Microbiota community structure in traditional fermented milk dadiah in Indonesia: Insights from high-throughput 16S rRNA gene sequencing

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Abstract

Dadiah is an Indonesian traditional fermented milk and is neither pasteurized nor boiled, but no food poisoning has been reported to date. Microbiota inhabiting dadiah has never been fully explored. In this study, we performed deep sequencing of 16S ribosomal RNA genes extracted from 11 dadiah samples and analyzed the dadiah microbiota at the genus level. We found that *Lactococcus*, *Lactobacillus*, and *Leuconostoc* were predominant among the dadiah microbiota. Unexpectedly, *Klebsiella* and *Chryseobacterium*, potential pathogens, were also found in some of the dadiah samples. There was little difference in the microbiota among samples taken from the same bamboo tube. In contrast, there were differences between the dadiah microbiota from different bamboo tubes, even those collected from the same sampling area. Furthermore, the composition of the dadiah microbiota showed large differences between sampling areas. We believe that our findings will lead to further improvement in the preparation of dadiah.

Key words: Dadiah, fermented buffalo milk, deep sequencing, 16S ribosomal RNA, microbiota

Introduction

Dadiah is an Indonesian traditional fermented milk (yogurt-like product) that is consumed in West Sumatra, Indonesia. Milk is safe to consume after pasteurization, but dadiah is produced by pouring fresh, raw, unheated (unpasteurized) buffalo milk into bamboo tubes covered with banana leaves. The bamboo tube is incubated at room temperature (28–32°C) for two days, allowing it to ferment spontaneously. Fresh bamboo tubes are used in each dadiah production. The most common lactic acid bacteria genera in raw dairy milk are *Lactococcus*, *Lactobacillus*, *Leuconostoc*, and *Enterococcus* [1], with *Lactobacillus*, *Lactococcus*, and *Leuconostoc* also being dominant in dadiah [2]. However, the composition of microbial communities (microbiota) inhabiting dadiah is still largely unknown.

Interestingly, there has been no product failure and to date, no food poisoning has been officially reported by people who have consumed dadiah [2], even though dadiah is produced from unpasteurized milk under (sub)tropical conditions. Therefore, our goals are to fully characterize the dadiah microbiota. To this end, in this study, we collected the bacterial composition data of 11 dadiah samples from four local areas in Indonesia. The dadiah microbiota populations were characterized by deep sequencing of 16S ribosomal RNA (rRNA) amplicons. Our results indicate the global and local variations in the bacterial communities of dadiah.

Materials and methods

Sample collection:

Samples were obtained from four local areas (Batusangkar, Alahan Panjang, Padang Panjang, and Agam) in West Sumatra, Indonesia during the period from October 2014 to October 2016, because dadiah is not produced continuously throughout the year (Figure 1A). Because dadiah is rarely manufactured as a large-scale dairy product, we obtained the samples from traditional markets in each area under (sub) tropical conditions. Samples from different local areas were produced from different buffalo milk sources. All the samples were transported by air to Okayama University, Japan, were kept below freezing temperature during transit, and were then stored at –20°C in the laboratory until they were required for DNA extraction.

Preparing 16S rRNA gene amplicons:

The 16S rRNA amplicon sequencing technique is based on the amplification of small fragments of one or two of the nine hypervariable

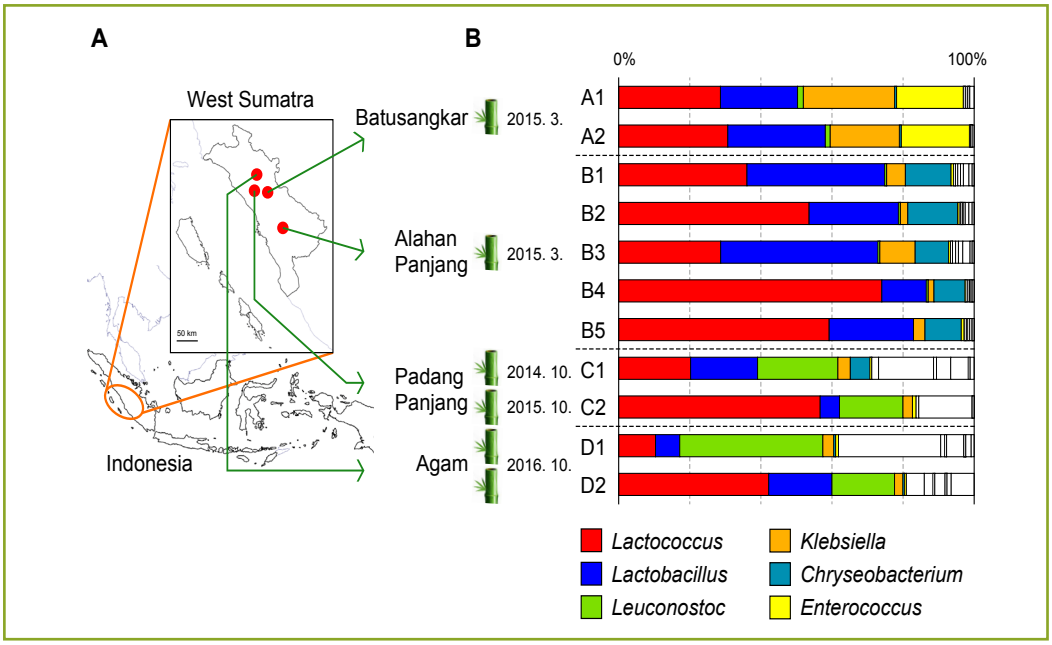

Figure 1: Sample collection and the dadiah microbiota. (A) Sampling areas. All 11 samples (six bamboo tubes) were obtained from four local areas in West Sumatra, Indonesia. Dates of sampling are also shown. (B) Composition of the dadiah microbiota at the genus level. To clarify the diversity of the microbiota, the remaining 10 genera (*Acetobacter*, *Acinetobacter*, *Bifidobacterium*, *Raoultella*, *Serratia*, *Corynebacterium*, *Staphylococcus*, *Stenotrophomonas*, *Frateuria*, and unclassified) are shown in white

regions in the 16S rRNA gene [3]. Bacteria and bacterial DNA were prepared using a modification of our previous method [4, 5]. DNA was purified by treatment with ribonuclease A (Wako), followed by precipitation with 20% PEG 6000 (Nacalai Tesque) in 2.5 M NaCl. The pelleted DNA was rinsed with 75% ethanol, and dissolved in TE buffer. The V3-V4 hypervariable region of the bacterial 16S rRNA gene was polymerase chain reaction (PCR)-amplified using the barcode-tag universal primer sets 341F–805R (341F 5'-CCTACGGGNGGCWGCAG-3' and 805R 5'-GACTACHVGGGTATCTAATCC-3') [6]. The PCR amplification was performed using the following program: 95°C for 3 min, 25 cycles (95°C for 30 s, 55°C for 30 s, 72°C for 30 s), 72°C for 30 s, then hold at 4°C. Agilent 2100 Bioanalyzer (Agilent Technologies) was used to determine the size (approximately 550 base pairs) of the PCR fragments. The PCR products were purified using Agencourt AMPure XP Beads (Beckman Coulter). Nextera XT Index Kit (Illumina) was used for labeling the amplicons with different dual barcodes.

Deep sequencing and data analysis:

Deep paired-end sequencing was performed using the Illumina MiSeq platform. The obtained raw sequence data comprising samples/reads (A1/147,498; A2/193,368; B1/227,094; B2/157,520; B3/174,554; B4/143,438; B5/181,710; C1/388,628; C2/157,214; D1/135,904; and D2/121,896) was initially processed using Mothur v1.38.1 [7] for barcode splitting. The resulting demultiplexed sequences were clustered into bins called Operational Taxonomic Units (OTUs) based on 97% sequence similarity, using the Ribosomal Database Project classifier [8]. Only genera containing over 1% of total OTUs were used for subsequent analysis. The taxonomy of each OTU was assigned using the SILVA rRNA database [9]. SPSS program v23.0 [10] was used for principal components analysis (PCA). The sequence data sets supporting the results of samples/reads (A1, A2, B1, B2, B3, B4, B5, C1, C2, D1, and D2) in this article are available in the NCBI Sequence Read Archive under accession number SRA556056.

Results and discussion

To investigate the bacterial composition in dadiah, we first collected 11 dadiah samples from six bamboo tubes, obtained from four local areas in West Sumatra, Indonesia (Figure 1A). To examine the diversity of the microbiota in an identical dadiah, samples from Batusangkar and Alahan Panjang were obtained from different sites inside the same bamboo tube without stirring. Moreover, to examine the differences in the microbiota between bamboo tubes, each sample from Padang Panjang and Agam was obtained from different bamboo tubes after stirring. The V3-V4 region of the 16S rRNA gene was amplified from the 11 samples and subjected to deep sequencing. We obtained a total of 2,028,824 reads and 39,677 high-quality filtered reads per sample. The high-quality filtered read was clustered into 1,054 phylotypes (OTUs: at 97% sequence identity), and their representative sequences were used in the taxonomic analysis. Thus, the OTUs were assigned to 154 genera, 16 of which showed over 1% of the total OTUs and were used for subsequent analysis.

The bacterial composition of the 11 samples was determined for each taxonomic level according to the read counts of the 16 genera in each sample. As expected, the microbiota of all the samples were highly abundant in *Lactococcus* and *Lactobacillus* (Figure 1B), which is consistent with a previous report [11]. This core microbiota (*Lactococcus* and *Lactobacillus*) accounted for more than 40% of the total reads of the 16 genera in all the samples except D1. In sample D1, *Leuconostoc* was the most predominant genus. These results indicate that indigenous lactic acid bacteria (*Lactococcus*, *Lactobacillus*, and *Leuconostoc*), which are derived from buffalo milk, bamboo tubes, and/or banana leaves, may be involved in the fermentation of dadiah without starter culture.

There were some subdominant genera enriched in dadiah. Unexpectedly, *Klebsiella* and *Chryseobacterium*, which are considered as potential pathogens, were also found in dadiah (especially samples A1–A2 and B1–B5) (Figure 1B). *Klebsiella* belongs to the *Enterobacteriaceae* family and is used as an indicator of fecal contamination [12], whereas *Chryseobacterium* has been found in soil and water environments [13]. *Enterococcus*, which is also an indicator of fecal contamination [14], was also found in dadiah, especially in samples A1–A2 (Figure 1B). Because dadiah is neither pasteurized nor boiled, it is possible that these potential pathogens could contaminate dadiah during the production process, which is not ideal, although no food poisoning has been officially reported to date [2]. Lactic acid and bacteriocin, which are produced by the core microbiota (*Lactococcus and Lactobacillus*), may contribute to the suppression of potential pathogens in dadiah. The

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presence of potential pathogens in dadiah remains to be evaluated.
Genus composition in dadiah varied substantially between the four sampling areas (Figure 1B). The dadiah microbiota from Batusangkar was abundant in *Klebsiella* and *Enterococcus*, as mentioned above, whereas the dadiah microbiota from Padang Panjang and Agam were abundant in *Leuconostoc*. The diversity of the dadiah microbiota from the four sampling areas was analyzed by PCA (Figure 2). There was little

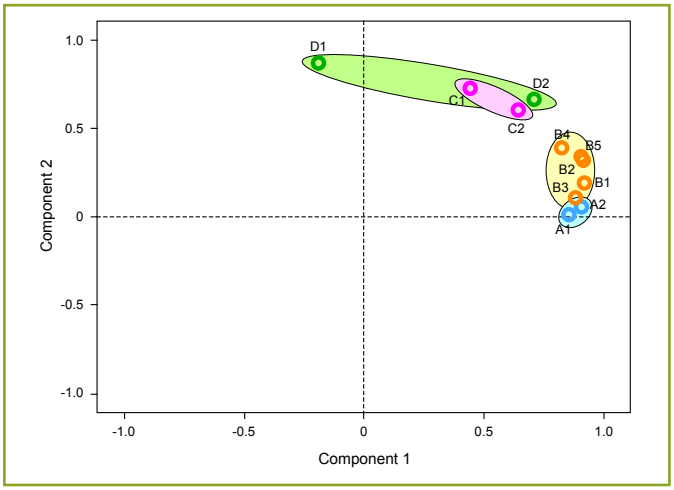

Figure 2: Principal component analysis of the dadiah microbiota. PCA was conducted with microbiota data from the 11 dadiah samples. PCA scores were plotted based on the presence and absence of individual OTU in individual samples. Circles with the same color represent samples obtained from the same sampling areas

difference among the samples (A1–A2 and B1–B5) obtained from the same bamboo tube (Figures 1 and 2), showing that the predominant microbiota in dadiah remains relatively constant in a single bamboo tube. In contrast, there were large differences between the samples (C1–C2 and D1–D2) obtained from different bamboo tubes, even those collected from the same sampling area (Figures 1 and 2). Interestingly, the microbiota of D2 more closely resembled that of C2 than D1 (Figure 2). These results indicate that the composition of dadiah microbiota is different in every bamboo tube. Because dadiah is still a homemade product made by traditional methods in Indonesia, different microbiota may be generated in every bamboo tube and every local area.
This study revealed variations in dadiah microbiota by deep sequencing. Besides the bamboo tube, fresh raw buffalo milk, fermentation temperature, and time are the important factors during spontaneous dadiah fermentation. However, detailed information about these factors is missing in this study, because a standard protocol for dadiah production is not well established. Further research is required to explore the main sources for the described dadiah microbiota characteristics.

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Conflict of interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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