

Profile of microbial community of naturally fermented Sumbawa mare's milk using next-generation sequencing

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Abstract

This study aimed to investigate the bacterial and fungal/yeast diversity in naturally fermented Sumbawa mare's milk through a next-generation sequencing approach, and evaluate the quality of fermented mare's milk based on the presence of pathogenic or undesirable microorganisms. Microbial density determined using plate count agar (total aerobic bacteria), de Man Rogosa Sharpe agar (*Lactobacillus*), M17 agar (*Lactococcus*) and yeast peptone dextrose agar supplemented with streptomycin 50 ppm (yeast). Nutritional content and acidity level of each fermented milk sample were also evaluated. Genomic DNA was extracted using FastDNA Spin (MPBIO). The total gDNA was further analyzed using illumina high-throughput sequencing (paired-end reads), and the sequence results were analysed using QIIME v.1.9.1 to generate diversity profiles. The difference in nutrient content of mare's milk was thought to affect the density and diversity of microbes that were able to grow. Fermented mare's milk samples from Sumbawa had the highest bacterial diversity compared to samples from Bima and Dompu. However, fermented mare's milk from Dompu had the best quality which was indicated by the absence of bacteria that have the potential to be pathogenic or food spoilage, such as members of the Enterobacteriaceae family (*Enterobacter*, *Klebsiella* and *Escherichia-Shigella*) and *Pseudomonas*. Genus of *Kazachstania* and *Kluyveromyces*, as well as family Dipodascaceae were frequently observed fungi/yeast from Sumbawa fermented mare's milk. The presence of potential pathogenic bacteria warrants special attention in improving the hygiene of manufacturing process.

Keywords: fermented mare's milk, high-throughput sequencing, lactic acid bacteria, metagenomic, QIIME, yeast

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Introduction

Fermented mare's milk, one of special commodity from Sumbawa Island, Province of West Nusa Tenggara, Indonesia, is made naturally without addition any starter cultures. The fresh milk is poured in a clean plastic container and then let it stand at room temperature for several days. The nutritional content of mare's milk is different with cow's milk. Mare's milk is easily digested with the nutritional content is almost similar to human milk. The optimum ratio of whey protein and casein has become the mare's milk is very suitable for infant diets (Uniacke-Lowe et al., 2010).

Recently, the indigenous microflora of Sumbawa mare's milk were studied based on dependent-culture method. As a result, some important and the uncultured microbes were not thoroughly investigated. The investigation of indigenous mare's milk microflora was still limited on lactic acid bacteria (LAB) with probiotic properties (Nuraida, 2015; Shi et al., 2012; Sujaya et al., 2008). Information on the other microbial groups such as fungi/ yeast has not been reported. The interaction between LAB and yeast in the naturally fermented milk products cannot be ignored (Altay et al., 2013; Jatmiko et al., 2012; Nyambane et al., 2014). Lactic acid bacteria in koumiss, product from Central Asia, play an essential role in developing flavor, texture and acidity level as well as

bring some health benefits, such as probiotics. Mean while, the ability of yeast in this product in fermenting lactose into alcohol has resulted a unique flavor (Mu et al., 2012).

Therefore, a metagenomic approach is essential to be elucidated to obtain comprehensive information of microbial groups in the fermented mare's milk. Next-generation sequencing (NGS) involving illumina high-throughput sequencing is the most applied in the metagenomic approach to profile the microbial diversity. In this research, the microbial diversity in naturally fermented Sumbawa mare's milk was investigated, and the quality of fermented mare's milk based on the presence of pathogenic or undesirable microorganisms was also evaluated.

Method

Sample Collection

Fresh mare's milk from Sumbawa was collected from a horse farm in three farming areas namely Dompu Regency, Bima Regency and Sumbawa Regency, Province of West Nusa Tenggara. One sample of fresh mare's milk was obtained from each farming location. Mare's milk was fermented naturally in a clean plastic container, and then incubated at room temperature for five days. The samples transported to Microbiology Laboratory, Faculty of Mathematics and Natural Sciences, Brawijaya University for further analysis.

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Microbial Isolation

Microbial isolation was performed by transferred 25 mL of fermented milk samples into 225 mL of saline water (NaCl 0.85%) served as 10^{-1} dilution. Serial dilution was conducted by taking 1 mL from each dilution until 10^{-6} . Samples from each dilution (0.1 mL) were inoculated into respective culture media on Petri dishes in duplicate. Media of plate count agar (PCA) for total bacteria, de Man Rogosa and Sharpe (MRS) agar for lactic acid bacteria and yeast peptone dextrose (YPD) agar supplemented with streptomycin 50 ppm for yeast. The incubation condition was conducted for each microbial group: 30°C for 24-48 h, 37°C for 24-48 h, 30°C for 48-72 h for total bacteria, LAB and yeast, respectively.

Determination of Nutritional Content and Acidity Level

Nutritional content (total protein, fat content, carbohydrate, total sugar, fiber, water content and ash) and acidity level of naturally fermented mare's milk was measured according to AOAC method.

Extraction of Genomic DNA and High-Throughput Analysis

To remove lipids, proteins and salts from fermented milk samples, 1 mL of fermented mare's milk was firstly emulsified with 9 mL of sterile trisodium citrate buffer (2%), and then incubated at 37°C for 5 min. After that, the mixture was homogenized using vortex at maximal speed for 1 min. Next, the samples were centrifuged at 12000 g for 1 min at room temperature (Aldrete-Tapia et al., 2014). The pellet was used for total DNA extraction using FastDNA Spin kit (MPBIO) by following the manufacturer's instructions. The quality and quantity of total DNA were checked using nanodrop spectrophotometer (ratio A260/ 280).

The next-generation sequencing analysis was performed at Macrogen Inc., South Korea using illumina (MiSeq) platform (paired-end reads). Primers used in this sequencing were V3-V4 region for bacteria (41F: 5'-

CCTACGGGNGGCWGCAG-3';805R: 5'GACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2013), and for fungi/ yeast was ITS1-F (CTTGGTCATTTAGA GGAAGTAA and ITS2-R (GCTGCGTTCTTCATCG ATGC) (Porter et al., 2011). In order to remove poor quality sequences, chimera, raw reads were filtered and further analyzed using QIIME v.1.9.1 to produce diversity profiles. The similarity of OTU was aligned with references from SILVA database.

Result

Microbial Density and Nutritional Content

The aerobic bacteria, lactic acid bacteria (LAB: *Lactobacillus* and *Lactococcus*) and yeast were isolated and enumerated using specific media. In general, the density of microbes in fermented Sumbawa mare's milk collected from three farming areas namely Dompou, Bima and Sumbawa was varies (Table 1). The highest aerobic bacterial density was observed in Dompou (3.73×10^5 CFU/mL), while the highest LAB density growing on MRS agar (*Lactobacillus* group) was mare's milk from Bima (1.43×10^4 CFU/mL). The highest number of LAB growing on M17 agar (*Lactococcus* group) and yeast were fermented mare's milk from Sumbawa, which were 4.75×10^5 and 6.50×10^4 CFU/mL, respectively.

Nutritional contents of fermented mare's milk among three farming areas was slightly different especially in terms of protein, fat, and carbohydrate (Table 1). Protein content of fermented mare's milk from Sumbawa was higher than those in Bima and Dompou. In contrast, fermented mare's milk from Sumbawa contained the lowest concentration of fat and carbohydrate. According to Indonesian National Standard No. 01-6054-199, the protein content of Sumbawa sample was in accordance with the standard (more than 0.2%). Fat content from Bima sample was the only sample which has fulfilled the standard (more than 1.3%). The acidity level of all milk samples was met the standard quality (pH > 3).

Table 1. Microbial density, nutritional content and acidity level of naturally fermented Sumbawa mare's milk

Parameters	SNI	Horse farming location		
	01-6054-1999	Dompou	Bima	Sumbawa
Total of aerobic bacteria (CFU/mL)	-	3.73×10^5	3.80×10^4	3.68×10^5
Total of LAB (MRS agar) (CFU/mL)	-	2.57×10^3	1.43×10^4	3.00×10^1
Total of LAB (M17 agar) (CFU/mL)	-	1.98×10^3	6.18×10^3	4.75×10^5
Total of Yeast (CFU/mL)	-	1.78×10^3	3.80×10^3	6.50×10^4
Protein total (%)	Min. 2.00	0.80	1.30	2.49
Fat total (%)	Min. 1.30	1.05	1.41	0.18
Water content (%)	-	93.09	91.46	96.94
Ash (%)	-	0.28	0.34	0.27
Carbohydrate total (%)	-	4.78	5.49	0.12
Sugar total (%)	-	2.18	2.42	2.18

Fiber (%)	-	0.37	n.d	0.73
pH	Min. 3.00	3.31	3.28	3.11

Bacterial Community Profile

In phylum level, Firmicutes and Proteobacteria were found in all fermented mare's milk samples (Figure 1). Phylum of Cyanobacteria (17%) and Actinobacteria (4%) were only found in fermented mare's milk from Sumbawa. Firmicutes was dominant (more than 90%) in fermented mare's milk from Dompu and Bima, while Proteobacteria was dominant in Sumbawa sample (22%) followed by Firmicutes (23%).

The number of bacterial genus obtained from fermented mare's milk was 11 genera (as well as three families) (Figure 2). *Lactobacillus* was the most dominant genus in both Dompu (90%) and Bima (81%) samples, while *Novosphingobium* (23%) was the dominant genus

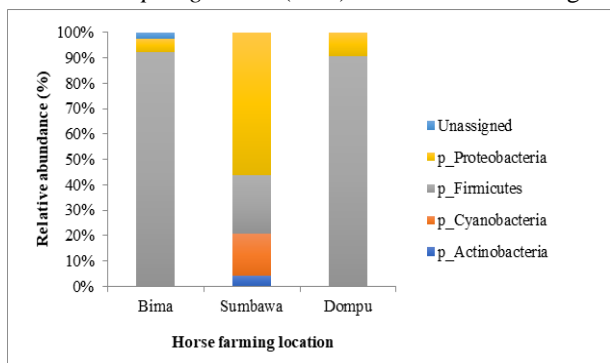


Figure 1. Relative abundance of bacteria sequence read at the phylum level

Fungal/Yeast Community Profile

The sample from Dompu was the only fungal/yeast DNA sample that meets the requirement of next generation sequencing. Therefore, there were two different horse farming from Dompu was utilized in this study, namely Dompu1 and Dompu2. A total of two phyla (Ascomycota and Basidiomycota) were found in all fermented mare's milk samples. At phylum level, all fermented mare's milk was dominated by phylum Ascomycota (100% for fermented mare's milk from Dompu1 and 84.5% for fermented mare's milk from Dompu2) (Figure 3).

The sequences of fungal/ yeast DNA classified until genus level, which was only two genera found in Dompu1 sample (*Kazachstania*-53% and *Kluyveromyces*-47.3%) (Figure 4). In Dompu2 sample, five genera (as well as one family and one order) have been detected namely family Dipodascaceae (53.2%), *Candida* 16.1%, *Trichosporon* 13.4%, *Kazachstania* 7.4%, order Saccharomycetales 5.2%, *Cyberlindnera* 0.6%, and *Malassezia* 0.6% (Figure 4). The yeast composition in Dompu2 sample was more diverse than Dompu1, as can be seen from the variety of family/genus found in Dompu2 sample.

in Sumbawa sample. The second largest genus in Bima and Dompu were *Lactococcus* (9%) and *Acetobacter* (9.2%), respectively. Genera of *Lactobacillus* and *Enterobacter* were found in equal abundance (14.6%) in Sumbawa sample. Other Enterobacteriaceae members in Bima sample were very low proportion (less than 10%) such as *Klebsiella* and *Escherichia-Shigella*. Genus of *Pseudomonas* in Sumbawa sample was also detected in a low number (4.4%). Acetic acid bacteria which also frequently observed in naturally fermented milk products was only detected in fermented mare's milk from Dompu by 9.2% representing for genus *Acetobacter*. In terms of bacterial composition (12 families or genera level), fermented mare's milk from Sumbawa Regency exhibited a high diversity compared to the other samples.

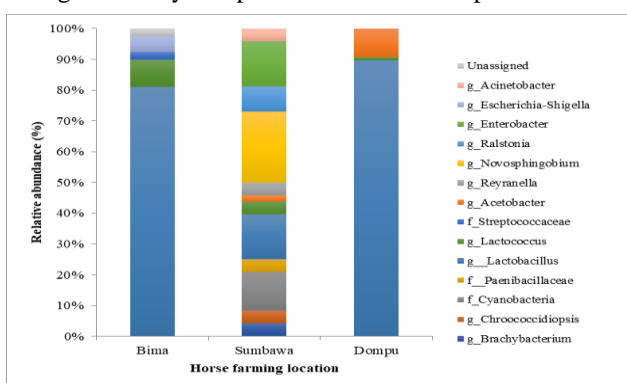


Figure 2. Relative abundance of bacteria sequence read at the genus level

Discussion

Naturally fermented Sumbawa mare's milk product from three regency (Dompu, Bima and Sumbawa) have been analyzed including nutritional contents as well as the acidity level and the total of microbes (aerobic bacteria, LAB and yeast). Nutritional contents affect the microbial density and diversity. The ability of microbes in utilizing carbon sources in form of protein, lipid and carbohydrate containing in the milk interfere the microbial growth rate. Furthermore, the manufacturing processes such as milking and packaging also affect the microbial composition in the products.

Lactic acid bacteria was a dominant bacterial group in the two samples of mare's milk (Dompu and Bima). The Indonesian fermented mare's milk has a similarity with koumiss in term of making process and the flavor. In koumiss, *Streptococcus* occupied the second largest after *Lactobacillus* (Zhong et al., 2016). Genus *Lactobacillus* also dominated tarag, a naturally fermented cow's milk from Mongolia and Northern China (Sun et al., 2014). The presence of family Enterobacteriaceae (genus *Enterobacter*) in fermented mare's milk from Sumbawa Regency with a relatively high proportion (around 14%) was also a concern in other fermented milk products (Hervert et al., 2017; Martin et al., 2016). *Pseudomonas*

as a psychrotrophic bacterial group is commonly found in raw milk causing spoilage of dairy products (Uraz and Citak, 1998). The presence of these bacterial groups indicate unhygienic conditions and contamination from

either fecal material or the dairy farm environment, such as water, equipment, plant materials and dirt (Martin et al., 2016).

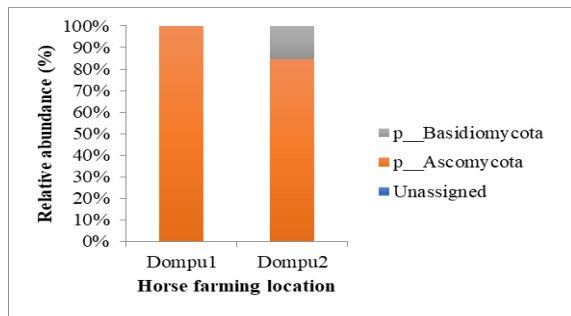


Figure 3. Relative abundance of yeast sequence read at the phylum level

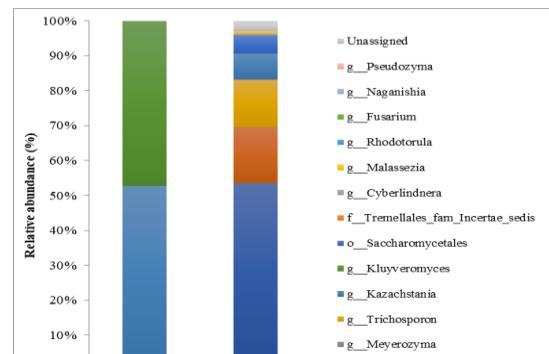


Figure 4. Relative abundance of fungal/yeast sequence read at the order/family level

Yeast in naturally fermented milk is commonly detected and contribute to the fermentation process. Ascomycotina was also a dominant phylum in homemade yoghurt from Xinjiang Ugyur, China (Xu et al., 2015). *Dipodascus geotrichum*, a member of Dipodascaceae family was frequently found in kefir grains (Dertli et al., 2017). *Trichosporon* was also commonly detected in milk samples, but its presence indicates a low hygiene standard (Sun et al., 2014). *Kazachstania unispora* was also detected in koumiss (Mu et al., 2012). The presence of *Candida* was reported in naturally fermented milk products of Indonesia, such as *Candida stelimalicola* in dadih (Jatmiko et al., 2012), and *Candida* sp. in dangke (Syah, 2012). The common feature of naturally fermented product is the microbial diversity varies among production time (Zhong et al., 2016). This can be seen from the fungal/yeast diversity observed in both Dompu samples. Although the horse farm location was similar, the different time of sampling of fermented milk product showed different fungal/yeast diversity. This might be due to different horse and environmental factors involving during milking and packaging.

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