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Full Paper

# Impact of vegetables on the microbiota of the rice bran pickling bed Nukadoko

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**Nukadoko, a fermented rice bran bed for pickling vegetables called nukazuke, has a complex microbiota. Within it, deep interactions between the microbiota of the pickled vegetables and nukadoko characterize and control the qualities of both products. To address this notion, we monitored the changes in the microbiota of nukadoko and nukazuke while pickling different vegetables. Raw or roasted rice bran was mixed with salted water and fermented at 24°C for 40 days, following which different species of vegetable, *Cucumis sativus* var. *sativus*, *Brassica oleracea* var. *capitata,* or *Raphanus sativus* var. *hortensis,* were pickled. The microbial composition of the washing solution of fresh vegetables, as well as that of the nukadoko and nukazuke for each vegetable, was analyzed by amplicon sequencing of 16S rRNA genes. Although the microbiota of nukadoko varied depending on the species of pickled vegetables, no transcolonization of any species of bacteria from fresh vegetables to nukadoko was observed. However, some lactic acid bacterium (LAB) species eventually dominated the microbiota of both nukazuke and matured nukadoko, although they were not detected in either the fresh vegetables or rice bran. Particularly, *Lactiplantibacillus plantarum* was dominant among all pairs of pickled vegetables and matured nukadoko, whereas the transcolonization of some other LAB species was observed in a pickled vegetable-specific manner. *Staphylococcus xylosus* was observed to some extent in each nukadoko, yet it was not detected in any nukazuke. Overall, a LAB-dominant microbiota was established in both nukadoko and nukazuke in an underlying process that was different but partly common among vegetables.**

**Key words: nukadoko, nukazuke, microbiota analysis, bacterial adhesion**

## INTRODUCTION

The fermented rice bran bed “nukadoko” is traditionally used for pickling vegetables in Japan. Various vegetables can be pickled in nukadoko, and the resulting pickled vegetables are called “nukazuke”. Nukadokos are prepared by kneading rice bran with salted water and adding seasonings such as chili pepper and are fermented with vegetables. The abundance of nutrients in nukazuke, compared with fresh vegetables, has been attracting attention. In particular, nukazuke contains vitamins, notably vitamin B1, at higher concentrations than in fresh vegetables [1], suggesting that the nutrients of rice bran are transferred to nukazuke [2]. In addition, the dietary fiber in nukazuke is suggested to improve various physiological functions, including lowering blood lipid levels [3, 4].

Nukazuke also has a stronger acidic and umami flavor than fresh vegetables. This unique flavor is derived from metabolite products produced within the interactions among various microorganisms, particularly lactic acid bacteria (LAB) and yeast, inhabiting in the complex microbiota of naturally fermented nukadoko. The microbiota of nukadoko changes depending on various factors, such as the conditions of nukadoko fermentation (salt content, pH, and temperature), differences in rice bran and seasonings used, the types of microbiota present on the hands of food handlers producing and maintaining the nukadoko, and the frequency of mixing nukadoko [5–7]. For example, the addition of Japanese pepper and chili pepper to nukadoko accelerates the growth of LAB and maintains a high prevalence of *Pediococcus pentosaceus* [5]. Furthermore, the microbiota differ among nukadokos prepared using rice bran collected from different production areas, whereas the abundance of *Lactiplantibacillus*

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*plantarum* commonly increases with the progress of fermentation and eventually dominates the microbiota [6].

During fermentation, the interplay between vegetables and the microbiota of the nukadoko acts as a source of nutrients. Evidently, carbohydrates are depleted in nukadoko after 30 days fermentation, and thereafter the nukadoko falls into a state of sugar deficiency [7]. Thus, the pickling vegetables must be an important source of valuable nutrients for the survival of microbes in nukadoko. In addition, various soil and environmental microorganisms adhere to the surfaces of vegetables and can affect the nukadoko microbiota, acting as sources of allochthonous bacterium members.

As mentioned above, the dynamics of the nukadoko microbiota depend on complex factors; however, they remain largely unexplored. Hence, in this study, we carefully monitored the succession of microbiota in both fermenting nukadoko and pickling vegetables and compared them among different types of rice bran and vegetables to gain insight into the transcolonization in their niche.

## MATERIALS AND METHODS

### *Nukadoko preparation, maintenance, and sampling*

The rice bran used in this study was made in Ishikawa prefecture in Japan. Roasted rice bran was prepared by heating the raw rice bran at 120°C for 2 hr. The raw or roasted rice bran (1,800 g) was kneaded with water (2 L) and NaCl (200 g) in a commercial plastic barrel (Shinkigosei Co., Ltd., Tokyo, Japan) and then covered with a plastic lid and incubated at 24°C for natural fermentation using wild bacteria and yeast attached to the rice bran and vegetables.

The sampling schemes used in this study are described in Fig. 1. When sampling the nukadoko and nukazuke, the nukadoko was homogenized by stirring with the hands while wearing latex gloves. The saline concentration and water moisture content were maintained by adding salt at 11, 19, 23, and 31 days and freshly prepared rice bran at 23 and 31 days.

Sampling of nukadoko was carried out on days 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, 34, 37, and 40. When the nukazuke was pickled in the nukadoko, the nukadoko was sampled after removing the nukazuke. Sampled nukadoko was stored at −80°C for microbiota analysis.

Three different species of vegetables, *Cucumis sativus* var. *sativus* (cucumber), *Brassica oleracea* var. *capitata* (cabbage), and *Raphanus sativus* var. *hortensis* (Japanese white radish), were used for producing nukazuke in this study. Each vegetable species was pickled every two days in batches of nukadoko prepared both from raw and roasted rice bran, respectively. When pickling cucumber, 20 cucumbers were cut into halves after cutting off the tips at both ends, and ten pieces (about 1,500 g) were pickled in each batch of nukadoko for 24 hr. When pickling cabbage, two cabbages were used, with each cut into twelve equal pieces, and two pieces (about 400 g) were pickled in each batch of nukadoko for 24 hr. When pickling Japanese white radish, the centers of three Japanese white radishes were cut into quarters, and one piece (about 300 g) was pickled in each batch of nukadoko for 24 hr. During the pickling process, fresh vegetable and nukazuke samples were collected at 1, 5, 7, 11, 13, 19, 25, 31, and 39 days and at 2, 6, 8, 12, 14, 20, 26, 32, and 40 days, respectively, for microbiota analysis. Furthermore, the nukazuke samples were rinsed under sterilized reverse osmosis water for 1 min to remove adhered nukadoko. The fresh vegetables and nukazukes were suspended in an equal amount of sterilized reverse osmosis water within a sterile Stomacher sample bag (AS ONE Corporation, Osaka, Japan). The vegetables were removed, and thereafter the suspension was centrifuged at 8,000 × g for 10 min at 4°C, followed by centrifugation of the pellet at 20,000 × g for 3 min at 4°C. The precipitate was stored at −80°C until processing for microbiota analysis.

### *Chemical analysis*

For chemical analysis, 10 g of each nukadoko was mixed with 10 g sterilized reverse osmosis water in a sterile Stomacher sample bag (AS ONE Corporation). Each nukazuke was crushed to obtain the juice from it in a sterile Stomacher sample bag (AS ONE Corporation). Rice bran and nukazuke were removed, and thereafter the suspension was used for salinity and pH measurement. The salinity of the nukadoko and nukazuke were analyzed by the Mohr method [8]. The suspension was centrifuged at 2,406 × g for 15 min at 24°C, and the supernatant was then filtered and used for organic and amino acid analysis.

Organic acid analysis was performed by direct detection using a high-performance liquid chromatograph (HPLC) equipped with an Agilent Hi Plex H column (8 µm, 7.7 × 300 mm; Agilent Technologies, Santa Clara, CA, USA) and a diode array detector HS (Agilent Technologies) [9].

Amino acid analysis was performed by pre-column derivatization using an HPLC equipped with an Agilent

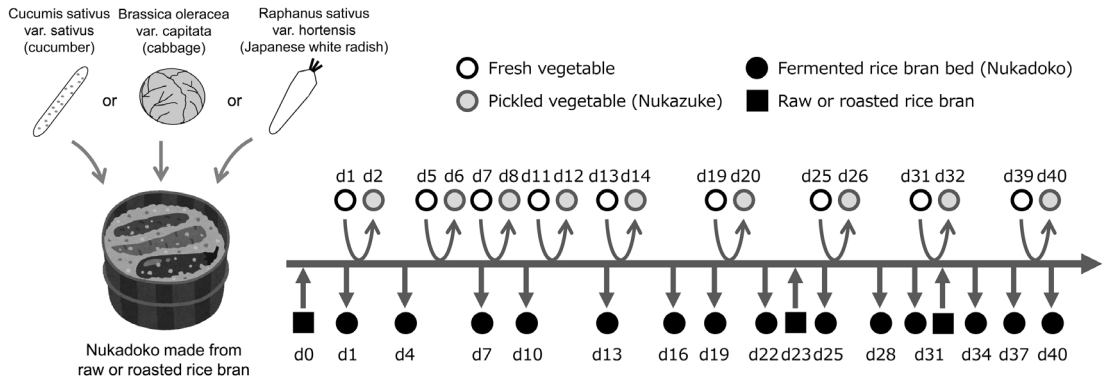


**Fig. 1.** Sampling scheme in this study.

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Poroshell 120 HPH-C18 column (2.7 µm, 3.0 × 100 mm; Agilent Technologies) [10]. The derivatization reagents used were *O*-phthalate aldehyde (Agilent Technologies) and 9-fluorenylmethyl chloroformate (Agilent Technologies).

### *Enumeration of LAB*

For enumeration of LAB, 10 g of each nukadoko was mixed with 10 g sterilized reverse osmosis water in a sterile Stomacher sample bag (AS ONE Corporation). Each vegetable and nukazuke was crushed to obtain the juice from it in a sterile Stomacher sample bag (AS ONE Corporation). Solids were removed, and thereafter the suspension was spread on MRS agar (Difco, BD, Sparks, MD, USA) and incubated at 30°C for 48 hr. The colonies grown on selected plates (30–300 colonies per plate) were counted as colony-forming units (CFUs).

### *DNA extraction*

A total of 200 mg of each nukadoko and each precipitate obtained from the fresh vegetables and nukazuke was mixed with 1 mL phosphate buffered saline (PBS) and vortexed [11]. After centrifugation at 20,000 × g for 5 min at 4°C, the supernatant was removed and washed twice with 1 mL of PBS buffer. Thereafter, 300 mg of glass beads (diameter, 0.1 mm) (TOMY SEIKO, Tokyo, Japan), 300 µL of Tris-SDS solution, and 500 µL of TE buffer-saturated phenol (Wako Pure Chemical Industries, Osaka, Japan) were added to the sample, which was then shaken vigorously at a speed of 5.0 m/sec for 30 sec using a Fast Prep-24 instrument (MP Biomedicals, Santa Ana, CA, USA). The supernatant was collected by centrifugation at 20,000 × g for 5 min at 4°C. Then, 500 µL of phenol-chloroform-isoamyl alcohol (25:24:1 v/v; Wako Pure Chemical Industries) was added to the supernatant, which was then shaken vigorously at a speed of 4.0 m/sec for 45 sec using a Fast Prep-24 instrument (MP Biomedicals). After centrifugation under the same conditions as previously mentioned, 250 µL of the supernatant was mixed with 25 µL of 3 M sodium acetate (pH 5.2; Sigma Aldrich, St. Louis, MO, USA) and 300 µL of isopropanol (Nacalai Tesque, Kyoto, Japan). The DNA sample was precipitated at 30°C for 30 min and centrifuged under the same conditions as mentioned previously to obtain a DNA pellet. Subsequently, the DNA pellet was washed once with 500 µL of 70% (v/v) ethanol (Wako Pure Chemical Industries) and air-dried prior to suspension in 20 µL Tris-EDTA (TE) buffer (pH 8.0), followed by storage at 30°C until use.

### *16S rRNA gene amplicon sequencing and processing of sequencing data*

The V3–V4 region of the bacterial 16S rRNA gene was amplified from total bacterial DNA using TaKaRa Ex Taq HS (Takara Bio, Shiga, Japan) and the universal primers Bakt_341F (5′-CGCTCTTCCGATCTCTGCCTACGGGNGGCWGCAG-3′) and Bakt_805R (5′-TGCTCTTCCGATCTGACGACTACHV GGGTATCTAATCC-3′) [12]. The amplified products were then used as a second PCR template for further amplification with barcode-tag primers. The second-PCR products were purified using FastGene Gel/PCR extraction (Nippon Genetics Co. Ltd., Tokyo, Japan), according to the manufacturer's protocol. The amplified DNA was quantified using a PicoGreen dsDNA Assay Kit (Life Technologies, Eugene, OR, USA), following the manufacturer's protocol. The purified DNA was sequenced using an Illumina MiSeq sequencer (Illumina Inc., San Diego, CA, USA).

The QIIME 2 platform [13] was used to create the amplicon sequence variants (ASVs), the taxonomy of which was identified with cut-off values higher than 0.7 to the SILVA database (release 138.1), from the obtained sequences. The relative abundance (%) of each taxon was calculated based on the number of corresponding reads per sample.

### *Beta diversity analysis*

Principal coordinate analysis (PCoA) was performed based on the genus composition of the samples by using the "rda" function in the vegan package of R (http://cran.r-project.org/package=vegan) [14]. Regressions of the number of observed operational taxonomic units (OTUs) to the PCoA ordination were calculated using the ordisurf function of the R vegan package.

### *Statistical analysis*

For beta diversity, permutational multivariate analysis of variance (PREMANOVA) was performed to calculate statistical differences in microbial composition between groups [15].

### *Accession number of 16S rRNA gene sequences*

Raw sequence data of 16S rRNA genes obtained in this study were deposited in the DNA Data Bank of Japan (DDBJ; https://www.ddbj.nig.ac.jp/index-e.html). The DDBJ Sequence read archive identifier was DRA017424 under BioProject no. PRJDB17018, which contains sampling data accession links under the Biosample identifiers SAMD00657159 to SAMD00657326.

## RESULTS

### *Changes in the chemical environment during maturation of nukadoko*

The salt content commonly accounted for approximately 6% of freshly prepared nukadokos and thereafter decreased with repeated pickling of the vegetables. The salt content of nukazukes ranged from 1 to 3% throughout the test, regardless of the used rice bran and pickled vegetables. During the 38 days of fermentation, the pH dropped from 6.0 to 4.0 in all tested samples of the nukadoko and nukazuke (Supplementary Figs. 1, 2).

The concentration of lactic acid increased up to 1% after fermentation in both nukadoko and nukazuke, while that of acetic acid increased up to 0.1%. Particularly, raw and roasted nukadoko pickling Japanese white radish showed the highest acetic acid concentrations (Supplementary Table 1).

The concentration was also significantly increased in most amino acids, with some exceptions. In particular, the concentration of glutamic acid increased by more than 10 times during the 38 days of fermentation in the nukadoko prepared from raw rice bran and increased by 2–3 times in nukadoko prepared from roasted rice bran. After fermentation, the concentrations of these amino acids tended to be higher in the nukadoko prepared from raw rice bran than those prepared from roasted rice bran, while the level of each amino acid tended to be lower in the nukadoko pickling cabbage (Supplementary Table 1).

### *Changes in the number of LAB*

The initial number of LAB differed between raw and roasted nukadokos. However, the numbers of both of them increased to over 8 log cfu/mL until 14 days after the start of fermentation, and thereafter the level was maintained until the end of this

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experiment. Although the number of LAB differed among the raw vegetables, it was constant at approximately 6 log cfu/mL in nukazukes after 8 days of fermentation in the nukadoko (Supplementary Fig. 3).

### *Analysis of microbiota of fresh vegetables before pickling in nukadoko*

Several bacteria, including *Pantoea*, *Sphingomonas*, and *Serratia*, were detected in all samples of the fresh vegetables before pickling. However, none of the species from the fresh vegetables exhibited an increase in occupancy during pickling (Figs. 2–5).

### *Succession of microbiota of the cucumber nukadoko and nukazuke*

In the case of nukadoko fermented from raw rice bran while pickling cucumbers, *P. pentosaceus* appeared to be the most dominant species on day 7 and was observed until the end of the experiment (day 40). Meanwhile, *L. plantarum* emerged at day 20, increased until the end of the experiment, and ultimately accounted for approximately 30% of the total microbiota (Fig. 2). Moreover, these two dominant bacteria co-existed in nukazuke; *P. pentosus* dominated in the early to middle stage, and thereafter, *L. plantarum* emerged toward the end of the experiment. In contrast, *Staphylococcus xylosus* appeared to be dominant on day 4 in the nukadoko and was observed until the end of the experiment. However, *S. xylosus* was observed to have low occupancy in the nukazuke compared with the nukadoko.

In the case of nukadoko fermented from roasted rice bran, consistent colonization of *S. xylosus* was observed; however, *P. pentosaceus* was not observed. On the contrary, *Weissella* species closely related to *Weissela confusa*, *Weissela cibaria* (*W. confuse/cibaria*), and *Lactococcus lactis* appeared at a stable level throughout the experiment. Similar to the case of nukadoko fermented from raw rice bran, *L. plantarum* appeared after day 10 and was dominant in both the nukadoko and nukazuke until the end of the experiment.

### *Succession of microbiota of the cabbage nukadoko and nukazuke*

*W. confusa*/*cibaria* appeared to be the most dominant species on day 4; however, it was subsequently overridden by *Lactobacillus sakei* (Fig. 3). Nevertheless, as observed for the cucumber nukadoko, *L. plantarum* ultimately emerged as the most dominant both in the nukadoko and nukazuke. The microbiota of the cabbage nukazuke exhibited the same transition as that of the nukadoko. In addition, the nukadoko prepared using the roasted rice bran exhibited the same microbiota changes as that using the raw rice bran. However, *L. sakei* was observed to be the most dominant species until the end of the experiment, while *L. plantarum* increased and *L. sakei* decreased over time.

### *Succession of microbiota of the radish nukadoko and nukazuke*

Three LAB species, namely *P. pentosaceus*, *W. confusa*/*cibaria*, and *Leuconostoc mesenteroides*, appeared in the early stage, and thereafter, *L. plantarum* increased to become the most dominant species toward the end of the experiment, with a pattern similar to that observed for nukadoko pickling cucumbers or cabbage (Fig. 4). The nukazuke microbiota was dominated by *W. confuse/cibaria*, *L. mesenteroides*, *P. pentosaceus*, and

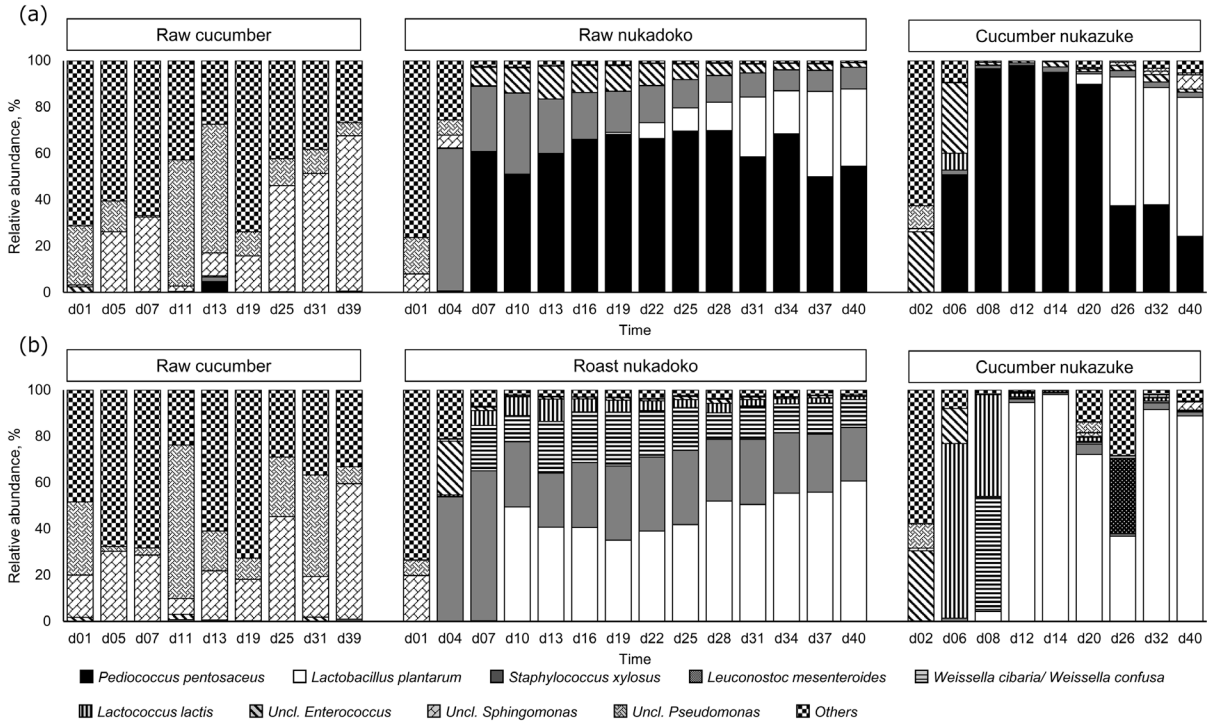


**Fig. 2.** Succession of microbiota of fresh *Cucumis sativus* var. *sativus* (cucumber) and their nukazukes and nukadokos.

The relative abundance of each bacterial species was determined based on the read count of the amplicon sequence variants (ASV) assigned to each species. The bacterial composition of the sample sets associated with nukadoko prepared using the raw rice bran (a) and the roasted rice bran (b), respectively.

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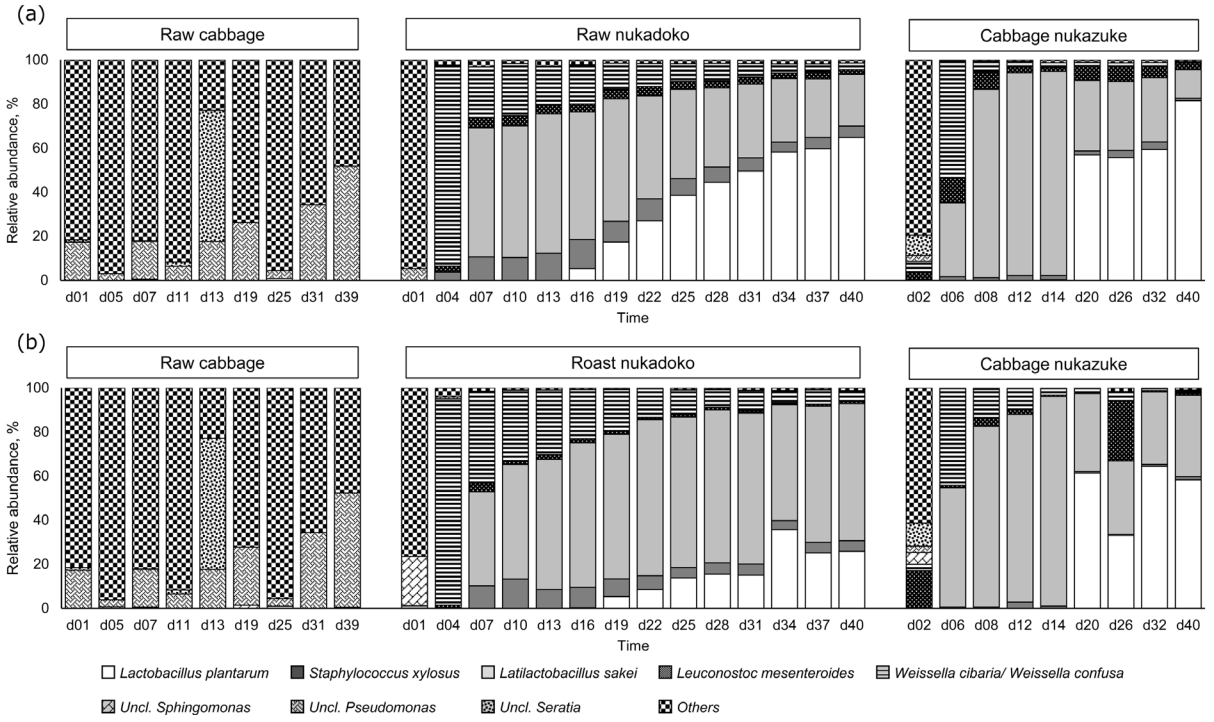


**Fig. 3.** Succession of microbiota of fresh *Brassica oleracea* var. *capitata* (cabbage), and their nukazukes and nukadokos.

The relative abundance of each bacterial species was determined based on the read count of the amplicon sequence variants (ASV) assigned to each species. The bacterial composition of the sample sets associated with nukadoko prepared using the raw rice bran (a) and the roasted rice bran (b), respectively.

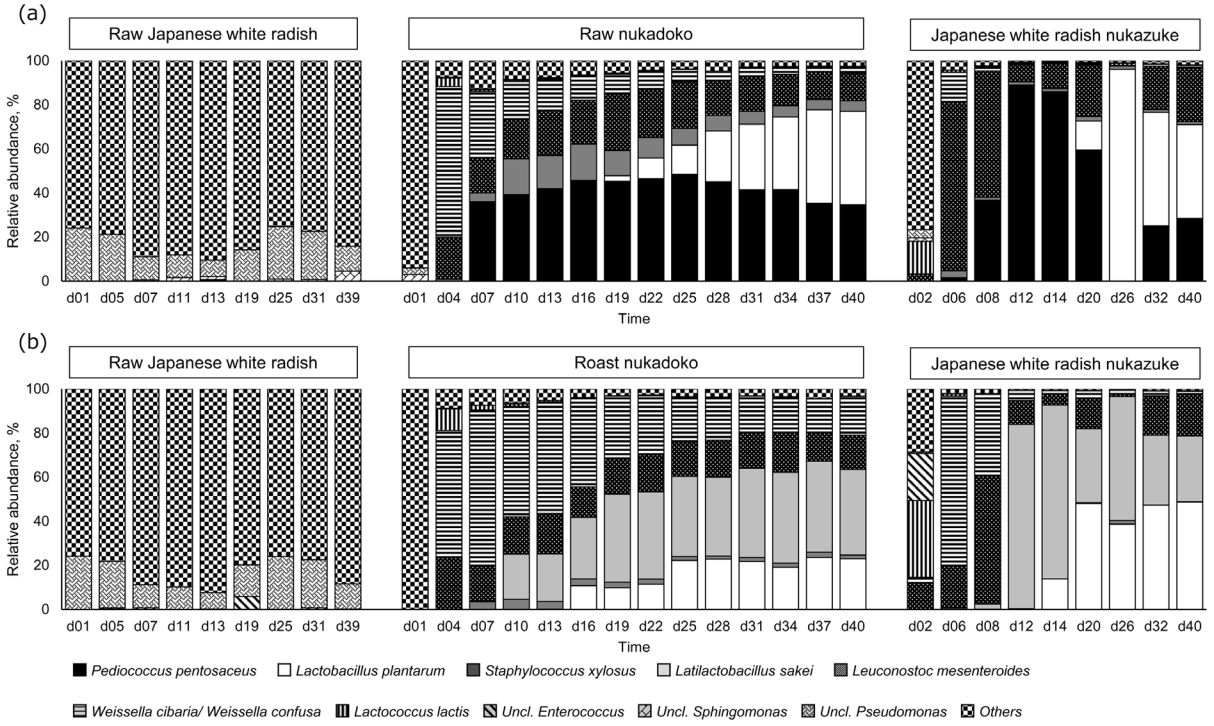


**Fig. 4.** The succession of microbiota of fresh *Raphanus sativus* var. *hortensis* (Japanese white radish), and their nukazuke and nukadoko.

The relative abundance of each bacterial species was determined based on the read count of the amplicon sequence variants (ASV) assigned to each species. The bacterial composition of the sample sets associated with nukadoko prepared using the raw rice bran (a) and the roasted rice bran (b), respectively.

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*L. plantarum* during the initial, middle, and late fermentation periods, respectively. In case of the roasted nukadoko, *L. sakei* occupied approximately 30% of the microbiota, in contrast to the nukadoko prepared from the raw rice bran, the dominant species in which was *P. pentosaceus*. *L. plantarum* increased from day 15 onwards and ultimately became the second most dominant species in the nukadoko and the most dominant species in the nukazuke.

***Overview of similarity in microbiota among the sets of vegetables, nukadoko, and nukazuke.***

To overview the microbiota similarity among the fresh vegetables, nukadoko, and nukazuke, the UniFrac distances between their microbiotas were calculated, and the principal coordination was plotted (Fig. 5). In all sets, fresh vegetable samples were clustered separately from nukadoko and nukazuke samples, which were clustered closely together. This result agrees with the above finding that the bacterial species in fresh vegetables were not shared with those in nukadoko and nukazuke, whereas species were largely shared between the pairs of nukadoko and nukazuke.

## DISCUSSION

In this study, we considered the pickling of vegetables as one of the major factors contributing to the changes in the microbiota of nukadoko and compared the microbiota of fresh vegetables with those of nukadoko and nukazuke as well as the microbiota of nukadoko with that of nukazuke.

The concentration of acetic acid in each nukadoko was higher in both the nukadokos prepared from raw and roasted bran with pickled Japanese white radish compared with cucumber or cabbage. Comparing the results for each microbiota, the occupancy of homofermentative lactobacilli, including *P. pentosaceus*, was high in the nukadoko with pickled cucumber and cabbage, the occupancy of heterofermentative lactobacilli, such as *L. mesenteroides* and *W. confusa/cibaria*, was higher in the nukadoko with pickled Japanese white radish. We considered that the difference in the fermentative type of LAB grown in these nukadokos may have caused the change in the concentration of acetic acid.

The dominant species, mostly LAB, were shared between nukadoko and nukazuke. However, this was not found in the comparisons between nukadoko and fresh vegetables. In the initial stages of development, some non-LAB species were commonly detected in the fresh vegetables and nukadoko. These results

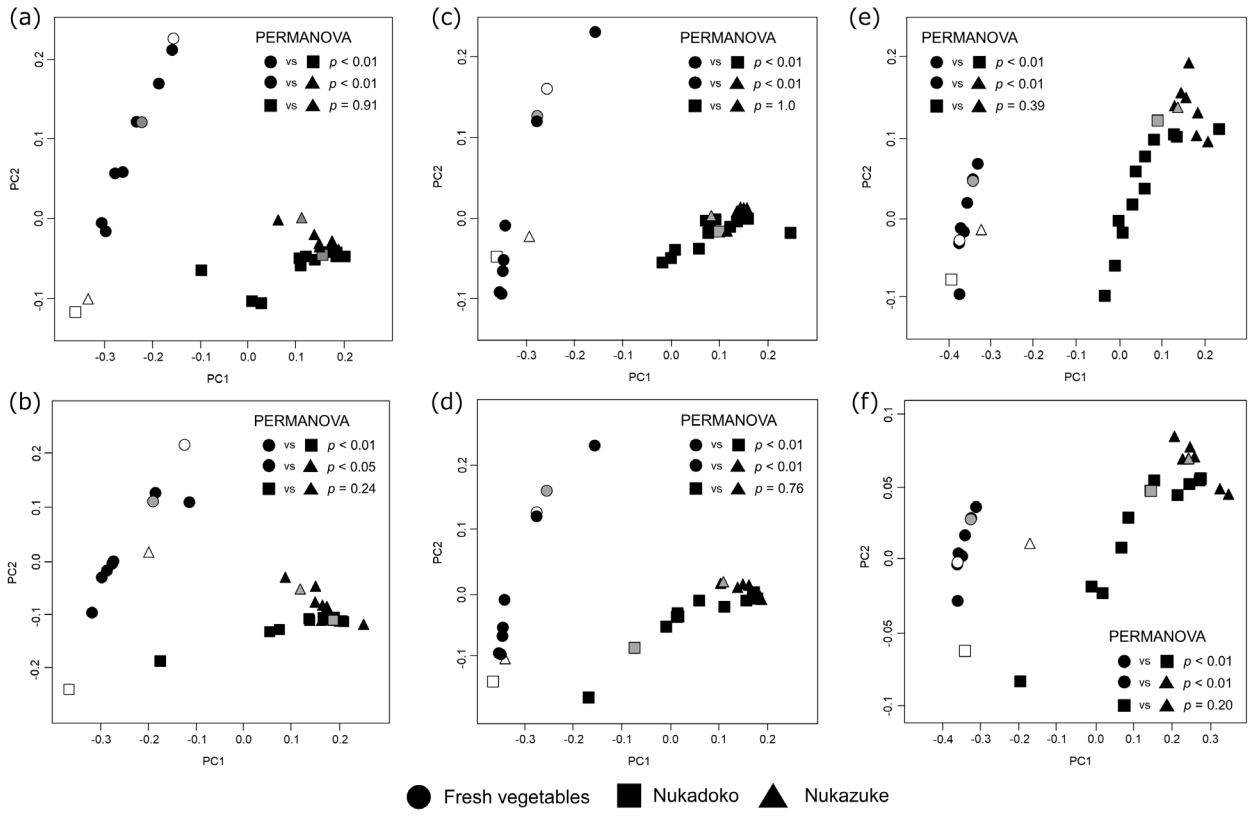


**Fig. 5.** Principal coordinate analysis of the microbiota of fresh vegetables and their nukazukes and nukadokos; the analysis was based on weighted UniFrac.

White markers depict the first-day samples, grey markers depict the last-day samples, and black markers depict other samples. (a) cucumber nukadoko made from the raw rice bran, (b) cucumber nukadoko made from the roasted rice bran, (c) cabbage nukadoko made from raw rice bran, (d) cabbage nukadoko made from the roasted nukadoko, (e) Japanese white radish nukadoko made from the raw rice bran, and (f) Japanese white radish nukadoko made from the roasted rice bran. Statistical differences between groups of sample origin were calculated by PERMANOVA with pairwise.adonis of the R vegan package.

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suggested that these non-LAB species transiently colonized the fresh vegetables and nukadoko but were outcompeted by the colonization of LAB species. After the nukadoko matured, a LAB microbiota was commonly established in all the tested samples. The maturation of nukadoko was associated with an increase in organic acids, particularly lactic acid and acetic acid, the concentrations of which increased to nearly 1% and 0.1%, respectively (Supplementary Table 1), resulting in the survival of acid-tolerant lactic acid bacteria species. Similar results have been observed in previous studies of nukadoko [5–7]. The microbiota of each nukadoko differed depending on the pickled vegetables and the type of rice bran used to prepare the nukadoko, even though it did not reflect the microbiota of the fresh vegetables subjected to pickling with nukadoko. This suggests that differences in the nutrients contained in the rice bran used in the production of nukadoko and the vegetables pickled in nukadoko affect the growth of certain bacterial species in the nukadoko. Bacterial growth commonly involves carbohydrates, essential amino acids, vitamins, and minerals. Amongst the vegetables used in this study, cucumbers had higher contents of copper and pantothenic acid than the cabbage and Japanese white radish [1]. In addition, cabbage had the highest carbohydrate content and also contained high levels of calcium, manganese, and essential amino acids. On the other hand, Japanese white radish also contained these nutrients, but it did not have any specific nutrients compared with cucumber and cabbage. These differences in the nutrients of the vegetables appear to be responsible for the differences in microbiota composition.

In our previous study, we investigated the effect of stirring nukadoko on its microbiota and showed that not stirring it causes a bias in the bacterial and fungal microbiota as well as metabolites between the upper and lower layers of each nukadoko we examined [16]. In other words, operations such as stirring the nukadoko change the nukadoko microbiota. In addition, Ono *et al.* [6] also found that the microbiota of nukadokos changed over time and that the predominant bacterial species differed in each nukadoko in the early stages of fermentation. Furthermore, the composition of the other LAB species, such as *P. pentosaceus* and *Latilactobacillus curvatus*, was observed to differ among nukadokos using rice bran from different regions. In the present study, the microbiota of nukadoko changed with different pickled vegetables. This suggested that vegetable nutrients, rather than vegetable-attached bacteria, were responsible for the changes in the microbiota. Collectively, the results of the present study and those of previous studies suggest that the microbiota of nukadoko depends on the type of rice bran and pickled vegetables used, which can affect the growth of certain bacterial species. The properties of the nukadoko also differ: The rice bran determines the composition of the nukadoko microbiota by providing adhered bacteria, and pickled vegetables promote the growth of specific bacterial species by providing nutritional components. With the addition of modification by the stirring process, the final characteristic nukadoko microbiota is established. In the present study, *L. sakei* was the dominant bacterial species in the microbiota of nukadoko pickling cabbage, which was consistent between the raw and roasted rice bran microbiota. Among lactobacilli, *L. sakei* has been reported to show a strong lactic acid-producing ability and to incorporate linoleic acid during growth [17]. Compared with cucumber and Japanese white radish, cabbage contains higher amounts of linoleic acid [1, 18, 19]. Therefore, the use of vegetables with characteristic nutrients may infer the predominant bacterial species, which could be used to ensure consistent quality in nukadoko and nukazuke in the food industry.

Among all the species in the microbiota analysis, the behavior of *S. xylosus* was particularly noteworthy. *S. xylosus* is a gram-positive, coagulase-negative *Staphylococcus* species [20]. In the food industry, this species is used as a fermentation starter to ripen and enhance meat flavor [21]. Although *S. xylosus* was detected to some extent in each nukadoko, it was not detected in the nukazuke. In contrast, LAB, specifically *L. plantarum* and *P. pentosaceus*, dominated the microbiota of nukazuke. Several studies have reported that bacteria grow on vegetable surfaces, and because *S. xylosus* was detected on vegetables that had undergone several washing processes [22, 23], it is assumed that the ability of this species to adhere to vegetables is not low. One of the factors involved in the bacterial adhesion mechanism is the hydrophobicity of the bacterial surface, and it has been reported that *Lactobacillus* species such as *L. plantarum* have high hydrophobicity of the cell surface [24, 25]. On the other hand, the hydrophobicity of the cell surface of *S. xylosus* is not as high as *Lactobacillus* species, although some strains have been reported to show hydrophobic cell surface and biofilm formation properties [26, 27]. The surfaces of plants are hydrophobic due to the cuticular wax on them and the microstructure of their cell walls [28]. These differences in hydrophobicity among the bacteria may have resulted in differences in hydrophobic interactions with plant cell surfaces and may have been one of the factors limiting the species transferred from the nukadoko to the nukazuke. More detailed verification of the adhesion function of these strains is needed; in particular, the adhesion factors on the surface layer of LAB, which are related to their adhesion to vegetables, are a subject for future research.

*L. plantarum* was detected in each nukadoko and nukazuke approximately 19 days after fermentation. This phenomenon was observed regardless of the pickled vegetables. Okada [29] observed a similar phenomenon when analyzing the microbiota of Goishicha, which is produced through the fermentation of molds and LAB, and Sunki, which is made from lactic acid fermentation of red turnips without salt. This phenomenon can be attributed to the cell wall peptidoglycan of *L. plantarum* being of the diaminopimeline type, which is speculated to be involved in the tolerance against catechins and tannins, which represent plant-derived antibacterial substances. In the same manner, the domination of *L. plantarum* in nukadoko may also be associated with the leakage of these antibacterial substances from bran and pickled vegetables.

In conclusion, this study revealed the transcolonization of LAB from nukadoko into the nukazuke microbiota but not from vegetables into nukadoko. Although the microbiota of nukadoko differed depending on the types of raw vegetables pickled, consistent colonization of bacterial species from the fresh vegetables in nukadoko was not observed. It is possible that the presence of minor species, the transcolonization of which was observed from vegetables, was below the detection limit in nukadoko in the 16S rRNA amplicon analysis. Moreover, some underlying factors, such as nutrients that determine the formation of the nukadoko microbiota, may be present in vegetables. Further studies are required to understand the relationship between pickled vegetables and the nukadoko microbiota, which may enable a more bio-controlled production of nukadoko and nukazuke.

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## CONFLICT OF INTEREST

None.

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