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Diversity analysis, identification, and bioprospecting of Lactic Acid Bacteria (LAB) isolated from Sumbawa horse milk

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Abstract. *Fidien KA, Manguntungi B, Sukmarini L, Mustopa AZ, Triratna L, Fatimah, Kusdianawati. 2021. Diversity analysis, identification, and bioprospecting of Lactic Acid Bacteria (LAB) isolated from Sumbawa horse milk. Biodiversitas 22: 3333-3340.* Sumbawa horse milk has a probiotic potential because of the presence of Lactic Acid Bacteria (LAB). The LAB present in Sumbawa horse milk has been reported to have antimicrobial activities against pathogenic bacteria, including *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Escherichia coli*, and *Vibrio cholerae*. However, the potential of LAB from Sumbawa horse milk as antioxidant and antidiabetic is still unexplored. Studies related to the diversity of indigenous bacteria in Sumbawa horse milk based on metagenomic analysis have not been widely studied either. Therefore, this study aimed to determine the diversity of species of indigenous bacteria in Sumbawa horse milk and to identify LAB bioprospecting from Sumbawa horse milk. The diversity of indigenous bacterial species was investigated by the 16S rRNA gene-targeted metagenomic approach from bacterial DNA isolated from Sumbawa horse milk. The identification of LAB was also carried out by the 16S rRNA gene identification method. LAB bioprospecting on antioxidant activity was determined using the DPPH method, while the antidiabetic activity was measured using the α-glucosidase inhibition assay. The diversity analysis of indigenous bacteria based on 16S rRNA gene-based metagenomic revealed at least 7 phyla were relatively abundant in Sumbawa horse milk. The greatest abundance was shown by the phylum Proteobacteria (0.641%) and Firmicutes (0.327%). *Enterococcus durans* (39.01%) was the species that had the highest abundance in Sumbawa horse milk, followed by *Lactococcus garvieae* (30.13%) and *Lactococcus lactis* (19.85%). Moreover, based on the identification of the 16S rRNA gene, eight LAB isolates had similarities to bacterial strains, including Enterococcus faecium DSM 20477, *E. faecium* NBRC 100486, *E. faecium* ATCC 19434, *E. durans* 98D, *E. faecalis* ATCC 19433, *E. faecalis* NRBC 100480, *Lactococcus lactis* subsp. *hordniae* NBRC 100931 and *L. garvieae* JCM 10343 with similarity levels of more than 98%. In terms of LAB bioprospecting, the antioxidant assay showed the highest DPPH radical binding activity by *L. garvieae* L.22PR (43%). Meanwhile, the highest inhibitory activity of α-glucosidase was shown by *E. faecium* G.6PR (45%).

Keywords: Bioprospection, diversity analysis, identification, metagenomics, Lactic Acid Bacteria, Sumbawa horse milk

INTRODUCTION

Sumbawa horse milk widely known as wild horse milk, is a special commodity from Sumbawa Island, West Nusa Tenggara, Indonesia. According to Sujaya et al. (2008a), Sumbawa horse milk has probiotic potential. It has been reported that this milk could increase cellular immunity against *Salmonella thyphimurium* infection (Reni et al. 2013). The presence of lactic acid bacteria (LAB) in Sumbawa horse milk provides beneficial health effects, such as growth modulation bacteria beneficial for health in the human digestion (Sujaya et al. 2012). Investigations on the diversity of indigenous bacteria in Sumbawa horse milk are still limited to LAB and its metabolites (Sujaya et al. 2008b; Setyowati et al. 2012; Nuraida 2015; Manguntungi et al. 2018). Moreover, the method used to identify bacteria present in this milk still depends on microbial cultivation. Metagenomics is a functional analysis method based on microbial DNA sequences in the sample (Handelsman 2004). Studies on the microbial community and metagenome functional analysis in milk have been conducted in human milk (Ward et al. 2013) and goat milk (Zhang et al. 2017a).

The study on LAB carried out by Sujaya et al. (2008b) revealed that the isolated LAB from Sumbawa horse milk was predominantly belongs to the *Lactobacilli*. Several LAB species that have been characterized include *Lactobacillus acidophilus*, *Lb. brevis*, *Lb. plantarum*, and *Lactococcus lactis* (Antara et al. 2009). Setyowati et al. (2012) and Manguntungi et al. (2018) reported that LAB isolated from Sumbawa horse milk had antimicrobial activity against pathogenic bacteria, including *Staphylococcus epidermidis*, *S. aureus*, *Escherichia coli*, and *Vibrio cholerae*. However, to the best of our knowledge, there has been no study yet on bioprospecting of other than antimicrobial activities of LAB from Sumbawa horse milk.

Thus, in this study, 16S rRNA gene-based metagenomic approach was employed to detect populations of

indigenous species of bacteria found in Sumbawa horse milk. LAB identification from Sumbawa horse milk was also carried out to determine the isolated species. In addition, bioprospecting on antioxidant and antidiabetic activities was also assessed to uncover further the potential of LAB from Sumbawa horse milk.

MATERIALS AND METHODS

Metagenomic DNA isolation of Sumbawa horse milk

The metagenomic DNA was isolated from horse milk collected from the Penyaring Village, Sumbawa District, West Nusa Tenggara. Isolation was carried out following the manufacture's instruction of ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research Corp.) with a slight modification. Briefly, a sample of 5 mL of Sumbawa horse milk was put into a 15 mL falcon and centrifuged at 2,000 rpm, 4°C for 10 min. The supernatant was taken and centrifuged again at 6000 rpm, 4°C for 10 min. The pellet so collected was suspended with 750 µL Lysis Solution, then transferred into the Lysis Tubes. The resuspension was then vortexed for ≥ 20 minutes then centrifuged at 10,000 rpm for 1 min. A total of 400 µL supernatant was transferred into a filter in a collection tube and centrifuged at 8,000 rpm for 1 min. A total of 1200 µL DNA Binding Buffer was added and mixed well. A total of 800 µL sample was transferred into the column and centrifuged at 10,000 rpm for 1 min. The supernatant was then discarded. This stage was carried out twice. After that, a total of 400 µL Wash Buffer 1 was added to the column in a new collection tube and centrifuged for 10 min, then the supernatant was removed. A total of 700 µL Wash Buffer 2 was added to the column and centrifuged at 10,000 rpm for 1 min then the supernatant was removed. A total of 200 µL Wash Buffer 2 was added back to the column on the Collection Tube and centrifuged at 10,000 rpm for 1 min. The column was transferred into a 1.5 mL microtube, and 50 µL DNase/RNase Free Water was added and then incubated for 1 min before centrifuged at 10,000 rpm for 1 min. The eluted DNA was then confirmed by electrophoresis with 1% agarose in 1x TAE Running Buffer at a voltage of 100 V. Sequencing was carried out by Novogene CO., Ltd, Japan using Next-Generation Sequencing (NGS) MiSeq instruments (Illumina, Inc.).

Isolation of Lactic Acid Bacteria

A total of 100 µL Sumbawa horse milk was cultivated in 5 mL of M17 broth media with various sugars (lactose (L) and glucose (G)) and incubated at 30 °C overnight. Then, 100 µL overnight culture was diluted with 900 µL 0.85% NaCl and resuspended. The 10^{-3} and 10^{-4} dilutions of M17 cultures were taken and spread on M17 Agar media with various sugars (lactose and glucose) and then incubated at 30 °C for 48 h. A single colony was retrieved and cultivated in M17 broth, then incubated at 30°C for 16 h for later use in genomic DNA isolation.

Genomic DNA isolation of Lactic Acid Bacteria

The genomic DNA isolation was carried out according to the method developed by Doyle and Doyle (1990) with a slight modification. Bacterial cell pellet collection was performed by transferring 1.5 mL of the M17 broth culture to a microtube and centrifuge at 6,000 rpm, 4 °C for 10 min. The supernatant was then removed. The pellets were suspended with 500 µL TE Buffer pH 8.0 containing 60 mg/mL lysozyme, then incubated at 37°C for 1 h. After incubation, 200 µL 10% SDS, 100 µL 5 M NaCl, and 80 µL 10% CTAB were added and the mixture was incubated at 60°C for 30 min (inverted every 10 min). A total of 500 µL chloroform was added and then centrifuged at 13,000 rpm 4°C for 15 min. The supernatant was transferred into a new microtube and 0.6 x volume isopropanol was added, then incubated at -20°C for 2 h. After centrifugation at 13,000 rpm 4°C for 10 min, the supernatant was removed and the pellet was washed with 1 mL of 70% ethanol. The DNA pellet was then dried overnight before dissolved using 27 µL ddH_2O and 3 µL RNase (1 mg/mL) and incubated at 37°C for 60 min.

Polymerase Chain Reaction (PCR) amplification of 16S rRNA gene

Amplification of the 16S rRNA gene was performed using a thermal cycler. Each PCR reaction (50 µL) containing: 2.5 µL of 100 ng DNA template; 1 µL for each 10 mM primer; 1 µL My Taq DNA Polymerase (Bioline), 10 µL 5x My Taq Hs Red Buffer (Bioline), and 34.5 µL ddH_2O. Oligonucleotides for bacterial PCR 16S rRNA include forward 8F primer (5'-AGA GTT TGA TCA TGG CTC AG-3 ') and 15R reverse primer (5'-AAG GAG GTG ATC CAA CCG CA-3'). The PCR conditions used were as follows: pre-denaturation for 5 minutes at 95°C, denaturation at 95°C for 1 min, annealing at 58°C for 1 min, an extension at 72°C for 30 sec, and a final extension at 72°C for 7 min. PCR was carried out in 35 cycles. PCR products were electrophoresed on 1% agarose gel in TAE 1 x Running Buffer at 100 V and stained with ethidium bromide. Electrophoresis gel was visualized with a UV-Vis transilluminator. The 1 kb DNA ladder marker was used to estimate the size of the DNA fragments. Sequencing for identifying 16S rRNA bacteria was carried out by 1st Base DNA sequencing service (Axil Scientific Pte Ltd.) The sequencing results were analyzed using MegaX and BLAST software.

Antioxidant activity assay

The ability of LAB as an antioxidant agent was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Shimada et al. 1992) with a slight modification. A total of 20 µL supernatant metabolites and 180 µL DPPH solution (0.2 mM in methanol) were mixed in a microplate and incubated for 30 min in a dark condition. A growth medium was used as a blank, while vitamin C (50 mg/mL) was a positive control. After incubation, the DPPH concentration was measured using a multiplate reader (Thermo Scientific) with an absorbance at 540 nm. The color change was caused by the reduction of DPPH to diphenylpicrylhydrazine, which is a non-radical compound. The remaining DPPH molecules were read for their absorbance at 540 nm after 30 min of incubation.

α-glucosidase inhibition assay

Inhibition of α-Glucosidase was determined by the method adopted from Zahratunnisa et al. (2017). Briefly, 15.51 µL (equivalent to 14.5 α-glucosidase enzyme units) of α-glucosidase was diluted with the addition of a phosphate buffer pH 6.8) up to 5 mL volume. The reaction mixture of the α-glucosidase inhibition assay consisting of 30 µL sample, 36 µL phosphate buffer pH 6.8, and 17 µL p-nitrophenyl-α-D-glucopyranoside/PNPG (5 mM). After pre-incubation at 39 °C for 5 min, 17 µL α-glucosidase (0.045 units / mL) was added and incubated at 39 °C for 15 min. The reaction was then stopped by adding 100 µL Na_2CO_3 (200 mM). The inhibition of α-glucosidase was measured at 405 nm. Acarbose was used as a positive control of α-glucosidase inhibition. The antidiabetic potential of each LAB isolate in Sumbawa horse milk was measured by calculating the inhibitory activity of the crude extract of LAB against the α-glucosidase enzyme.

Data analysis

The data of antioxidant activity assay and α-glucosidase inhibition assay obtained were then analyzed using One Way ANOVA with Duncan's continued test using an α value of 0.05.

RESULTS AND DISCUSSION

Diversity analysis of the indigenous bacteria of Sumbawa horse milk

Analysis of the diversity of bacteria indigenous Sumbawa horse milk was performed on samples from Penyaring Village, Sumbawa District, West Nusa Tenggara, Indonesia. The diversity of phyla based on taxa phylum obtained from the NGS analysis showed differences in the relative abundance of each phylum in the sample of Sumbawa horse milk (Figure 1). The largest relative abundance belonged to Proteobacteria (0.641%) and Firmicutes (0.327%).

The bacterial community profile based on the 10 genera with the greatest abundance (33.13%) is shown in a taxonomic tree (Figure 2). It shows the abundance of bacterial communities in Sumbawa horse milk at the phylum level detected, including Firmicutes, Bacteriotedes, Fusobacteria, and Proteobacteria. The class of the Proteobacteria detected was gamma-proteobacteria. Compare to other classes, *Bacilli* was the most detected bacteria. Furthermore, the orders detected with the largest to the smallest relative abundance were Lactobacillales, Bacillales, Bacteriodales, Clostridiales, Enterobacteriales, and Fusobacteriales.

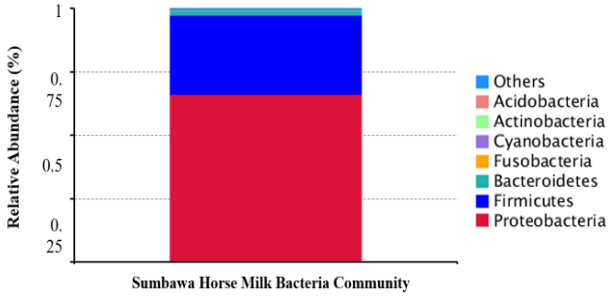

Figure 1. Composition of the Sumbawa horse milk bacteria community

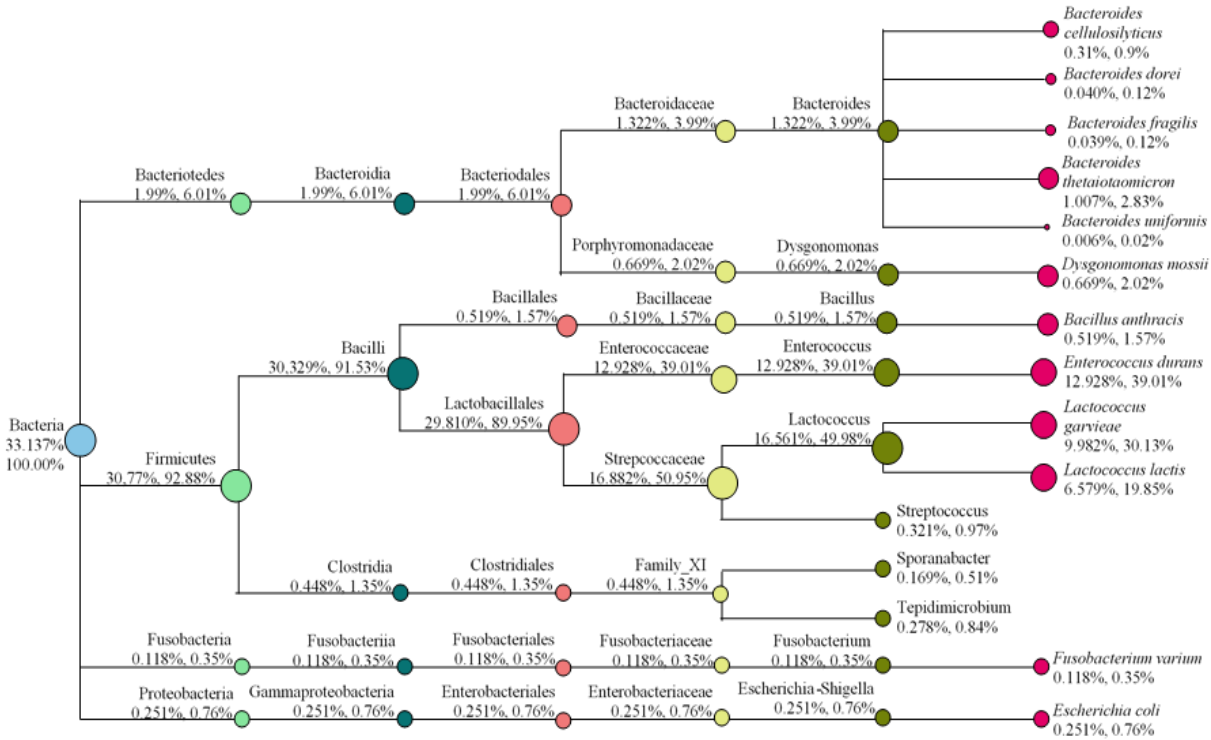

Figure 2. Taxonomic tree of indigenous bacteria of Sumbawa horse milk

Identification of LAB isolated from Sumbawa horse milk

LAB identification was carried out using bacterial isolates obtained from horse milk samples Sumbawa horse milk in Sumbawa District, West Nusa Tenggara is mainly produced in Penyaring and Lenangguar villages. The sample collection of Sumbawa horse milk from different locations aimed to compare LAB distribution from these two regions. The morphology of the LAB colony grown on the media was verified based on shape, edges, and color. The colonies obtained from the isolation were round, smooth edges, and white. The morphology of these isolates was in accordance with the morphology of LAB reported by Laily et al. (2013).

Identification of LAB in Sumbawa horse milk was carried out by a molecular analysis based on the 16S rRNA gene. Based on the results of electrophoresis, the amplified DNA fragment was about 1500 bp. The sequencing results of LAB identification using 16S rRNA sequence were as follows (Table 1).

As seen in Table 1, eight identified LAB isolates from Sumbawa horse milk were from two bacterial genera, namely *Enterococcus* and *Lactococcus*. Six LAB isolates G.5PR, G.6PR, G.18LG, G.20LG, G.19PR, and L.24PR have similarities with *E. faecium* NBRC 100486 (99.18%), *E. faecium* DSM 20477 (99.59%), *E. durans* 98D (99.1%), *E. faecium* ATCC 19434 (99.45%), E. *faecalis* ATCC 19433 (99.05%), and *E. faecalis* NBRC 100480 (98.95%), respectively. While two isolates L.19G and L.22PR shared 99.93% and 98.40% identities with *L. lactis* subsp strain. *hordniae* NBRC 100931 and *L. garvieae* JCM 10343, respectively. The presence of *E. faecium, E. faecalis,* and *L. garvieae* in Sumbawa horse milk was also reported by Kusdianawati et al. (2020).

The 16S rRNA sequences of each analyzed isolate were then used to see the level of kinship. Moreover, the kinship analysis of LAB isolates from Sumbawa horse milk is presented in a phylogenetic tree (Figure 3). The eight strains of LAB isolates were grouped into four groups, where the strains in each group were similar based on the sequence.

Antioxidant activity of LAB isolated from Sumbawa horse milk

The samples used for the antioxidant assay were supernatant metabolites extracted from the culture broth of LAB strains in this study. The results of the calculations from the assay performed are shown in Table 2. The DPPH radical scavenging test showed that *L. garvieae* L.22PR metabolite had the highest free radical scavenging activity of 43%, followed by two strains of *E. faecium* G.6PR and G.5PR at 42% and 41%, respectively. The lowest percentage of DPPH inhibition was shown by *L. lactis* subsp hordniae L.19LG metabolite (18%). The antioxidant activity of the LAB metabolites from Sumbawa horse milk was lower than that of the control vitamin C (79%).

Table 1. Species of LAB in Sumbawa horse milk

Isolate code	Species	Percent identity	Accession number
G.5PR	*Enterococcus faecium* NBRC 100486	99.18%	NR_113904.1
G.6PR	*Enterococcus faecium* DSM 20477	99.59%	NR_114742.1
G.18LG	*Enterococcus durans* 98D	99.19%	NR_036922.1
G.20LG	*Enterococcus faecium* ATCC 19434	99.45%	NR_115764.1
G.19PR	*Enterococcus faecalis* ATCC 19433	99.05%	NR_115765.1
L.19LG	*Lactococcus lactis subsp. hordniae* NBRC 100931	99.93%	NR_113958.1
L.22PR	*Lactococcus garvieae* JCM 10343	98.40%	NR_113268.1
L.24PR	*Enterococcus faecalis* NBRC 100480	98.95%	NR_113902.1

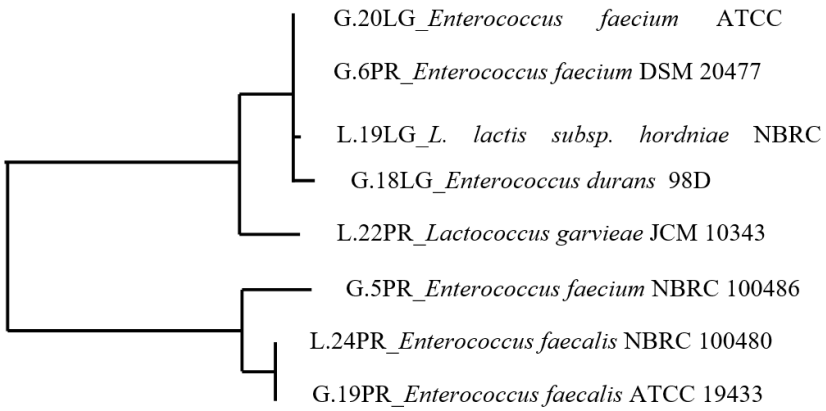

Figure 3. Phylogenetic tree of LAB isolates of Sumbawa horse milk based on 16S rRNA sequence

Antidiabetic activity of LAB isolated from Sumbawa horse milk

The inhibitory activities obtained in the present study were ranged between 26 to 45% (Table 3). The highest inhibitory activity was shown by the strains *E. faecium* G.6PR and *L. garvieae* L.22PR at 45% and 42%, respectively. The strain *L. lactis* L.19LG showed the lowest α-glucosidase enzyme inhibitory activity, which was 30%. The activity of the control acarbose in inhibiting the α-glucosidase enzyme was 74%.

Discussion

Next-generation sequencing (NGS) using Illumina is the most widely applied technique in metagenomic studies for analyzing microbial diversity and functional analysis profiles (Jatmiko et al. 2019). NGS is the most effective approach technology for individual classification to particular taxa and allowed finer characterization of bacterial genomes as well as deeper taxonomic recognition of complex microbiomes (Nimnoi and Pongsilp 2020). The hypervariable V3-V4 region in the 16S rRNA gene is a target of PCR amplification that can provide information about the bacterial community profile more accurately than the hypervariable V1-V3 region (Teng et al. 2018).

Table 2. Results of antioxidant assay of LAB isolated from Sumbawa horse milk

Isolate code	Sample	Inhibition
Control +	Vitamin C	79% ± 0.013^{d}
Control -	M17 *Broth* media	0%
G.5PR	*Enterococcus faecium* NBRC 100486	41% ± 0.054^{c}
G.6PR	*Enterococcus faecium* DSM 20477	42% ± 0.23^{c}
G.18LG	*Enterococcus durans* 98D	31% ± 0.052^{bc}
G.20LG	*Enterococcus faecium* ATCC 19434	38% ± 0.106^{c}
G.19PR	*Enterococcus faecalis* ATCC 19433	13% ± 0.146^{a}
L.19LG	*Lactococcus lactis* subsp. *hordniae* NBRC 100931	18% ± 0.071^{ab}
L.22PR	*Lactococcus garvieae* JCM 10343	43% ± 0.150^{c}
L.24PR	*Enterococcus faecalis* NBRC 100480	38% ± 0.056^{c}

Note: Result followed by the same letter show no significant difference in the one-way ANOVA test, α = 0.05

Table 3. Results of α-glucosidase inhibition assay of LAB Isolated from Sumbawa horse milk

Isolate code	Sample	Inhibition
Control +	Akarbosa	74% ± 0,044^{d}
Control -	M17 *Broth* media	0%
G.5PR	*Enterococcus faecium* NBRC 100486	34% ± 0,016^{b}
G.6PR	*Enterococcus faecium* DSM 20477	45% ± 0,028^{c}
G.18LG	*Enterococcus durans* 98D	30% ± 0,017^{ab}
G.20LG	*Enterococcus faecium* ATCC 19434	31% ± 0,024^{ab}
G.19PR	*Enterococcus faecalis* ATCC 19433	35% ± 0,030^{b}
L.19LG	*Lactococcus lactis* subsp. *hordniae* NBRC 100931	30% ± 0,037^{ab}
L.22PR	*Lactococcus garvieae* JCM 10343	42% ± 0,007^{c}
L.24PR	*Enterococcus faecalis* NBRC 100480	31% ± 0,041^{ab}

Note: Result followed by the same letter show no significant difference in the one way anova test, α = 0,05

Research on the bacterial community in Sumbawa horse milk conducted by Jatmiko et al. (2019) also detected Lactobacillales and Bacillales. The abundant families that dominate bacteria in Sumbawa horse milk include Enterococcaceae and Streptococcaceae. At the genus level, the enormous bacteria belong to *Lactococcus*. Moreover, the abundance of bacteria at the species level in Sumbawa horse milk was dominated by LAB *Enterococcus durans* as the largest species found, followed by *L. garvieae* and *L. lactis. Lactococcus* and *Enterococccus* spp. are found in abundant quantities in dairy products (Hou et al. 2015). Both genera have been reported to dominate the bacterial population in breast milk (Mehanna et al. 2013) and Colombian fermented cow's milk (Chaves-López et al. 2011). The abundance of these genera in livestock milk can be affected by environmental conditions on the farm. A study on the effect of animal husbandry practices on the abundance of bacteria in goat milk (Tormo et al. 2015) reported that *Enterococcus* sp. was abundant in milk products from most farms that did not separate the pen from the milking location. On the other hand, farms that are separated between cages and milking sites have a high abundance of *Lactococcus* spp. Direct contact between the milking site and the pen or the presence of hay in the pen seems to increase the inoculation of *Enterococcus* in milk.

Enterococcus is often the dominant genus of LAB found in fermented food and milk products, which *E. faecalis, E. faecium*, and *E. durans* are common species, especially in dairy products (Franz et al. 2011). *E. faecium* has antibacterial and antifungal activity against pathogenic microbes in the mouth, *Streptococcus mutans* and *Candida albicans* (Ng et al. 2020). *E. faecium* isolated from goat's milk is known to produce bacteriocins which can inhibit the growth of *Listeria* (Chanos and Williams 2011). Bacteriocins can cause cell membrane damage as indicated by the destabilization of cell membrane permeability (Todorov et al. 2010). A nosocomial bacteria *E. faecalis* is also known to produce a bacteriocin compound that inhibits hyphal morphogenesis, biofilm formation, and *C. albicans* virulence (Abrantes et al. 2011; Chanos and Williams 2011; Graham et al. 2017). Meanwhile, *E. durans* isolated from traditional fermented Mongolian cream has been reported to express an enzyme from the cholylglycine hydrolase family (Li et al. 2018). The enzyme catalyzes the glycine and taurine bile salts which are conjugated to amino acid and bile acid residues; thus, it can reduce cholesterol levels in the body (Begley et al. 2006).

Lactococcus is a typical bacterial genus present in plant milk products and biofilms in milking machines (Dalmasso et al. 2009). *L. lactis* is a bacterial strain from the genus *Lactococcus* that has the status of Qualified Presumption of Safety (QPS) and Generally Recognized as Safe (GRAS) (Yazdankhah et al. 2016). In its use in the food and health sectors, *L. lactis* has few reports as an opportunistic pathogen (Chien and Lee 2007). This strain produces the lactic acid compound that can suppress the growth of pathogens. Thus, *L. lactis* is widely used as starter bacteria in fermented milk products (Volzing et al. 2013). Moreover, bacteriocin nisin from *L. lactis* is widely used as a food preservative (Liu et al. 2017). In the field of genetic

and medical engineering, *L. lactis* is widely used as a host cell in heterologous protein expression (Noreen et al. 2011). *Lactococcus garvieae* is a type of LAB found as a pathogen in fish and various other marine and freshwater species. These species have also been isolated from other animals, such as ruminants with subclinical mastitis and pigs with pneumonia (Tejedor et al. 2011). Although many have been reported as pathogens, there are strains of *L. garvieae* known to produce bacteriocins, including *L. garvieae* LG34 (Gao et al. 2015). This strain has a bacteriocin, namely garvieacin LG34, which can inhibit the growth of *Listeria monocytogenes* and several other strains of *L. garvieae* (Tosukhowong et al. 2012). A study by Ayyash et al. (2020) reported that *L. garvieae* produced exopolysaccharides which had potential as antitumor, antioxidant, antidiabetic, and antipathogenic.

As mentioned earlier, the antioxidant activity of LAB in Sumbawa horse milk was determined using the DPPH method. The DPPH method has several advantages, including easy, simple, fast, reproducible, suitable for samples with a particular polarity, sensitive, and only requires a small number of samples (Ridho 2013). Free radical scavenging is done by measuring the decrease in absorbance of DPPH in ethanol solvent by the test sample, which converts the purple color of DPPH into yellow. The decrease in the absorbance of DPPH is proportional to the number of radicals captured by the test sample (Astuti 2009). The ability of free radical scavenging by the test sample can be influenced by the extraction method of LAB cells and the concentration of metabolite compounds used (Zhao et al. 2015). Manguntungi et al. (2020) reported that a high antioxidant activity (71%) of LAB isolated from masin (a fermented sauced) was shown by the highest concentration (500 µg/mL) of masin.

Compounds that thought to play a role in binding DPPH free radicals are exopolysaccharides derived from LAB (Seo et al. 2015). The antioxidant activity of *L. garvieae* in dairy products has been reported by Ayyash et al. (2020). The exopolysaccharide (EPS) produced by *L. garvieae* had a DPPH inhibitory activity of 51.92% at a 5 mg/mL concentration and increased to 67.52% when the EPS concentration was increased to 10 mg/mL. The DPPH inhibitory activity of the EPS produced by *E. faecium* was 38.4% at a sample concentration of 8 mg/mL (Abdhul et al. 2014). Exopolysaccharides can bind reactive oxygen and free radicals (Zhou et al. 2019). EPS in *L. lactis* subsp. *lactis* showed the same antioxidant activity as control ascorbic acid in tests conducted by Pan and May (2010). Kodali and Sen (2008) reported strong superoxide and hydroxyl radical activity in vitro on EPS of the probiotic strain *B. coagulans*. However, the effect of the amount of EPS production on LAB in antioxidant activity has not been further studied yet (Zhang et al. 2017b).

The inhibition ability of α-glucosidase activity was influenced by the concentration of the metabolite compounds used (Pratama et al. 2015). The high concentration of the compound increase the chance of the inhibitor to close the active site of the enzyme and bind to the enzyme and the enzyme-substrate complex. The inhibition of α-glucosidase by LAB isolated from fermented milk was reported previously by Graham et al. (2019) with the value of 33.41%. An investigation by Ramchandran and Shah (2008) reported that the inhibitory activity of α-glucosidase from the TCA filtrate of *L. casei* 2607, *L. acidophilus* 33200, *L. delbrueckii* ssp. *Bulgaricus* 1092, and *B. longum* 5022 were good with high α-glucosidase inhibitory activities (>80%). It seemed that the inhibitory activity of α-glucosidase resulted from exopolysaccharides produced by LAB (Ramchadan and Shah 2009). The principle mechanism of EPS in inhibiting α-glucosidase activity is the same as acarbose acting. EPS on LAB in the digestive system can attach to the intestinal mucosa surface to suppress the growth of pathogenic bacteria (Patten and Laws 2015). In addition to EPS, other compounds that are thought to play a role in α-glucosidase inhibition are proteins (Lacroix and Li-Chan 2013). Muganga et al. (2015) showed that the presence of α-glucosidase inhibitory activity from cell extracts of several *Lactobacillus* sp. grown in skim milk media with varying results was due to the different proteolytic properties of each isolate.

In conclusion, analysis of the diversity of indigenous bacteria in Sumbawa horse milk detected at least 7 types of phylum, with the two greatest relative abundance phylum including Proteobacteria (0.641%) and Firmicutes (0.327%). Of the 11 species detected based on the 10 largest genera, *Enterococcus durans* (39.01%) had the highest abundance, followed by *Lactococcus garvieae* (30.13%) and *Lactococcus lactis* (19.85%). Eight LAB isolates from Sumbawa horse milk identified as different strains, including *E. faecium* DSM 20477, *E. faecium* NBRC 100486, *E. faecium* ATCC 19434, *E. durans* 98D, *E. faecalis* ATCC 19433, *E. faecalis* NRBC 100480, *L. lactis* subsp. *hordniae* NBRC 100931, and *L. garvieae* JCM 10343. The highest antioxidant activity was shown by the *L. garvieae* L.22PR strain with a DPPH radical binding percentage of 43%, while the highest antidiabetic activity was shown by the *E. faecium* G.6PR strain with 45% α glucosidase inhibition.

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