Effects of Japanese pepper and red pepper on the microbial community during nukadoko fermentation

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Nukadoko is a fermented rice bran bed traditionally used for pickling vegetables in Japan. To date, the production of both homemade and commercial nukadoko has depended on natural fermentation without using starter cultures. Spices, Japanese pepper, and red pepper, are added to nukadoko empirically, but the functions of spices in nukadoko have not been fully elucidated. To investigate the effects of Japanese pepper and red pepper on nukadoko fermentation, we compared the chemical and microbiological changes during 2 months of fermentation of a laboratory model nukadoko with or without spices. The successive pH values and colony counts in the first 10 days showed that the spices promoted lactic acid bacteria (LAB) growth and fermentation in the nukadoko niche. The successive bacterial communities during natural fermentation of nukadoko were carefully monitored by pyrotag 16S rRNA analysis, and the effect of spices on the development and maintenance of the nukadoko microbiota was investigated. It was shown that addition of Japanese peppers and red peppers shortened the pre-lactic acid fermentation phase, during which *Staphylococcus saprophyticus* grew dominantly, and promoted the development of a microbiota that LAB dominated. Notably, the growth of the dominant LAB, *Pediococcus pentosaceus*, was improved by adding either Japanese pepper or red pepper. The differences in the LAB species, which were associated with the differences in chemical composition of the nukadoko, were dependent on the type of pepper used. We conclude that the spices used can affect the bacterial community and modulate its metabolic profile in nukadoko.

Key words: nukadoko, spice, microbiota, 16S rRNA gene, pyrotag sequencing, lactic acid fermentation

INTRODUCTION

Nukadoko is a fermented rice bran bed used to produce nukazuke, a type of Japanese pickled vegetables. Nukadoko gives the pickled vegetables a salty and sour taste, a special flavor [1], and adds vitamins B1 and B2 [2]. Nukadoko is initially prepared by natural fermentation of rice bran. Once well-fermented nukadoko is prepared, it can be used as a seed for another batch of nukadoko. When nukadoko becomes stale, it can be renewed by refermentation with additional fresh rice bran. This refreshment process 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.

Fermented nukadoko harbors a number of species of lactic acid bacteria (LAB) [2, 3] that are thought to contribute to the considerable resistance of nukadoko to spoilage. In our previous studies, we analyzed the change in the microbiota during the natural fermentation of four nukadokos for 2 months and found two types of LAB: the first one included *Pediococcus* or *Weissella* species and appeared in the first 1–2 weeks of fermentation, and the other is *Lactobacillus plantarum*, which commonly appeared in the post-fermentation stage [4]. We also investigated the microbial community of nukadoko aged over 150 years and found two dominant *Lactobacillus* species, *Lactobacillus acetotolerans* and *Lactobacillus namurensis* [5, 6]. Interestingly, these two species show different growth profiles in nukadoko: *L. namurensis* appears as a main lactic acid fermenter a few days after

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refreshment, whereas *L. acetotolerans* grows very slowly and dominates the nukadoko microbiota after a few weeks of ripening, at which point other LAB cannot survive in the lowered pH [6]. As long as the aged nukadoko was used as an inoculants for refreshment, the relay of these two dominant LAB species appeared [5, 6], suggesting that the balance between these two lactobacilli is a key in the stabilization of nukadoko during refreshment.

It is common for nukadoko to be fermented with some spices and seasonings. Notably, Japanese pepper (*Zanthoxylum piperitum*) and/or red pepper (*Capsicum annuum*) are conventionally added to nukadoko. Since the antimicrobial effect of these spices is known [7, 8], they may be involved in microbiological control as well as enrichment of flavor. However, the effect of these spices on nukadoko fermentation is not well understood. To address this, we carefully monitored the effect of spices on the bacterial community and chemical composition using a laboratory model nukadoko with or without spices in the present study. Bacterial composition was investigated using the pyrosequencing-based 16S rRNA profiling technique, which allows top-down profiling of bacterial composition [9–12].

MATERIALS AND METHODS

Nukadoko preparation and sampling

The effect of spices was investigated using laboratory model nukadokos in which fresh rice bran was naturally fermented without inoculating any starter culture or fermented nukadoko [4]. The spices used in this study were Japanese pepper fruit and round sliced red pepper produced in Japan. We prepared four kinds of nukadoko: without spices (control), with Japanese pepper (J), with red pepper (R) and with Japanese pepper and red pepper (JR). The nukadoko compositions are shown in Table 1. The quantity of pepper to be added was determined by referring to the traditional method, in which the pepper concentration was generally chosen for dietary purposes. The rice bran paste was incubated at 24°C for 60 days, with daily mixing using a sterilized latex gloved hand. The paste was used to pickle an eggplant and a cucumber every 2 days, and 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 1, 3, 5, 8, 10, 12, 15, 19, 22, 26, 31, 37, 43, 47, 54 and 60 days. Samples for pyrotag sequencing and chemical analysis were stored at −80°C until use, whereas those for bacterial cultures were processed immediately after sampling.

Table 1. Compositions of the nukadoko samples used in this study

Component	Nukadoko sample name			
	Control	J	R	JR
Rice bran (g)	1200	1200	1200	1200
Salt (g)	150	150	150	150
Water (g)	1650	1650	1650	1650
Japanese pepper (g)	0	30	0	30
Red pepper (g)	0	0	10	10

Enumeration of LAB in nukadoko

The numbers of microorganisms in the nukadoko samples were determined by a standard plate count procedure. Ten grams of nukadoko was diluted in 90 ml sterile NaCl solution (0.85%, w/v), spread on MRS (*Lactobacillus*) agar (Difco, BD, Sparks, MD, USA) and incubated at 30°C for 48 hr. The resultant colonies (30–300 per plate) were counted as colony-forming units.

Amplification of the 16S rRNA V6–V8 region

The V6–V8 fragment of the 16S rRNA gene was amplified from total bacterial DNA using universal primers Q-968F-# (5′-CWSWSWWSHTWACGCGARGAACCTTACC-3′) and Q-1390R-# (5′-CWSWSWWSHTTGACGGGCGGTGWGTAC-3′) (# indicates a series of 64 barcode tags underlined in the sequence). Polymerase chain reaction (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 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 of 98°C for 15 sec, 54°C for 30 sec and 72°C for 20 sec; and 72°C for 5 min. The amplified products were purified using a QIAquick PCR Purification Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol and quantified using a NanoDrop Lite Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Pyrotag sequencing

Bacterial composition in each nukadoko sample was investigated using pyrosequencing-based 16S rRNA profiling. The V6–V8 region of 16S rRNA was amplified by PCR using barcode-tag universal primers from genomic DNA extracted from each nukadoko sample and was then mixed and applied to the pyrosequencing.

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 GS FLX Titanium LV emPCR Kit v2 according to the manufacturer's protocol (454 Life Sciences, Branford, CT, USA). Beads carrying amplified DNA were pooled and loaded onto half of a GS FLX Titanium PicoTiterPlate, and 454 pyrosequencing was conducted using an FLX Genome Sequencer (454 Life Sciences).

The analysis of pyrosequencing data was performed according to a previously published method [13, 14]. 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, 302,686 sequences were assigned to 64 nukadoko samples, with an average of 4,729 ± 1,184 reads per sample.

To prepare a nonredundant sequence set, 302,686 sequences were dereplicated within 99% nucleotide sequence identity 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 10,315 nonredundant operational taxonomic units (OTUs) was obtained. PCR 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). Following the chimera check, 8,000 OTUs corresponding to 280,752 reads were selected for further analysis as nonchimeric sequences.

In order to get taxonomic information, the 8,000 selected OTUs were subjected to two web-based searches, the Ribosomal Database Project (RDP) classifier [15] and RDP SeqMatch [16] (http://rdp.cme.msu.edu/). In the RDP classifier search, the cutoff value of the confidence threshold for taxonomic classification was set at 80% as generally recommended. The RDP SeqMatch k-nearest-neighbor tool was employed to gain data on the composition of the 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 data on species composition [14]. 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 the others shown in parentheses: *L. plantarum* (*Lactobacillus pentosus*, *Lactobacillus*

paraplantarum), *Enterococcus faecalis* (*Enterococcus faecium*, *Enterococcus lactis*, *Enterococcus durans*), *Weissella cibaria* (*Weissella confusa*), *Staphylococcus saprophyticus* (*Staphylococcus xylosus*, *Staphylococcus succinus*). The data collected on the microbiota in each nukadoko sample were profiled based on the catalogue of 8,000 nonredundant 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.

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. The salinity of the nukadoko was analyzed by the Mohr method [17].

Organic acid and sugar concentrations were determined by high-performance liquid chromatography. 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 and a flow rate of 1.0 ml/min. The sugar concentration was analyzed using an Asahipak NH2P-50 4D column (Shodex) coupled to a refractive index (RI) detector at 30°C with 75% acetonitrile as the mobile phase and a flow rate of 0.8 ml/min.

RESULTS

Effect of spices on total LAB count

To examine the effect of spices, rice bran was naturally fermented without inoculating any starter culture or seed nukadoko. Figure 1 shows the changes in colony count of LAB during fermentation of nukadoko. In the nukadokos with spices (J, R and JR), the LAB count increased logarithmically until day 8, while that in the control without spice increased at the same rate in the first 5 days and then slowed down but continued until day 12. At day 8, the LAB counts in J, R and JR were approximately one-order higher than those in the control nukadoko. After day 10, there was no significant difference in the LAB count between samples.

Effect of spices on bacterial composition

The pyrotag analysis of a total 64 samples provided 280,752 quality-filtered reads clustered into 8,000 OTUs, each sharing 99% sequence identity. Based on taxonomic annotation of each OTU, the composition of the bacterial community in each sample was analyzed (Appendix Table 1). In all nukadoko sample, the maximum number of species was observed until day 5, although the
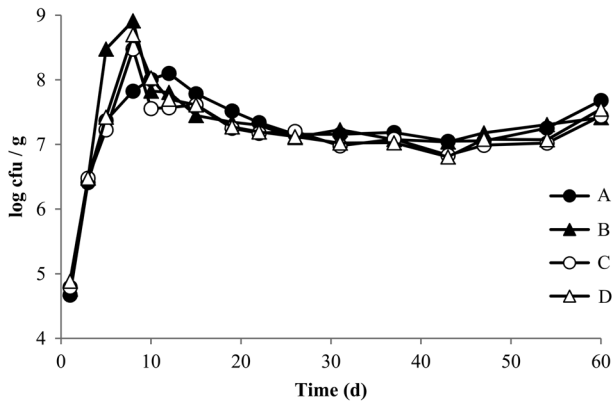
Fig. 1.   Changes in the number of lactic acid bacteria during the initial batch fermentation of nukadoko samples
(A) control nukadoko (no spices), (B) Japanese pepper nukadoko, (C) red pepper nukadoko and (D) Japanese pepper and red pepper nukadoko. CFU, colony-forming units.

LAB group was underrepresented, suggesting a higher bacterial diversity in fresh rice bran under less dense and less competitive conditions. In J, R and JR nukadoko samples, the observed numbers of LAB species were lower than that in the nonspice control.

Figure 2 shows time-dependent variations in the composition of the nukadoko bacterial community at the family level. *Staphylococcaceae* organisms were observed at the initial stages of fermentation for all samples. In the control sample, these bacteria were dominant until day 54, while *Lactobacillaceae* gradually increased from day 10 to day 60, eventually becoming dominant on day 60. Figure 2 clearly shows that the onset and subsequent domination of LAB was accelerated by adding spices. In the J nukadoko samples, *Staphylococcaceae* organisms were replaced by *Lactobacillaceae*, which gradually dominated the microbiota from day 8 to day 60. Similarly, the relative abundance of *Staphylococcaceae* organisms in the R and JR nukadoko samples rapidly decreased, and *Lactobacillaceae* and *Leuconostocaceae* dominated the
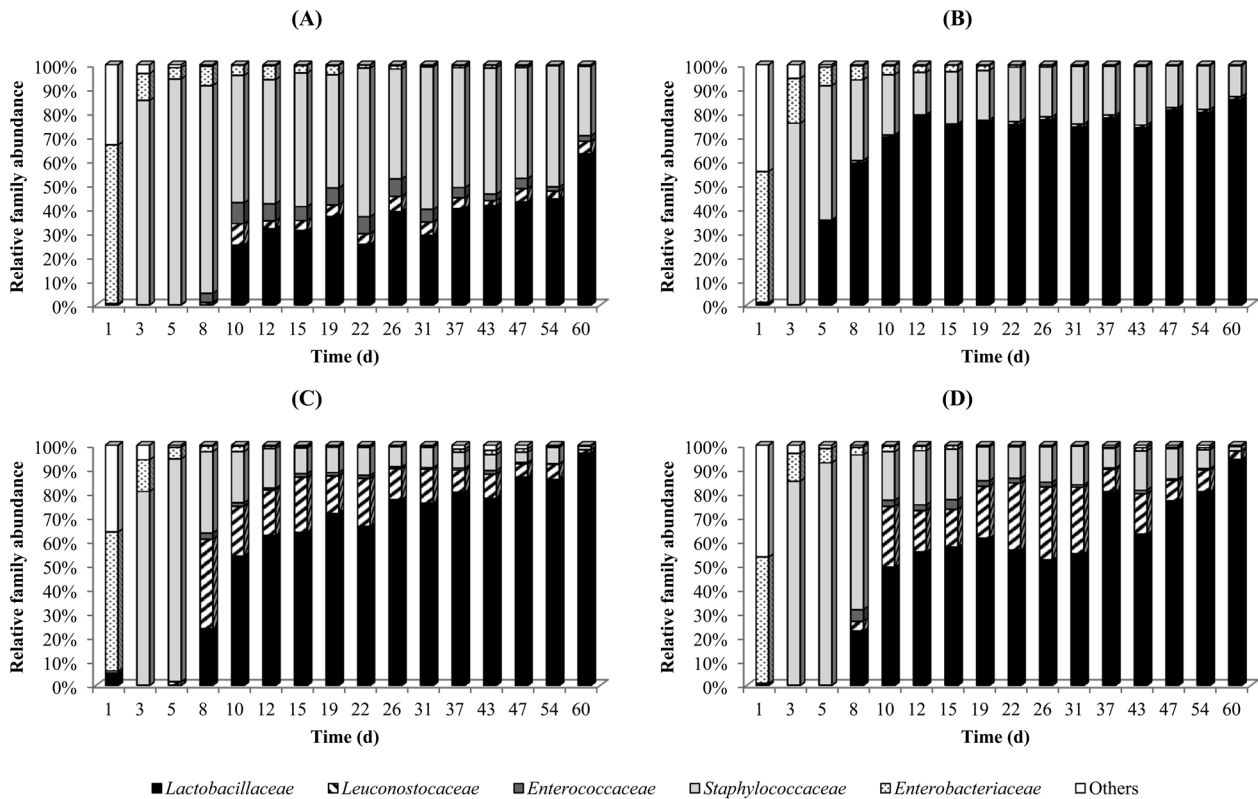

Fig. 2.   Dynamic changes in the familial structure of the bacterial community during the initial batch fermentation of nukadoko samples
(A) control nukadoko (no spices), (B) Japanese pepper nukadoko, (C) red pepper nukadoko and (D) Japanese pepper and red pepper nukadoko. The relative abundance of each bacterial family was determined based on the read counts of each operational taxonomic unit (OTU) assigned to the family. OTUs assigned to chloroplasts were removed from the analysis.
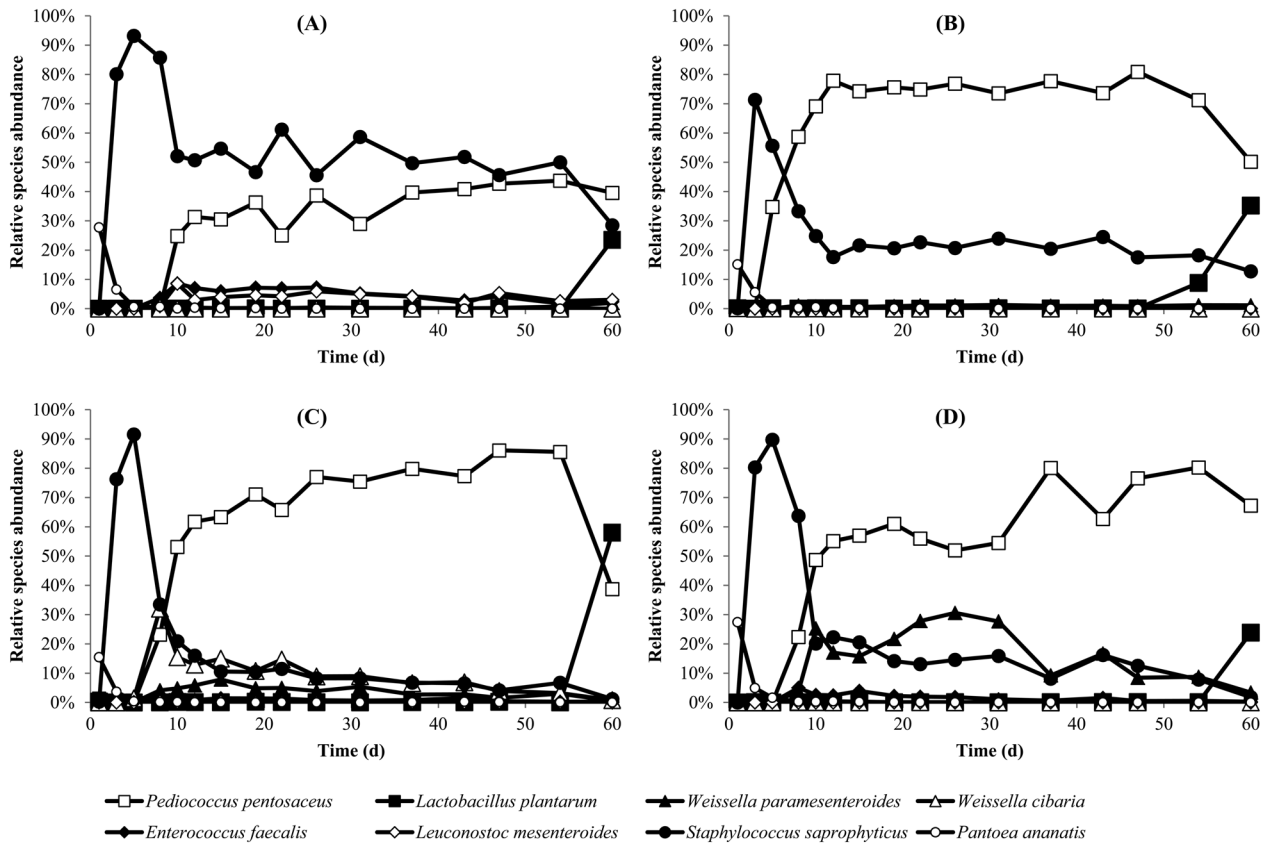
Fig. 3. The relative abundance of predominant bacterial species during the initial batch fermentation of nukadoko samples
(A) control nukadoko (no spices), (B) Japanese pepper nukadoko, (C) red pepper nukadoko and (D) Japanese pepper and red pepper nukadoko. The relative abundance of each species was determined based on the read counts of each operational taxonomic unit (OTU) assigned to the species. OTUs assigned to chloroplasts were removed from the analysis. The species in each OTU were determined by the Ribosomal Database Project RDP SeqMatch based on the V6–V8 sequences following seqmatch Q400 data processing (for details, see Materials and Methods).

microbiota of these nukadoko samples from day 8 to day 60 and from day 10 to day 60, respectively.

The dominant species detected by the pyrotag sequencing are shown in Fig. 3. In all samples, *S. saprophyticus* was dominant at the initial stage of fermentation (until day 5). The relative abundance of *L. plantarum* appeared in the post-fermentation stage from day 54 to day 60. In the control samples, *S. saprophyticus* was dominant from day 3 to day 54, and then *Pediococcus pentosaceus* became dominant by day 60. In addition, an increase in the relative abundance of *Leuconostoc mesenteroides* was observed from day 10. In contrast, in the J, R and JR samples, *P. pentosaceus* became dominant between day 5 and 10. In the R samples, increases in *W. paramesenteroides* and *W. cibaria* were detected from day 8, whereas an increase in the relative abundance of *W. paramesenteroides* was detected in the JR samples from day 10.

Effect of spices on chemical change

Changes in pH values and the concentrations of organic acids and sugars were also monitored in these models (Fig. 4). In all nukadokos, pH dropped to between 4 and 5 in the first 2 weeks. This acidification was consistent with the increase in lactic acid and acetic acid in all nukadokos. The onset of the pH drop was evidently earlier in nukadoko with spices compared with the nonspice control. The production level of acetic acid differed among samples and was especially low in J nukadoko compared with the other samples. Sugars were detected in all nukadoko samples, with glucose, fructose, and sucrose initially present at 35 mM, 20 mM and 120–155 mM respectively. In all nukadoko samples, glucose and fructose increased from day 1 to day 5; however, glucose exhaustion occurred earlier in the J, R and JR samples than in the control samples. In addition, fructose exhaustion occurred earlier in the J and JR samples than
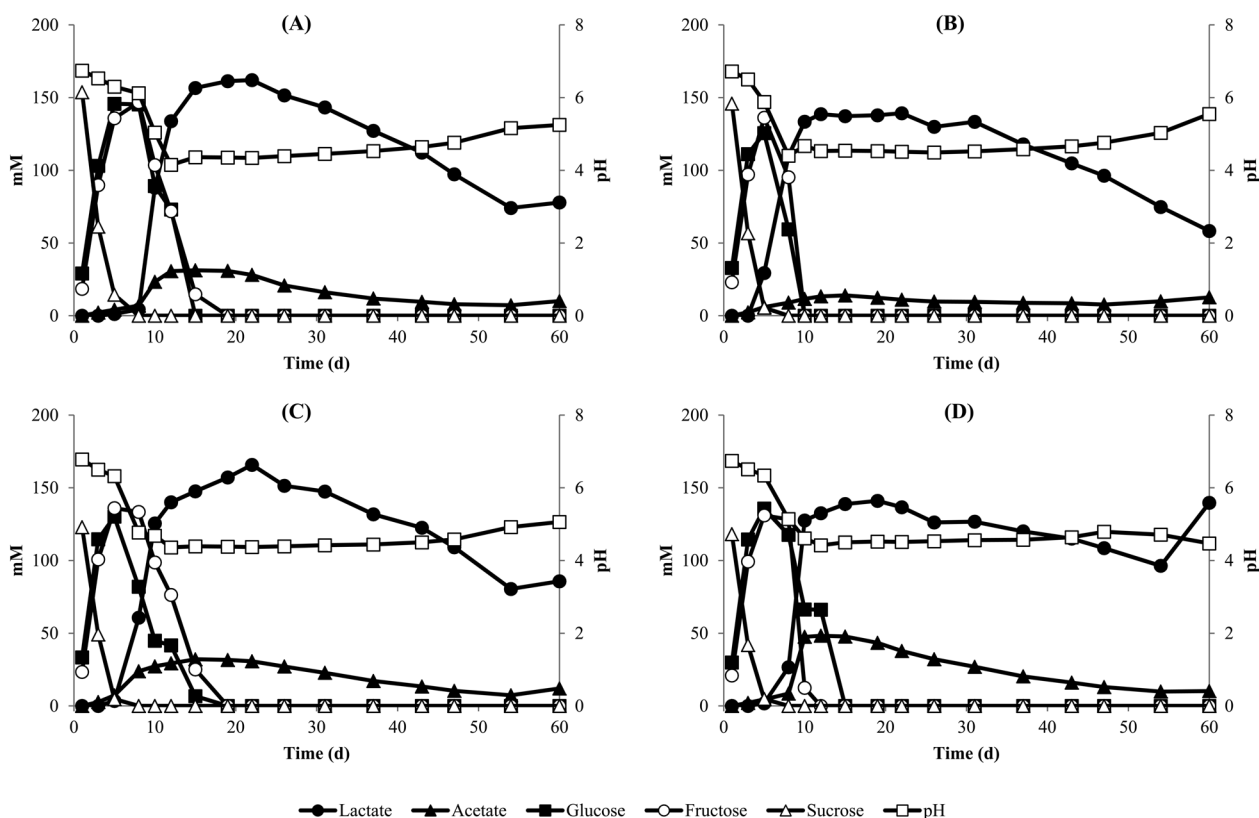
Fig. 4.   Changes in pH and the concentrations of organic acids and carbohydrates during the initial batch fermentation of nukadoko samples (A) control nukadoko (no spices), (B) Japanese pepper nukadoko, (C) red pepper nukadoko and (D) Japanese pepper and red pepper nukadoko.

in the control and R samples. In contrast, sucrose started decreasing immediately and was exhausted in all samples within 1 week. Sugar exhaustion coincided with the end of the increase in lactic acid, suggesting that lactic acid production in nukadoko mostly depended on the fermentation of these sugars.

DISCUSSION

Previous studies have reported that Japanese pepper and red pepper exhibit antibacterial activity against *Staphylococcus aureus* [7, 18]. In the present study, we found that Japanese pepper and red pepper suppressed the growth of *S. saprophyticus*, which was the initial colonizer in nukadoko, and development of a LAB-dominant microbiota was accelerated by the addition of spices. It appears that *S. saprophyticus*, the initial colonizer in nukadoko, competitively hampers the growth of LAB and suppresses lactic acid fermentation in nukadoko. This finding is in line with results published by Revilla et al., which showed that red pepper accelerates the ripening process of dry sausage by promoting of LAB

growth [19], and Sharma et al., which showed that red pepper enhanced L-lactate production by *Lactobacillus acidophilus* in curd formation [20].

In the present study, the spices also appeared to have an inhibitory effect on some LAB species. *L. mesenteroides* was only detected in the nonspice control, suggesting this species is sensitive to both peppers. *Weissella* species were detected only in the nukadoko containing red pepper, suggesting that *Weissella* species are tolerant to red pepper but not to Japanese pepper and that red pepper promoted growth of *Weissella* species. Japanese pepper and red pepper contain different antibacterial substances: Japanese pepper contains a polymeric procyanidin, ZP-CT-A [7], and red pepper contains capsaicin (8-methyl-N-vanillyl-6-nonenamide) [21]. Cichewicz et al. showed that red pepper has antibacterial activity against *Bacillus cereus*, *Bacillus subtilis*, *Clostridium sporogenes*, *Clostridium tetani* and *Streptococcus pyrogenes* [8]. On the other hand, the antibacterial spectrum of Japanese pepper has not been well elucidated, except for *S. aureus* [7]. Jeong et al. also showed that kimchi with red pepper powder contained much higher proportions of *Weissella*

than kimchi without red pepper powder [22]. *W. confusa* has been detected in raw red pepper [23]. These findings suggest that the microbiota of nukadoko is influenced by differences in the antimicrobial spectrum of spice and bacteria species inhabiting each of the spices.

We found that lactate and acetate gradually decreased from the middle to late fermentation stages in nukadoko. This may be caused by endogenous yeast. In all nukadoko samples, yeast increased logarithmically from day 3 to day 8, and the number of yeast was more than 10^5 cfu/g from day 8 to 60 (data not shown). It is known that some species of yeast utilizes lactate and acetate as carbon resources [24]. Our data showed that the sugars in nukadoko were exhausted in the late fermentation stage. It appears that those endogenous yeast utilize lactate and acetate as carbon resources in the late fermentation stage. We also found that the concentration of acetic acid was different among nukadoko samples after day 10. This difference appeared to be reflected by the differences in fast-growing LAB species among the nukadoko samples. In the control without spices, R and JR nukadoko samples, the heterofermentative LAB *L. mesenteroides*, *W. paramesenteroides* and *W. cibaria* were the subdominant species at day 10. In contrast, the homofermentative LAB *P. pentosaceus* was the dominant species in J nukadoko at day 10. Therefore, heterofermentative LAB growing in the first 10 days appear to contribute to acetate accumulation in nukadoko. These results suggest that the microbiota of nukadoko in the first 10 days is influenced by the addition of spices. It appears that the spices manipulate the bacterial community and modulate its metabolic profile in nukadoko.

In conclusion, we found that the addition of Japanese and/or red peppers promotes lactic acid fermentation in nukadoko by shaping LAB flora. Determining the correlation between nukazuke/nukadoko flavor and the LAB composition established with different spices and seasonings would be of interest.

ACKNOWLEDGEMENT

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Appendix Table 1. Number of operational taxonomy units (OTUs) of nukadoko samples[a]

Sample name	Fermentation day	Total			LAB		Sample name	Fermentation day	Total			LAB	
		Reads	No. of OTUs	No. of species	%	No. of species			Reads	No. of OTUs	No. of species	%	No. of species
Control	1	5426	442	103	0.2	6	R	1	5622	369	69	0.2	2
	3	5260	396	62	0.0	0		3	3230	297	68	0.1	2
	5	2318	231	48	0.2	3		5	4649	359	60	1.5	4
	8	4095	349	59	4.5	6		8	3336	450	49	57.9	11
	10	3813	304	56	40.6	13		10	3841	464	51	70.2	10
	12	4162	404	64	37.1	13		12	4294	459	51	76.8	12
	15	3451	313	54	39.5	10		15	5557	516	48	85.3	10
	19	4761	377	63	44.5	11		19	4683	392	32	85.8	9
	22	2592	235	35	36.3	11		22	5142	466	40	85.8	10
	26	5823	358	53	51.6	11		26	4540	351	36	89.1	8
	31	2693	229	37	39.5	10		31	4276	331	37	89.4	7
	37	3938	309	45	48.0	10		37	3512	243	49	86.7	9
	43	4635	361	55	45.9	16		43	3458	268	58	82.9	11
	47	5294	338	41	52.5	9		47	5689	442	73	88.7	15
	54	5310	367	35	48.8	10		54	3269	210	33	91.6	9
	60	5272	335	32	70.4	11		60	6716	323	28	98.2	11
J	1	4475	337	68	0.1	5	JR	1	3511	269	64	0.1	2
	3	5132	478	91	0.1	3		3	3830	349	66	0.1	2
	5	2891	283	49	31.2	7		5	4838	423	69	0.1	4
	8	5972	404	57	56.7	5		8	1930	176	40	29.0	9
	10	5108	372	46	60.1	7		10	3264	261	49	69.7	8
	12	5484	328	46	70.0	5		12	3280	272	42	69.1	7
	15	5017	331	48	70.6	6		15	3204	240	38	72.0	9
	19	6816	401	50	73.3	9		19	3151	209	26	83.8	7
	22	4474	272	31	75.1	3		22	4054	280	35	85.4	9
	26	6045	314	37	77.0	6		26	3092	238	31	83.7	9
	31	5240	301	36	74.3	5		31	3366	205	31	82.3	7
	37	5781	310	35	78.4	6		37	4307	292	44	87.0	6
	43	5008	266	26	74.6	8		43	2195	152	34	80.0	6
	47	6750	357	27	81.6	5		47	3861	247	48	85.3	11
	54	4285	252	27	81.3	9		54	3228	199	47	89.2	9
	60	6626	332	29	86.8	10		60	3880	243	31	97.4	13

[a] Taxonomic information was obtained using the 16S rDNA V6–V8 sequences from pyrosequencing and database searches using Ribosomal Data Project SeqMatch, as described in the Materials and Methods. Percentage values were calculated by dividing the number of classified reads by the total read number for each sample. No. of species indicates the number of different species in each sample.