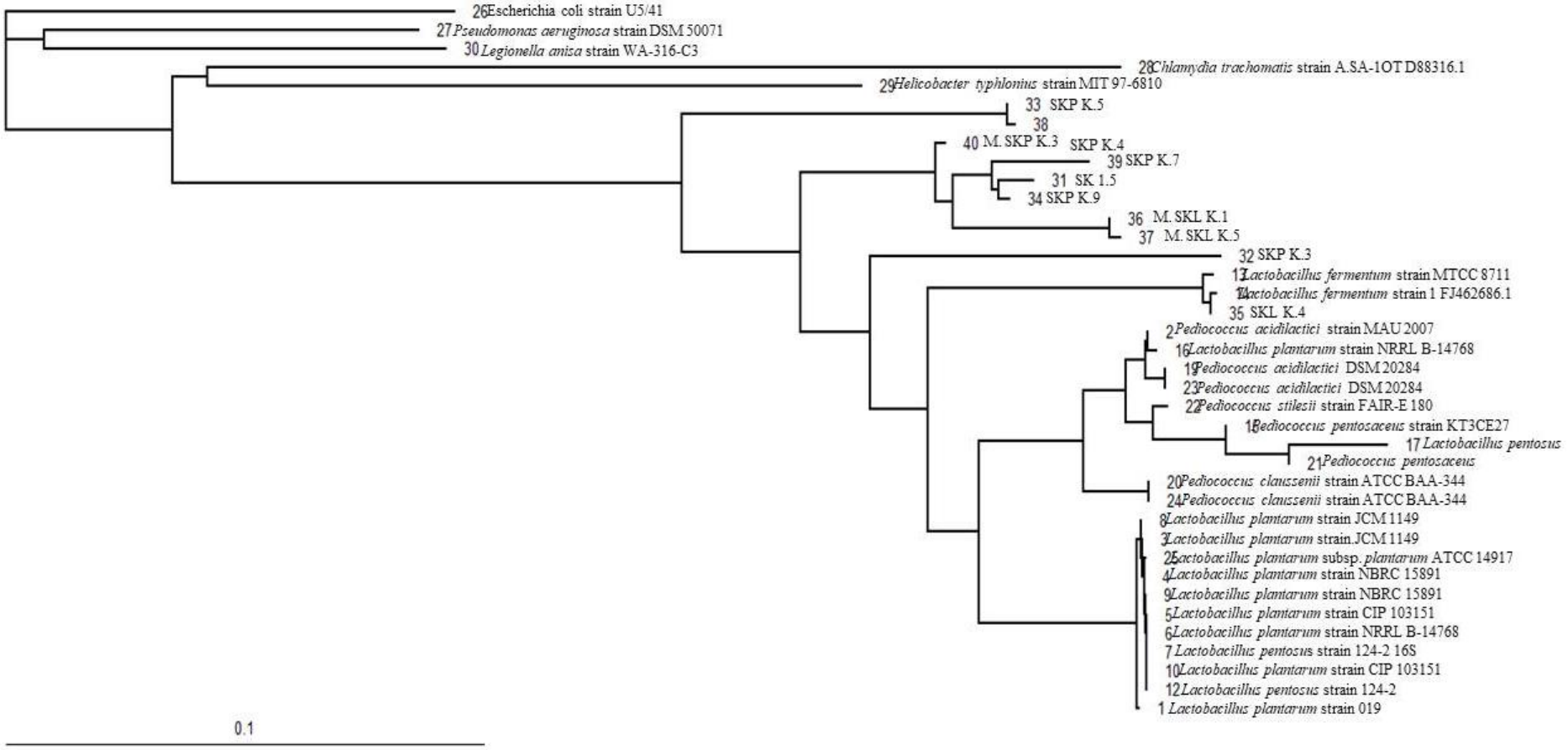

Figure 6. Phylogenetic tree based on 16S rRNA sequence analysis of LAB strains isolated from Sumbawa horse milk (this study) and several Gram-positive and Gram-negative bacteria obtained from GenBank accession.

Isolates of SK 1.5 (*E. faecium*), SKP K.9, SKP K.7, M.SKP K.3 (*E. thailandicus*), M.SKL K.1 and M.SKL K.5 (*E. faecalis*) are Gram-positive lactic acid bacteria belonging to the genus *Enterococcus*. *Enterococcus* is one of the normal components of microbiota found in raw milk (Giménez-Pereira 2005). *Enterococcus* strains group have been isolated from raw milk are *E. faecalis* and *E. casseliflavus* (Gelsomino et al. 2002), *E. lactis* (Bauer et al. 2009), *E. italicus*, and *E. faecium* (Gaaloul et al. 2014). *E.* occurs in different foods of dairy products, for instance, cheeses and raw milk (Morandi et al. 2012; Elmoslih et al. 2017). *Enterococcus* is found in a variety of artisanal cheeses made from raw or pasteurized milk from goats, sheep, buffaloes, and cows (Cogan et al. 1997; Franz et al. 1999). The most common *Enterococcus* in cheese is *E. faecalis* and *E. faecium* (Dave et al. 1980; Centeno et al. 1995; Arizcun et al. 1997; Cogan et al. 1997; Stiles et al. 1997; Suzzi et al. 2000). From the current regulatory point of view, the genus *Enterococcus* is neither recommended for the QPS list nor has GRAS status. The development of highly adapted methods and legislations for *Enterococcus* strains are still required (Hanchi et al. 2018).

SKP K.3 isolate belongs to *W. confusa* species. This genus includes Gram-positive, catalase-negative, and non-endospore forming coccoid or rod-shaped LAB (Björkroth et al. 2014). *W. confusa* (Cys2-2) produces active substances (bacteriocin) with inhibitory potential. The result revealed that *W. confusa* (Cys2-2) bacteriocin exerted its bactericidal effect by weakening the membrane integrity of target cells leading to cell death (Tenea and Lara 2019).

SKP K.5 isolate belongs to *L. garvieae* species. *L. garvieae* is one of the genera of *Lactococcus* species (Cai et al. 2011). *L. garvieae* isolated from raw milk and dairy products have been reported to inhibit indicator strains due to the production of bacteriocin (Villani et al. 2001). The study of Suneel dan Basappa (2013) showed that the *L.garvieae* produce bacteriocin that inhibits a wide variety of pathogenic microbes which suggested to be used as an alternative type of antibiotic.

SKP K.4 isolate belongs to *L. petauri* species. This bacterium was successfully isolated from a healthy human gut (Ou et al. 2020). *L. petauri* has the closest relationship with *L. garvieae* and is a Gram-positive, non-motile, non-spore forming, and facultatively anaerobic (Goodman et al. 2017). *L. peauri* comes from the genus *Lactococcus*. Most members of this genus are found in fermented milk products such as cheese, yogurt, and butter (Fusco et al. 2019).

SKL K.4 isolate belongs to *L. fermentum* species. Traditional fermented dairy products including fermented yak, mare, goat, and cow milk were accurately identified as *L. fermentum* (42 strains) (Yu et al. 2011). Mikelsaar and Zilmer (2009), that *L. fermentum* strain ME-3 has the function of antimicrobial activity against intestinal pathogenic bacteria (*S. typhimurium*) and high antioxidant activity that it is potentially be used as a probiotic (Georgieva et al. 2015).

Based on genetic diversity result, that LAB was a predominant species founded in Sumbawa horse milk as mentioned in Table 3. Those LAB species may have great potency for probiotics or as a source for antimicrobe substance. Based on empirical evidence, it was believed that Sumbawa horse milk give benefit in term of the well-being of local people who consume it, yet there is no clear scientific based evident until recently. According to Hermawati (2005), such benefits of Sumbawa horse milk consumption in the local community were the ability to cure digestive-related diseases, tuberculosis, anemia, lung inflammation, and cancer.

The result demonstrates that LAB from different sources of horse milk in Sumbawa (Lenangguar Village and Penyaring Village) from RAPD analysis showed 5 clusters resulted from GTG5 and LB2 primers and based on 16S rRNA revealed a complex composition of LAB species. Such diversity of LAB provides useful information for further studies such as a selection of probiotic strain and starter culture design or antimicrobial for the industrial production of traditional fermented milk. The diversity of LAB contained in Sumbawa horse milk has the potential to be used as an antimicrobial and probiotic.

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