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Monitoring of the microbiota profile in nukadoko, a naturally fermented rice bran bed for pickling vegetables

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Nukadoko is a fermented rice bran mash traditionally used for pickling vegetables in Japan. To date, the production of both homemade and commercial nukadoko depends on natural fermentation without using starter cultures. Here, we monitored chemical and microbiological changes in the initial batch fermentation of nukadoko. Nukadoko samples were prepared by spontaneous fermentation of four different brands of rice bran, and microbiome dynamics were analyzed for 2 months. In the first week, non-*Lactobacillales* lactic acid bacteria (LAB) species, which differed among the samples, grew proportionally to pH decrease and lactate increase. Thereafter, *Lactobacillus plantarum* started growing and consumed residual sugars, causing further lactate increase in nukadoko. Finally, microbial communities in all tested nukadoko samples were dominated by *L. plantarum*. Taken together, our results suggest that the mixture of the fast-growing LAB species and slow-growing *L. plantarum* may be used as a suitable starter culture to promote the initial fermentation of nukadoko.

[**Key words:** Nukadoko; Fermented rice bran; Microbiota; Lactic acid fermentation; 16S rRNA; Pyrotag sequencing; *Lactobacillales*; *Lactobacillus plantarum*]

Nukazuke is a type of pickled vegetables in Japan. Nukadoko is a fermented rice bran bed used for nukazuke that gives pickling vegetables a salty and sour taste and a special flavor (1); it also has high levels of vitamins B1 and B2 (2). Nukadoko is initially prepared by natural fermentation of vegetables in rice bran until a fermenting culture has been established; the well-fermented nukadoko can be renewed by adding fresh rice bran. This refreshment is performed every few months, and in some cases, nukadoko has been maintained for more than 100 years without spoilage, even though the ingredients, including vegetables and rice bran, are not sterilized.

The fermented nukadoko harbors a number of lactic acid bacteria (LAB) species (2,3), which are thought to be responsible for its remarkable resistance to spoilage. In our previous study, we investigated the mechanism of this superlative preserving property of nukadoko by analyzing the microbial community structure in the aged nukadokos. Two *Lactobacillus* species, *Lactobacillus acetotolerans* and *L. namurensis*, were found to be the dominant species (4). These two species showed different growth profiles in nukadoko: *L. namurensis* grew fast (generation time of 6.2 h) and was a key lactic acid producer in the LAB consortium, whereas *L. acetotolerans* grew very slow (generation time of 92.9 h) and dominated the nukadoko microbiota after a few weeks of riping (4). As long as the ultra-aged nukadoko was used as a starting culture for refreshment, these two LAB species displayed the same growth kinetics (4,5), suggesting that the balance between these two lactobacilli is a key factor in the stabilization of nukadoko during the refreshment.

However, little information is available about the microbial composition of nukadoko starting cultures, which can show high variability depending on the ingredients and fermentation conditions that are difficult to control even in the laboratory experiment. In this study, we employed pyrotag sequencing (6–9), together with biochemical and culture-based methods to analyze the composition of bacterial communities in nukadoko starting cultures. We prepared four laboratory model nukadoko samples by using different brands of rice bran produced in various prefectures in Japan. The obtained information on the microbiota in the initial batch of nukadoko fermentation will aid in the generation of a starter culture for the control and stabilization of the microenvironment and nutritional quality in nukadoko and nukazuke.

MATERIALS AND METHODS

Preparation and sampling of nukadoko Four brands of rice produced in Niigata (NG), Yamagata (YG), Oita (OT), and Saitama (ST) prefectures in Japan were milled to obtain rice bran by using the same milling machine. As shown in Table 1, nutrition in rice bran did not differ among the samples. Each rice bran sample (1050 g) was kneaded with water (1800 ml) and NaCl (150 g). The resulting rice bran paste was incubated at 24°C for 60 days, with mixing by hand covered with sterilized latex gloves once a day and pickling an eggplant and a cucumber every

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TABLE 1. Composition of nukadoko samples used in this study.

Component[a]	Nukadoko sample[b]			
	NG	YG	OT	ST
Rice bran brand (prefecture)	Koshihikari (Niigata)	Hagenuk (Yamagata)	Koshihikari (Oita)	Milky Queen (Saitama)
Nutrition in rice bran (%)				
Moisture	13.0	14.0	12.5	13.3
Protein	11.5	12.7	13.4	13.0
Fat	20.5	18.8	18.0	18.5
Ash	7.2	8.1	7.5	7.3
Carbohydrate	47.8	46.4	48.6	47.9

[a] Moisture, protein, fat, ash, and carbohydrate were analyzed by air oven method, Kjeldahl, acid decomposition, direct ashing, and subtraction methods, respectively.
[b] Nukadoko samples were prepared by mixing the indicated rice bran (1050 g) with water (1.8 L) and NaCl (150 g).

2 days. The salt concentration was maintained in the range of 4–5% by adding NaCl three times during the experiment.

Each batch of nukadoko was sampled (20 g) at 2, 5, 8, 11, 15, 18, 22, 25, 29, 32, 36, 40, 44, 50, 54, and 60 days. Samples for the pyrotag sequencing and chemical analysis were stored at −80°C until use, whereas those for bacterial cultures were processed immediately after sampling.

Chemical analysis Ten grams of nukadoko was blended in 90 ml sterile NaCl solution (0.85%, w/v) and used for pH measurement and chemical composition analysis. Salinity in nukadoko was analyzed by the Mohr method (10).

Organic acid and sugar concentrations were determined by high performance liquid chromatography (HPLC). Organic acid analysis was performed using an RSpak KC-811 column (Shodex, Tokyo, Japan) coupled to a UV detector, at 60°C with 3 mM $HClO_4$ as the mobile phase at a flow rate of 1.0 ml/min. Sugar concentration was analyzed using Asahipak NH2P-50 4D (Shodex) coupled to a refractive index (RI) detector, at 30°C with 75% acetonitrile as a mobile phase at a flow rate 0.8 ml/min.

DNA isolation from nukadoko Total genomic DNA from nukadoko samples was extracted according to a previously described procedure combined with the bead beating method (4) by using the QIAamp DNA stool Mini Kit (Qiagen, Hilden, Germany). Nukadoko (200 mg) was washed twice with 1 ml phosphate-buffered saline (PBS, pH 7.4) and re-suspended in 1 ml PBS. The suspension was transferred to a 2.0-ml screw-capped tube containing 0.3 g of zirconium beads (0.1 mm in diameter; As One Corporation, Osaka, Japan). The tube was beaten at 2700 rpm for 3 min at 4°C by using a bead-beater instrument (Multi-beads Shocker; Yasui Kikai, Osaka, Japan). Subsequently, 300 μl of phenol-chloroform-isoamyl alcohol (25:24:1) was added to the lysed suspension, and the mixture was shaken at 2700 rpm for 40 s at 4°C and centrifuged at 20,000 ×*g* for 1 min at 4°C. The upper layer was then applied onto a QIAamp Spin Column (Qiagen), washed, and eluted with 100 μl of distilled water according to the manufacturer's instructions.

Amplification of 16S rRNA V6–V8 region The V6–V8 fragment of the 16S rRNA gene was amplified from total bacterial DNA by using universal primers Q-968F-# (5′-CWSWSWWSHTWACGCGARGAACCTTACC-3′) and Q-1390R-# (5′-CWSWSWWSHTTGACGGGCGGTGWGTAC-3′) (number sign (#) indicates a series of 64 barcode tags underlined in the sequence). PCR was performed in a total volume of 50 μl containing 10 ng DNA template, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphate (dNTP) mixture, 10 pM of each primer, and 1.25 U TaKaRa Ex Taq HS DNA polymerase (Takara Bio, Shiga, Japan). The cycling conditions were as follows: 98°C for 2.5 min; 20 cycles at 98°C for 15 s, 54°C for 30 s, and 72°C for 20 s; and 72°C for 5 min. The amplified products were purified using the QIAquick PCR Purifications Kit (Qiagen) according to the manufacture's protocol and quantified using a NanoDrop Lite microphotometer (Thermo Scientific, Waltham, MA, USA).

Pyrotag sequencing Equal amounts (100 ng) of amplicons from different samples were pooled and purified by conventional ethanol precipitation. The mixed DNA was clonally amplified by emulsion PCR using the GS FLX Titanium LV emPCR Kit v2 according to the manufacturer's protocol (454 Life Science, Branford, CT, USA). Beads carrying amplified DNA were pooled and loaded onto one-half of a GS FLX Titanium PicoTiter Plate, and 454 pyrosequencing was conducted using an FLX Genome Sequencer (454 Life Science).

The analysis of pyrosequencing data was performed according to a previously published method (11,12). The obtained 454 batch sequence data were sorted into each sample batch using the QIIME split_library.py script (http://qiime.org/scripts/split_libraries.html) with barcode sequences. As a result,296,607 sequences were assigned to 64 nukadoko samples, with an average of 4634 ± 1265 reads per sample.

To prepare a non-redundant sequence set, 296,607 sequences were dereplicated within 99% nucleotide sequence identity by using the pick_otus_through_otu_table.py script of QIIME (http://qiime.org/1.6.0/scripts/pick_otus_through_otu_table.html). As a result, a set of 12,803 non-redundant operational taxonomic units (OTUs) was obtained. Chimeras were removed using the Chimera.uchime program in Mothur 1.25.1 (http://www.mothur.org/wiki/Download_mothur) and the 16S rRNA sequence dataset gg_97_otus_4feb2011.fasta (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi). As a result of chimera check, 9821 OTUs corresponding to 278,779 reads were selected for further analysis as non-chimera sequences.

In order to get taxonomic information, the selected 9821 OTUs were subjected to two web-based searches, the Ribosomal Database Project (RDP) classifier (13) and RDP SeqMatch (14) (http://rdp.cme.msu.edu/). In the RDP classifier search, the cut-off value of confidence threshold for taxonomic classification was set at 80% as generally recommended. The RDP SeqMatch k-nearest-neighbor tool was employed to gain the data on composition of bacterial community at the species level. The seqmatch Q400 algorithm can be used to find the 20 closest 16S rRNA neighbors of the cultured strain in the RDP database and to convert the RDP SeqMatch results to the data on species composition (11). In seqmatch Q400, the species showing the best match were assigned to the query sequence, and if more than two species showed the same similarity score, the one with the highest count among the 20 closest neighbors was selected. Because of the high similarity in the V6–V8 region, some species observed in this study could not be discriminated from others shown in parentheses: *Lactobacillus plantarum* (*Lactobacillus pentosus*, *Lactobacillus paraplantarum*), *Lactobacillus curvatus* (*Lactobacillus sakei*), *Enterococcus faecalis* (*Enterococcus faecium*, *Enterococcus lactis*, *Enterococcus durans*), and *Weissella cibaria* (*Weissella confusa*). Thus we added group after the species name. The data collected on the microbiota in each nukadoko sample were profiled based on the catalog of 9821 non-redundant taxonomically annotated OTUs. The relative abundance of each taxonomic group was calculated by dividing the read count of identified sequences by the total read count in each sample.

Microbiota analysis by the culture method Ten grams of nukadoko was suspended in 90 ml sterile NaCl solution (0.85%, w/v), diluted, spread on MRS (*Lactobacillus*) agar (Difco, BD, Sparks, MD, USA), and incubated at 30°C for 48 h. The colonies grown on the selected plates (30–300 colonies per plate) were counted as colony-forming units (CFUs). Ten colonies were randomly picked.

The isolated LAB was identified by analyzing the 16S rRNA and recA gene sequences, and using API 50 CHL system (Sysmex bioMérieux, Lyon, France). The V1–V4 region of 16S rRNA was amplified from total bacterial DNA by using primers 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and Q-926R (5′-CCGTCAATTCCATTTRAGTTT-3′). PCR was performed in a 25 μl containing 10 ng extracted DNA, 10 mM Tris–HCl (pH 8.2), 200 mM NaCl, 20 μM EDTA, 0.2 mM DTT, 200 μM dNTP mixture, 200 nM of each primer, and 0.025 U PrimeSTAR HS polymerase (Takara Bio). The cycling conditions were as follows: 98°C for 2.5 min; 30 cycles at 98°C for 10 s, 55°C for 5 s, and 72°C for 60 s; and 72°C for 5 min. The amplified products were purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's protocol and analyzed by Sanger sequencing in Operon Biotechnologies in Japan. To distinguish *L. plantarum* from *L. pentosus* and *L. paraplantarum*, for isolates that have been identified as *L. plantarum* by 16S rRNA sequence, recA gene was analyzed by multiplex PCR assay as previously described (15). API 50 CHL system was performed according to the manufacturer's protocol. Lack of growth in the medium containing glycerol as a sole carbon source distinguished *L. plantarum* from *L. pentosus*.

RESULTS

Chemical changes during the initial nukadoko fermentation The changes in pH values and concentrations of organic acids and sugars were monitored in the initial batch fermentation of four nukadoko samples made from different brands of rice bran (Fig. 1). Within the first 2–3 weeks, the pH quickly declined from the initial value of 6.5 to the low level between 4.0 and 4.2; the drop was particularly dramatic in the first week. Accordingly, the level of lactic acid increased in the range between 150 and 200 mM in all nukadoko samples. The production of acetic acid, however, differed among the samples. In NG and YG samples, acetate increased gradually in the first 3 weeks up to 10 and 20 mM, respectively, and this level was sustained until the end of the experiment. In contrast, in OT and ST nukadoko samples, acetate increased rapidly to approximately 40 mM in the first 10 days but then started gradually decreasing until the end of the experiment.

Sugars, mainly glucose, fructose, and sucrose were detected in all nukadoko samples at the initial concentrations of 50 mM, 25 mM, and 40–90 mM, respectively. In all nukadoko samples, glucose and fructose showed an increase from day 2 to 5, whereas sucrose started decreasing from the beginning and was exhausted in 1 week. Sugar exhaustion coincided with the end of lactate