紀州鯖なれずしの網羅的および単離法による微生物菌叢の解析

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Analysis of Bacterial Biota Determined Using MiSeq Sequencing and Culture-based Investigations in *Kishu saba-narezushi* (mackerel *narezushi*)

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The microbiota of *Kishu saba-narezushi* (mackerel, *Scomber japonicus*), which is manufactured in the Hidaka region of Wakayama Prefecture, Japan, was analyzed using MiSeq sequencing and culture-based investigations. The dominant microbiota in *Kishu saba-narezushi* was *Lactobacillales* (98%), and all isolated strains were identified as *Lactobacillus plantarum*. From these findings, we concluded that *Lactobacillales* including *Lb. plantarum* is the major component of microbiota in late stage for fermentation of *Kishu saba-narezushi*.

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Key words : *Kishu saba-narezushi, the Hidaka region of Wakayama Prefecture, bacterial biota, lactic acid bacteria, Lactobacillus plantarum*
紀州鯖なれずし，和歌山県日高地方，微生物菌叢，乳酸菌，*Lactobacillus plantarum*

Narezushi is a fermented food made from salted fish, mammal, or bird flesh with a cooked carbohydrate, usually rice, and it is found only in regions of Southeast and Northeast Asia[1]. In Japan, *narezushi* is a traditional local food, and thus several types of *narezushi* have been developed by the indigenous populations using various kinds of local fish, *e.g.*, *samma* (saury, *Cololabis saira*)[2], *ayu* (sweetfish, *Plecoglossus altivelis altivelis*)[3], *aji* (horse mackerel, *Trachurus japonicas*)[4)~7)], *saba* (mackerel, *Scomber japonicus*)[8)~10)], and other fishes, and these recipes have been handed down to successive generations in each of the regions for many years.

Generally, *narezushi* is processed by lactic acid fermentation. In this fermentation process, lactic acid bacteria play an important role. Lactic acid bacteria produce a large amount of lactic acid in various *narezushi*, and the pH of the *narezushi* is kept low after the fermentation process. Therefore, *narezushi* utilizes one of the best preservation methods for perishable proteinaceous foods, especially fishes[11]. Indeed, bacterial biotas in several types of *narezushi* from some regions have been analyzed, and lactic acid bacteria were the dominant organisms in all of these *narezushi*[2)~9)].

Kishu saba-narezushi is mainly manufactured in people's homes during the autumn season in the Hidaka region of Wakayama Prefecture (*Kishu*), Japan. In the case of *Kishu saba-narezushi*, salted *saba* is placed on cooked rice, wrapped up in leaves of *Arundo donax* (Fig. 1), and then placed in layers in a wooden tub while being weighted down. *Kishu saba-narezushi* is fermented from a week to several months under anaerobic conditions. As a result of this process, the flavor of *Kishu saba-narezushi* is distinctive compared with that of other *narezushi*.

Fujii *et al*. reported on the bacterial biota in *Kishu saba-narezushi*, and its dominant microbial flora was

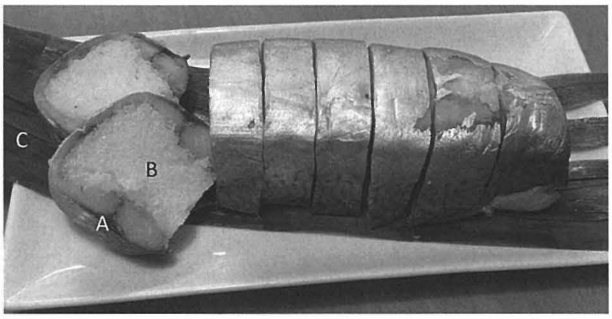

Fig. 1 Image of *Kishu saba-narezushi* (*narezushi* made of mackerel)

(A) fish portion (*saba* [mackerel]), (B) rice portion, and (C) leaves of *Arundo donax*.

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§ Corresponding author, E-mail : t_nakaga@gifu-u.ac.jp composed of *Lactobacillus* (53%) and *Streptococcus* (33%) strains[9]. However, these strains were identified only by phenotypic characteristics, and there is no detailed data on the microbial population in the *Kishu saba-narezushi*.

In this study, we analyzed microbial biota of *Kishu saba-narezushi* in the Hidaka region of Wakayama Prefecture, using MiSeq sequencing and conducted culture-based investigations using the 16 S rRNA gene sequence.

Materials and Methods

1. *Kishu saba-narezushi* sample and contents of organic acids

Kishu saba-narezushi, the fermentation and maturation periods of which were 5 days (2015, Nov 26-30), was collected from a local manufacturer in the Hidaka region of Wakayama Prefecture.

Contents of organic acids of *Kishu saba-narezushi* were analyzed by the method described in the Standard Methods of Analysis in Food Safety Regulations[12]. In this study, we used two samples of *Kishu saba-narezushi* ($n = 2$). Thus data was shown as average of measured values and absolute error.

2. Isolation and identification of lactic acid bacteria

Samples (10 g) of *Kishu saba-narezushi* were emulsified in 90 mℓ of sterile water and blended for 60 s (Stomacher 400 Circulator, Seward, London, UK). The sample suspensions were diluted in sterile water and appropriate dilutions were spread on MRS plate (Lactobacilli MRS Agar ; Becton, Dickinson and Company, Franklin Lakes, NJ).

The 16 S rRNA genes of isolated lactic acid bacteria were amplified from the colonies via the colony PCR method using the primers 20F and 1500 R[13].

3. MiSeq sequencing

From samples of *Kishu saba-narezushi*, we extracted total DNA using a NucleoSpin Soil kit (Macherey-Nagel GmbH & Co. KG, Germany). The V3-V4 variable region of the 16S rRNA gene was amplified from the total DNA using the 16 S metagenomic sequencing library protocol (Illumina, San Diego, CA)[14]. The paired-end reads were assembled using fastq-join (ea-utils version 1.1.2-537). The primary processing of sequencing reads and clustering into operational taxonomic units (OTUs) were accomplished in the context of Quantitative Insights Into Microbial Ecology (QIIME, version 1.8.0)[15].

Results and Discussion

At first, we performed a general chemical analysis, *i.e.*, the pH and contents of organic acids. The pH of *Kishu saba-narezushi* was 3.93 ± 0.08. Fujii *et al.* previously reported that the pH of *Kishu saba-narezushi* was 3.95 (fish portion) and 3.75 (rice portion)[9]. Additionally, the organic acid contents such as lactic acid and acetic acid in the fish portion were 9.85 ± 0.15 mg/g and 0.51 ± 0.02 mg/g, respectively, and those contents in the rice portion were 8.70 ± 0.00 mg/g and 0.48 ± 0.05 mg/g, respectively. These findings are similar to the data obtained from commercially available samples reported by Chang *et al.* (lactic acid, 11.4 ± 0.76 mg/g ; and acetic acid, 2.06 ± 0.12 mg/g)[10]. These results suggest that lactic acid fermentation occurred in *Kisyu saba-narezushi* and that the lactic acid contributes a sour taste to *Kisyu saba-narezushi*, as it does in other *narezushi*.

Next, we tried to determine the count of viable lactic acid bacteria in *Kishu saba-narezushi*. In *Kishu saba-narezushi* samples, the maximum viable plate counts were $3.3 \times 10^9 \pm 2 \times 10^8$ CFU/g.

We also identified the microbial biota in *Kishu saba-narezushi*. For our sample of *Kishu saba-narezushi*, 83,959 sequences were obtained and analyzed phylogenetically. These clones were classified into 336 OTUs. However, only two major orders were found in the bacterial biota of *Kishu saba-narezushi* : the dominant order was *Lactobacillales* (98%) and the other was *Bacillales* (Table 1). The most dominant family was found to be *Lactobacillaceae* (85.4%), followed by *Leuconostocaceae* (3.7%) and *Streptococcaceae* (3.6%) (Table 1). However, the genus for most reads belonging to *Lactobacillaceae* could not be identified (78.9%), although these reads did include the genus *Pediococcus* (4.0%), *Lactobacillus* (2.5%), and *Weissella* (0.1%) (Table 1). From these results, it was suggested that *Kishu saba-narezushi* contains a *Lactobacillales* - dominant microbiota.

Finally, we attempted to identify the dominant species in *Kishu saba-narezushi*. We randomly selected 12 single colonies from the *narezushi* using the MRS plate, and the 16S rRNA sequences of all strains had high homologies with the sequence of *Lb. plantarum* subsp. *plantarum* NBRC 15891^T (> 99%) (Fig. 2). On the other hand, strains of genera *Leuconostoc*, *Streptococcus* and *Pediococcus* were not isolated from *Kishu saba-narezushi*, although 16 S

Table 1 Read statistics of *Kishu saba-narezushi*

Order	(number of reads)	Family	(number of reads)	Genus	(number of reads)
Lactobacillales	82,259 (98%)	*Lactobacillaceae*	71,688 (85.4%)	not identified	71,688 (78.9%)
				Pediococcus	3 ,394 (4.0%)
				Lactobacillus	2 ,072 (2.5%)
				Weissella	79 (0.1%)
		Leuconostocaceae	3 ,107 (3.7%)	not identified	2 ,582 (3.1%)
				Leuconostoc	446 (0.5%)
		Streptococcaceae	3.015 (3.6%)	*Lactococcus*	3 ,004 (3.6%)
		Enterococcaceae	604 (0.7%)	*Enterococcus*	604 (0.7%)
Bacillales	1 ,607 (1.9%)	*Staphylococcaceae*	1 ,601 (1.9%)	*Staphylococcus*	1 ,601 (1.9%)

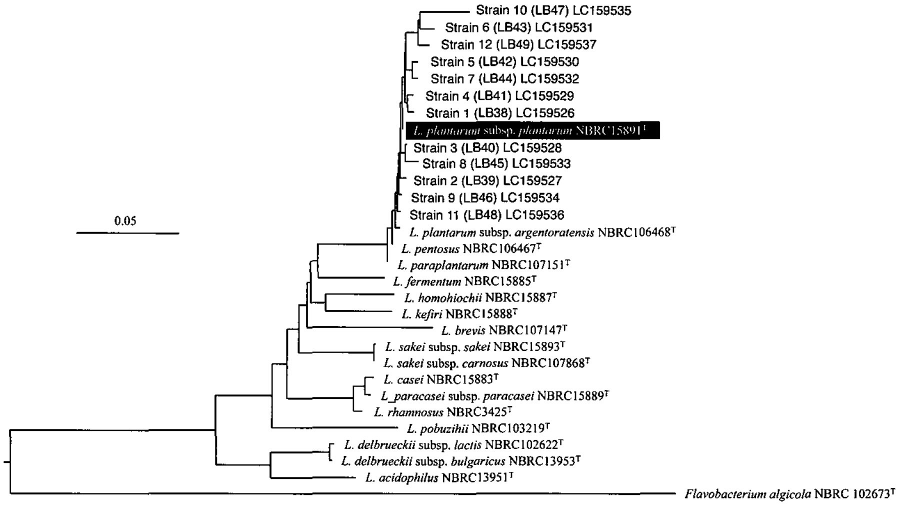

Fig. 2 Phylogenic relationships of the isolated strains from *Kishu saba-narezushi* and several related species based on 16S rRNA gene sequences

The branching pattern was generated by the neighbor-joining method.
The bar indicates 0.05 nucleotide substitutions per site. The sequence data are cited from GenBank.

rRNAs from these genera were identified in the sample. These findings support the result of metagenomic analysis that is a *Lactobacillales* - dominant microbiota in *Kishu saba-narezushi*.

In this study, we showed that the dominant microbial biota in *Kishu saba-narezushi* was lactic acid bacteria, *Lactobacillales* (98%). Recently, the microbiota of several *narezushi* have been reported; in *Mie saba-narezushi*[8)], *Mie-narezushi*[2)], *Mie ayu-narezushi*[3)], *Noto aji-narezushi*[4)~7)], and *funazushi*[16)] samples, *Lactobacillales* was the dominant microbial biota just as it was in *Kishu saba-narezushi*. Taking these reports together, our results indicate that bacteria belonging to the *Lactobacillales* may play important roles in the fermentation of various types of *narezushi*.

Moreover, all strains isolated from *Kishu saba-narezushi* were identified as *Lb. plantarum* (Fig. 2). From all findings in this study, we concluded that *Lactobacillales* including *Lb. plantarum* is the major component of microbiota in late stage for fermentation of *Kishu saba-narezushi*. It was reported that the dominant microbial flora of *Kishu saba-narezushi* was *Lactobacillus* (53%) strains, and they were identified as *Lb. plantarum, Lb. alimentaria*, and *Lb. coryniformis* via the colony picking method[9)]. In contrast, MATSUI *et al*. reported that the most abundant species found in *Mie saba-narezushi* was *Lb. curvatus* (51.1%)[8)]. At this time, we are not able to determine the exact cause for the difference in microbial biota between *Mie* and *Kishu saba-narezushi*. We speculate that one of the causes for this difference is related to the leaves, *Arundo donax*, which are used for wrapping up the salted *saba* that is placed on the cooked rice. Our next study will focus on ① identifying the origin of the microbial biota of *Kishu saba-narezushi*, ② observing transitions of microbial biota in *Kishu saba-narezushi* during its fermentation process, and ③ refermenting the *saba-narezushi* using isolated *Lb. plantarum* strains.

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紀州鯖なれずしの網羅的および単離法による微生物菌叢の解析

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本研究では，和歌山県日高地方の紀州鯖なれずしの微生物菌叢について，網羅的および単離法により同定した。紀州なれずしでは，ラクトバチルス科が98％を占める支配的な微生物群であり，単離法にて同定した12株すべてが*Lactobacillus plantarum*であった。これらのことから，*Lb. plantarum*を含む*Lactobacillus*が紀州なれずしの主要発酵菌であることが明らかになった。

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