Microbial diversity of '*narezushi*' from Wakayama Prefecture, western Japan

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Abstract

'*Narezushi*' is a Japanese traditional fermented food made of salted raw fish and cooked rice. We surveyed microbial community of four independent *narezushi* of mackerel (three samples) and saury (one sample) from Wakayama Prefecture, western Japan, by 16S ribosomal RNA gene sequencing. V4 region (254 bp) of the gene was amplified by PCR using barcoded primers and sequenced by Illumina MiSeq. Qualified sequences were clustered into total 150 operational taxonomy units (OTUs) with the threshold of 97 % identity, and actual OTU numbers observed in each sample were between 127 and 141. These OTUs were classified at the genus level using Ribosomal Database Project (RDP) classifier, and further assigned to species by the BLAST search. In all samples, Lactobacillales was dominant (86–93 % of analyzed sequences), confirming favorable lactic acid fermentation. The most abundant OTU was the same in all samples, which is represented by *Lactobacillus sakei*, *L. graminis*, *L. curvatus*, *L. plantarum*, and *L. brevis*. By contrast, the second most abundant OTU was represented by different coccus in each sample, such as *Leuconostoc pseudomesenteroides*, *Pediococcus pentosaceus* and *Lactococcus lactis* subsp. *lactis*. Small numbers of possible pathogens and putrefactive bacteria, such as *Yersinia*, *Aeromonas*, *Vibrio* and *Acinetobacter*, were also detected.

Key words: lactic acid bacteria, traditional fermented food, metagenomics.

1. Introduction

'*Narezushi*', a traditional type of Japanese *sushi*, was historically made to preserve raw fish fillets by lactic acid fermentation with cooked rice. Similar fermented foods are widely found in the eastern region of Asian Continent. Prior to the Nara Period (~AD 700), this type of fermented food was imported from Asian Continent to Japan, and was developed to Japanese *narezushi*. In the Edo Period (AD 1600~), modern type of *sushi* made of vinegared raw fish and rice was developed and spread to all over the country mainly in urban areas. *Narezushi* is now restrictedly found in peripheral regions in Japan such as Tohoku District, the Noto Peninsula, the area surrounding Lake Biwako, and the Kii Peninsula (including Wakayama Prefecture), from north to south (Fig. 1). Therefore, Wakayama is one of the southernmost localities of *narezushi* production. In Wakayama, mackerel (*saba*) is mainly used in the northern part (Fig. 2a) and saury (*samma*) is in the southern part (Fig. 2b). They are generally made as following processes: 1) fresh fish fillets is deeply salted for more than one week; 2) desalted in tap water for overnight; 3) put on the top of rod-shaped rice cooked with approximately 3 % salt water; 4) wrapped with the leaves of giant reed, *Arundo donax*, in the case of *saba narezushi*, or the leaves of ferns such as *Gleichenia japonica* in the case of *samma narezushi*; 5) packed in a wooden box and soaked by 3 % salt water to make anaerobic condition; 6) fermented generally at room temperature for more than 10 days, usually around 2 weeks. The fermentation period of *narezushi* in Wakayama is relatively shorter than those in the other localities, such as *funazushi* around Lake Biwako, which is fermented for more than 6 months.

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In recent years, several papers were published on microflora of *narezushi* from the Noto Peninsula (Hokuriku District, central Japan) and Mie Prefecture (eastern part of the Kii Peninsula); the former was performed by metagenomic strategy, whereas the latter clone library method[1-4]. However, no report has so far been published on *narezushi* from Wakayama. In the present study, we analyzed the microflora of four types of *narezushi* from Wakayama in detail using metagenomic 16S rRNA gene sequencing strategy.

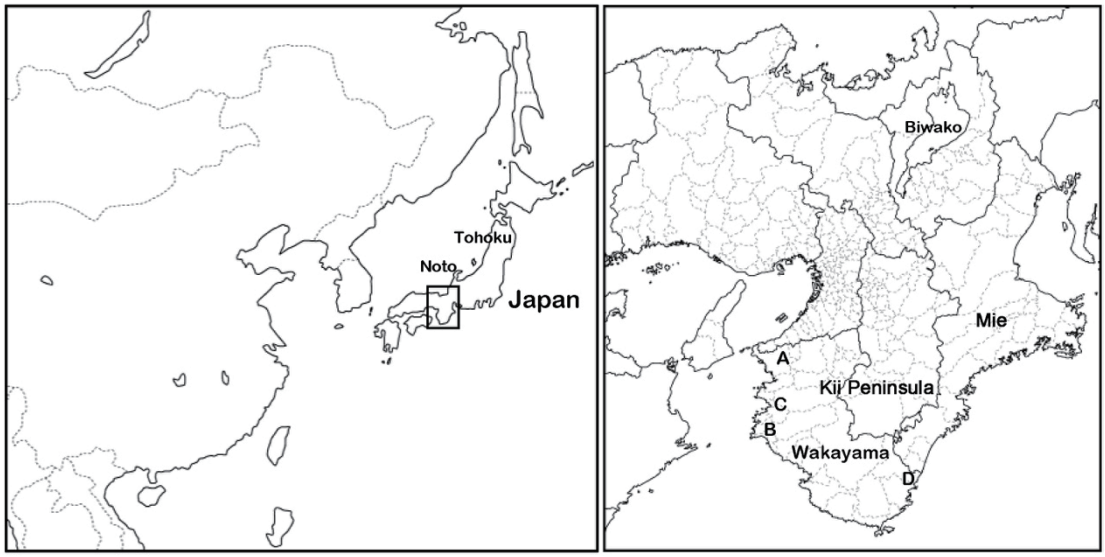


Fig. 1. Localities where the *narezushi* samples (A–D) were obtained.

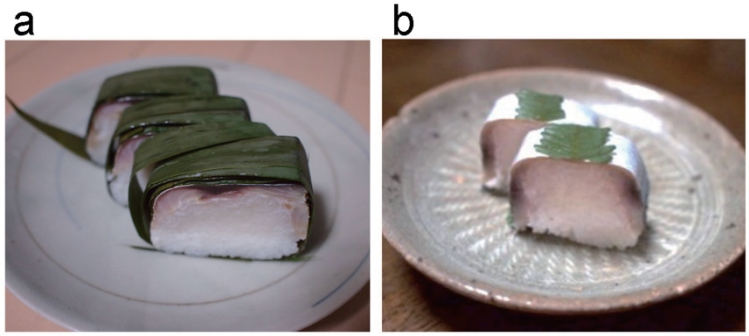


Fig. 2. *Narezushi* from Wakayama Prefecture, Japan: (a) *Saba* (mackerel) *narezushi*; (b) *Samma* (saury) *narezushi*. Fish fillets on the top of rod-shaped rice is wrapped with the leaves of giant reed (a) or fern (b).

2. Materials and methods

2.1. DNA extraction from *narezushi* samples

Four *narezushi* samples were purchased from different manufacturers in Wakayama Prefecture. Samples A (obtained in Wakayama-shi), B (Gobo-shi) and C (Aritagawa-cho) were prepared with *saba* (mackerel), and sample D (Shingu-shi) was with *samma* (saury) (Fig. 1). Each sample (1 g) was suspended in 2 ml of 150 mM NaCl thoroughly. After standing for 5 min at 25 °C, the supernatant was collected by pipetting, and then centrifuged at 4,000 *g* for 15 min. The precipitation was collected for DNA extraction with NucleoSpin Tissue (Macherey-Nagel GmbH & Co. KG, Germany) following the instruction manuals.

2.2. Amplification of V4 region of 16S rRNA gene

V4 region (254 bp) of 16S rRNA gene was amplified using KOD-Plus-ver. 2 (Toyobo, Japan) and the extracted DNA samples as templates. The primers used were 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). V4 region has been reported to be sufficient for taxonomy assignment[5]. Twenty cycles were performed and each cycle consisted of 10-s denaturation at 98 °C, 30-s annealing at 55 °C, and 60-s extension at 68 °C. A 2-min predenaturation at 94°C and an additional 3-min extension at 68 °C were applied before and after the cycles, respectively. Then the 254-bp bands were excised and purified with Wizard SV Gel and PCR Clean-Up System (Promega Co., WI, USA) and used as the template for the second PCR.

In the second PCR, 515F2 (5'-AATGATACGGCGACCACCGATCTACACTATGGTAATTGTGTGCCAGCMG CCGCGGTAA-3') and R_806_rcbc#### (5'-CAAGCAGAAGACGGCATACGAGATNNNNNNNNNNNNAGTCA GTCAGCCGGACTACHVGGGTWTCTAAT 3') were used to add the specific adapter sequences required for Illumina MiSeq platform (both ends) and Golay barcode (3'-end). Twelve bases with underline were individually indexed Golay barcodes for each sample as follows: TTCGCAGATACG of R_806_rcbc1882 for sample A, TTAGCCCAGCGT of R_806_rcbc1889 for sample B, GCTATATCCAGG of R_806_rcbc1908 for sample C, GCGCGTGTATCT of R_806_rcbc1934 for sample D. The reaction was performed basically the same as the first one except the extension temperature was 72 °C. The amplified fragments were excised, purified, and mixed (80 ng each). The sample was qualified with the Agilent 2100 Bioanalyzer and then applied to MiSeq sequencing.

2.3. Sequencing

Sequencing was performed with Illumina MiSeq system[6] and MiSeq Reagent Kit v3 (Illumina Inc., Ca, USA). Sequencing library was constructed and sequenced under 50 % denatured Phix concentration. Sequencing data was processed with MiSeq Control Software v2.3.0.3, Real Time Analysis v1.18.42, and Consensus Assessment of Sequence and Variation v1.8.2. Obtained reads were sorted by the barcode sequence and analyzed farther.

2.4. Taxonomic assignment

Paired-end reads were assembled into contigs, demultiplexed, and quality filtered with CD-HIT-OTU v0.0.1 (http://weizhong-lab.ucsd.edu/cd-hit-otu/)[7]. The operational taxonomy units (OTUs) were constructed from the library by clustering with the threshold of 97 % identity. Taxonomy was assigned based on the GreenGenes gg_13_5[8] by Ribosomal Database Project (RDP) classifier v2.2 (http://rdp.cme.msu.edu/index.jsp)[9] using QIIME v1.7 (http://qiime.org/)[10] with bootstrap score over 0.8. The representative sequences of each OTU were applied to similarity search against DDBJ 16S ribosomal RNA database v.2014_02_11 using the BLAST program v2.2.20.

2.5. Statistical analysis

Rarefaction analyses were performed with QIIME[10]. Rarefaction curves were calculated and α-diversity metrics of Chao1 and Shannon index were determined. Heatmap was created with the stats package of R (http://www.R-project.org/).

3. Results

3.1. Qualification of sequencing data and α-diversity estimates

Total DNA was extracted from four *narezushi* samples obtained in Wakayama Prefecture, Japan (Fig. 1), and V4 region of 16S rRNA gene was amplified and sequenced. Sequencing reads obtained ($1.1–1.6 \times 10^6$ reads per each

sample) were demultiplexed and quality filtered, resulting in 4.5–6.7 × 10^5 sequences per sample. The α-diversity metrics were analyzed for each sample (Table 1). The values of Chao1 richness were calculated between 133 and 145 (140.4 ± 5.1, mean ± SD). The values of Chao1 richness for all samples were larger by the number of 4 to 6 than the actual observed OTU numbers, indicating diversity with some rare reads in all the samples. Shannon indices were calculated between 1.9 and 2.2 (2.05 ± 0.13, mean ± SD), suggesting no significant difference in α-diversity.

The reads were clustered with threshold of 97 % identity and 150 OTUs were totally constructed. Numbers of observed OTUs were 127, 140, 141 and 134 for sample A, B, C and D, respectively (Table 1). Rarefaction curves plotted for all samples were saturated at around 3 × 10^5 reads for both Chao1 richness (Fig. 3a) and Shannon index (Fig. 3b), indicating adequate numbers of reads were taken for data analyses.

Table 1. Summary of sequence analysis and α-diversity metrics

Product (fish)	Total reads	Analyzed reads	OTUs	α-Diversity metrics	
				Chao1 richness	Shannon index
A (mackerel)	1,493,598	642,424	127	133.316	2.174
B (mackerel)	1,584,296	677,952	140	145.399	1.933
C (mackerel)	1,137,960	454,323	141	145.245	1.916
D (saury)	1,178,410	472,444	134	137.800	2.206

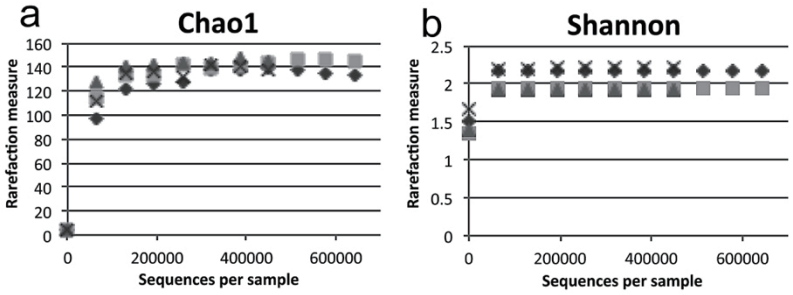


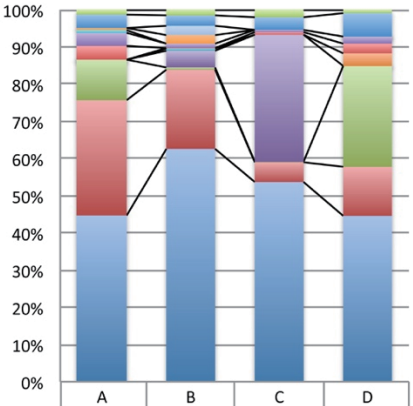
Fig. 3. Rarefaction curves for α-diversity metrics: (a) Chao1 richness; (b) Shannon index. Curves were plotted for *narezushi* samples A (◆), B (■), C (▲) and D (×).

3.2. Bacterial diversity

The major OTUs were classified to the genus level fundamentally by RDP classifier with bootstrap < 0.8 (Fig. 4). The most abundant OTU found in all four products (44.3, 61.2, 50.5 and 44.4 % for product A, B, C and C, respectively) were the same and assigned to family level, Lactobacillaceae, by RDP classifier. Therefore, several representative sequences in this OTU were similarity searched using the BLAST program and identified as *Lactobacillus sakei*/*L. graminis*/*L. curvatus*/*L. plantarum*/*L. brevis* with E-value of 1.00E–141 (Fig. 4), and thus indicated as *Lactobacillus sakei/graminis* (Fig. 5). Two other OTUs without genus level assignment were identified likewise, *Lactobacillus herbinensis* (Fig. 5) and *Enterobacter* sp. (Fig. 5) with E-values of both 1.00E–139. Genera classified as "Others" were less than 2.0 % of each sample (Fig. 4), which include Clostridiales, *Arcobacter*, *Shewanella, Proteus*, and *Halomonas*. Mitochondrial sequences from plants accounted for 3–6 % of either samples. Since the rice portion of *narezushi* was included and used for DNA extraction, it was possible that the plant DNA

was contaminated and further sequenced simultaneously.

Lactobacillus was predominant in all four samples, accounted for 44.7, 62.6, 53.7 and 44.6 % of total flora of samples A, B, C and D, respectively. *Leuconostoc* followed 31.0 and 21.3 % of samples A and B, respectively. For samples C and D, *Leuconostoc* was accounted for 5.3 and 13.2 %, respectively, as the third most abundant genus. *Pediococcus* was the second most frequent genus in sample C (34.2 %) and the third most in sample B (4.5 %). *Lactococcus* followed *Lactobacillus* in sample D (27.1 %) and was the third most abundant in sample A (10.9 %). The order Lactobacillales was accounted for 86.6, 89.7, 93.3 and 88.3 % of sample A, B, C and D, respectively.



	A	B	C	D
Others	1.3	1.5	2.0	0.9
plant (mitochondria)	3.6	2.8	3.4	6.3
Vibrio	0.0	2.5	0.0	0.0
Acinetobacter	0.6	2.3	0.0	0.0
Aeromonas	0.7	0.0	0.0	0.0
Enterobacter	3.5	1.2	0.6	1.9
Yersinia	3.7	0.0	0.7	2.6
*Enterococcus**	0.0	0.1	0.0	3.4
*Weisella**	0.0	0.6	0.0	0.0
*Pediococcus**	0.0	4.5	34.2	0.0
*Lactococcus**	10.9	0.6	0.1	27.1
*Leuconostoc**	31.0	21.3	5.3	13.2
*Lactobacillus**	44.7	62.6	53.7	44.6
total Lactobacillales	86.6	89.7	93.3	88.3

Fig. 4. Relative abundance of bacterial phylogenetic groups at the genus level in *narezushi* from Wakayama. Phylogenetically assigned groups at the genus level that reached an abundance ≥ 0.5 % of all classified sequences were labeled. All minor components (< 0.5 %) are clustered into "Others". Total percentage of the order Lactobacillales including six genera (*) were indicated at the bottom of the figure.

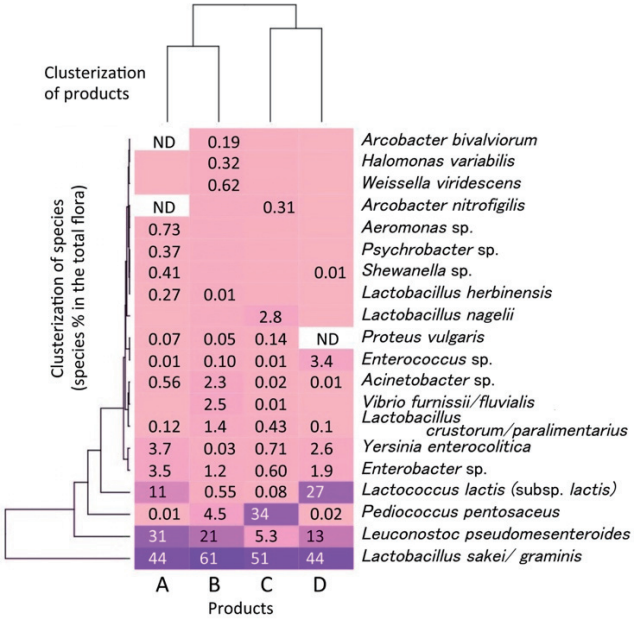


Fig. 5. Heatmap of the bacterial composition in *narezushi* from Wakayama. Clustering of the four samples and of the 20 species was performed based on the Euclidean distance calculated from the species as percentages (indicated on each box in the figure), and the resulting dendrograms are shown on the x and y axes, respectively. Blank boxes indicate value of < 0.01 %; ND means no read was detected for the corresponding species.

Table 2. Identification of the major OTUs with the BLAST program.

OTU ID	RDP classifier	BLAST	E-values (1.00E)
146	*Arcobacter*	*Arcobacter bivalviorum*	–135
133	*Halomonas*	*Halomonas variabilis*	–139
103	*Weisella*	*Weisella viridescens*	–139
144	*Arcobacter*	*Arcobacter nitrofigilis*	–139
104	*Aeromonas*	*Aeromonas* sp.	–137
128	*Schewanella*	*Psychrobacter* sp.	–139
125	*Schewanella*	*Schewanella* sp.	–139
134	*Lactobacillus*	*Lactobacillus herbinensis*	–139
66	*Lactobacillus*	*Lactobacillus nagelii*	–139
149	*Enterobacter*	*Proteus vulgaris*	–139
62	*Enterococcus*	*Enterococcus* sp.	–139
64	*Acinetobacter*	*Acinetobacter* sp.	–139
63	*Vibrio*	*Vibrio furnissii / fluvialis*	–139
67	*Lactobacillus*	*Lactobacillus crustorum / paralimentarius*	–139
56	*Yersinia*	*Yersinia enterocolitica*	–139
54	*Enterobacter*	*Enterobacter* sp.	–139
8	*Lactococcus*	*Lactococcus lactis* (subsp. *lactis*)	–140
11	*Pediococcus*	*Pediococcus pentosaceus*	–140
6	*Leuconostoc*	*Leuconostoc pseudomesenteroides*	–141
3	*Lactobacillus*	*Lactobacillus sakei / graminis*	–141

Twenty OTUs belonging to the 11 genera and "others" of Fig. 4 were identified to the species level with the BLAST program. *Schewanella*, *Halomonas* and *Arcobacter* are included in "others".

3.3. Identification in species level and diversity clustering

OTUs belonging to the 11 major genera and 3 minor ones (*Schewanella*, *Halomonas* and *Arcobacter*) in "others" (Fig. 4) were then classified at the species level by using the BLAST program (Table 2). OTUs classified as the family Lactobacillaceae except for *Enterococcus* were well assigned to the species with E-values of 1.00E–139 to 141. Other OTUs of *Halomonas, Proteus, Vibrio,* and *Yersinia* were also assigned to the species (E-values of 1.00E–135 to 139). OTUs of *Aeromonas, Psychrobacter, Shewanella, Acinetobacter* and *Enterobacter,* all categorized to the class Gammaproteobacteria, were not assigned at the species level even with the BLAST program.

Heatmap analysis of these 20 species was shown (Fig. 5). The dominant OTU of all four samples was the same and contained *Lactobacillus sakei*, *L. graminis*, *L. curvatus L. plantarum* and/or *L. brevis*, which are hardly distinguishable from each other using V4 region. In addition, *Lactobacillus* including *L. herbinensis, L. nagelii* and *L. crustorum/ L. paralimentarius* were found as minor contents (~2.8 % of the total flora). *Leuconostoc pseudomesenteroides* was relatively abundant (5–31 %) in all the samples. *Pediococcus pentosaceus* was detected in sample C (34 %) as the second most abundant, whereas 4.5 % in sample C and 0.01–0.02 % in samples A and D. *Lactococcus lactis* was the second most abundant species in sample D (27 %) and also major in sample A (11 %), though the percentages were much lower in samples B (0.55 %) and C (0.08 %). Small numbers of *Weissella viridescens* were detected in all samples, and the percentage was significantly larger in sample B (0.6%).

Clustering analysis indicated that samples A and B, and samples C and D composed clusters. Either three OTUs/species out of four belonging to the family Lactobacillaceae (*Lactobacillus sakei/graminis, Leuconostoc pseudomesenteroides, Pediococcus pentosaceus*, and *Lactococcus lactis*) were major microbial components of *narezushi* samples, and more than 14 minor species were observed in all the samples.

4. Discussion

In this study, microflora of four *narezushi* samples from Wakayama Prefecture were analyzed using V4 region of 16S rRNA gene. Numbers of the obtained data were sufficient for discussing the diversity, indicated by rarefaction analyses (Fig. 3). Shannon indices were around 2 for all of the samples without any significant differences, suggesting similar diversities of all samples. These data are similar to the previously reported Shannon indices, 1.23–2.60, of various *narezushi* samples from other areas[1]. Among 150 OTUs determined, 114 were common in all the samples, 19 in any three samples, 12 in any two and 5 were unique (1, 2 and 2 OTUs in samples B, C and D, respectively). The order Lactobacillales accounted for 86–93 % of the total reads in each sample, demonstrating these bacteria are essential for fermentation of *narezushi* in Wakayama area as was reported for those from Hokuriku District[12]. The most dominant OTU common in all the samples (44–61 %) was classified to the family Lactobacillaceae without any specified genus by RDP classifier. Further BLAST search revealed that this OTU contained *Lactobacillus sakei*, *L. graminis*, *L. curvatus*, *L. plantarum* and *L. brevis*. Because V4 regions of these *Lactobacillus* species are nearly identical, other valuable region in 16S ribosomal RNA gene should be sequenced for precise identification. The second most abundant species were different cocci in each sample: *Leuconostoc pseudomesenteroides* in samples A and B (31 and 21 % respectively), *Pediococcus pentosaceus* in sample C (34 %), and *Lactococcus lactis* in D (27 %). This suggests that cocci are exclusive of each other.

Lactobacillus sakei was first isolated from Japanese *sake* starter and originally described as *Lactobacillus sake*[11]. This species is widely found on plants as well as fresh meat and fish. It was also reported that *Lactobacillus sakei* was dominant in several *narezushi* samples such as *aji* (horse mackerel) *narezushi* from the Noto Peninsula[1] and *kaburazushi*, another type of lactic acid fermented *sushi*, from Ishikawa Prefecture[12], whereas different *Lactobacillus* species such as *L. plantarum* and *L. acidipiscis*, and also *Pediococcus ethanolidurans* were most abundant in other *aji narezushi* samples[1]. The frequency (44–61%) of the dominant species in our study was relatively lower than these reports (51–91%). This might be because shorter fermentation periods (around 2 weeks) for *narezushi* from Wakayama area. It was reported that *Lactobacillus* population increased approximately 11-fold by the end of the fermentation (41-day) as compared with the population at 7-day[13].

Lactobacillus sakei and *Lactobacillus curvatus* were most abundant in saury and mackerel *narezushi*, respectively, from Mie Prefecture, adjacent area of Wakayama, which were analyzed by the 16S rRNA gene clone library method[2,4]. Although we couldn't distinguish *Lactobacillus sakei* and *Lactobacillus curvatus* using V4 region of rRNA gene as a target sequence, the predominant species of *narezushi* samples from these adjacent areas are found to be closely similar. This might be due to the similar process of production. In our analyses, only sample D was of saury. Diversity clustering analysis showed that sample C is closer to D than to the cluster of samples A and B (Fig. 5). Therefore, microflora is not dependent on the fish ingredient, but rather on manufacturer and their production process.

As in the previous reports of others, we confirmed that Lactobacillales is also dominant in the flora of *narezushi* from Wakayama, which were fermented without any starter culture. Salt tolerance of *Lactobacillus sakei/curvatus* [14] and other Lactobacillales species might be the keys for predominant growth of these species in *narezushi*. It is obvious that major growth of Lactobacillales lowers the pH of the product to inhibit the growth of harmful bacteria. However, it is noteworthy that the DNA of possible pathogens and putrefactive bacteria were detected in the samples in significant proportions: i.e., *Yersinia*, *Aeromonas*, *Vibrio,* and *Acinetobacter*. Although we don't aware whether

they are alive in the final products because V4 region can be amplified from dead cells, we must pay attention to these bacteria.

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和文抄録

和歌山県産本なれ鮓の微生物叢解析

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本なれ鮓は、塩漬けにした生の魚肉を米飯とともに発酵させた日本の伝統食品である。和歌山県産のサバ本なれ鮓（３試料）とサンマ本なれ鮓（１試料）の合計４試料について、メタゲノム解析により菌叢を明らかにした。16S リボソーム RNA 遺伝子 V4 領域の 254 塩基対を、試料識別のためのバーコードを含むプライマーを用いた PCR により増幅させ、イルミナ MiSeq システムでシークエンスした。各試料から得られた十分な数の良質なシークエンスデータについて、相同性の閾値を 97%に設定しクラスタリングしたところ、合計 150 の OTU (operational taxonomy unit)が構築された。各試料に含まれる OTU 数は 127〜141 の範囲で、試料間に有意な差は無かった。これらの OTU を RDP (Ribosomal Database Project) classifier により属レベルで同定し、さらに BLAST により種レベルの同定を試みた。全ての試料において、乳酸菌目 Lactobacillales が総リードの 86–93 %を占めており、良好な乳酸発酵が示唆された。最も優勢な OTU は、いずれの試料においても *Lactobacillus sakei*、*L. graminis*、*L. curvatus*、*L. plantarum*、*L. brevis* に代表されるものであった。一方、次に優勢な OTU は試料間で異なり、*Leuconostoc pseudomesenteroides*、*Pediococcus pentosaceus*、*Lactococcus lactis* subsp. *lactis* などの乳酸球菌がそれぞれ代表種であった。また、ごく少数ながら、*Yersinia*、*Aeromonas*、*Vibrio*、*Acinetobacter* などの腐敗菌や食中毒菌の DNA が検出された。

キーワード：乳酸菌、伝統発酵食品、メタゲノム解析

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