Note

Comparison of the Bacterial Community Structures of *Ayu-narezushi* Produced by Two Different Manufacturers

HIROKI MATSUI^{1*}, ERIKO SAKA², YUKA ISOBE², AND MIYO NARITA²

¹Graduate School of Bioresources and ²Faculty of Education, Mie University,
Kurimamachiya-cho 1577, Tsu, Mie 514-8507, Japan

Received 11 December, 2009/Accepted 26 March, 2010

We analyzed and compared the bacterial communities in *ayu-narezushi* (*narezushi* made using the sweetfish) produced by two different manufacturers in Mie Prefecture, Japan, with the 16S ribosomal RNA gene clone library method. The bacterial diversity of *ayu-narezushi* 1 (Library 1) was higher than that of *ayu-narezushi* 2 (Library 2). The predominant lactic acid bacteria were *Lactobacillus fuchuensis*, *Lactococcus piscium*, and *Leuconostoc gelidum* in Library 1 and *Lactobacillus sakei* in Library 2, suggesting that these species play important roles in the fermentation of the 2 samples of *ayu-narezushi*. The results of LIBSHUFF indicated a significant difference between 2 libraries ($P = 0.001$).

Key words : *Ayu-narezushi*/Fermented food/Lactic acid bacteria/16S rRNA gene clone library.

Narezushi is a traditional fermented food found in various regions of Japan. It is made by fermenting salted fish meat with cooked rice, which acts as a carbohydrate source for the fermentation. This type of fermented fish product is widespread in Southeast and Northeast Asia (Ishige, 1993). In Mie Prefecture of Japan, several kinds of *narezushi* made out of a variety of fish are produced as ritual or celebratory foods.

Recently, we analyzed the microbial diversity in *samma-narezushi* by using the 16S ribosomal RNA (rRNA) gene clone library method and showed that *Lactobacillus sakei* was the predominant bacterial species, making up 72% of the total number of clones; this suggests that this lactic acid bacterium plays an important role in the fermentation of *samma-narezushi* (Matsui et al., 2008). To the best of our knowledge, this is the only published study investigating the microbial diversity of *narezushi* by using a culture-independent approach. Thus, information regarding the bacterial diversity in *narezushi* is still limited.

In the present study, we chose *narezushi* of *ayu* (sweetfish, *Plecoglossus altivelis altivelis*) from a variety of *narezushi* produced in Mie Prefecture. The purpose of this study was to use a 16S rRNA gene clone library to investigate and compare the diversity of bacteria in *ayu-narezushi* produced by two different local manufacturers in order to better understand the microorganisms involved in the fermentation of *narezushi*.

One sample (*ayu-narezushi* 1) was obtained from a local manufacturer in Kiho town, Mie Prefecture, Japan, in 2004, where it was produced as follows: After the removal of the head and internal organs, the sweetfish was salted for 6 months, after which the salted sweetfish was pickled in a tub along with cooked rice for about 3 weeks in winter. The other *ayu-narezushi* sample (*ayu-narezushi* 2) was obtained from a local manufacturer in Ise city, Mie Prefecture, Japan, in 2005. This fish was salted for 2 months, after which the salted sweetfish was pickled together with cooked rice for 3 weeks in winter. Sample treatment, DNA extraction, PCR amplification and cloning were carried out as described in Matsui et al. (2008). Briefly, Each sample was homogenized in a sterilized food processor. The sample was

*Corresponding author. Tel : +81-59-231-9593, Fax : +81-59-231-9540, E-mail: matsui (a) bio.mie-u.ac.jp.

immediately suspended in acetone at a final concentration of 70% to avoid the degradation of DNA (Fukatsu, 1999) and stored at -80°C until analysis. The stored samples were washed twice with sterilized physiological saline by centrifugation to remove acetone. DNA was extracted by the bead-beating method with the FastPrep instrument (Bio 101, Vista, CA) as described by Godon et al. (1997). The crude DNA was purified with Genomic-tip 100/G (QIAGEN, Hilden, Germany) and dissolved in TE buffer. The DNA concentration was adjusted to $10\text{ ng}/\mu\text{l}$. The 16S rRNA gene fragments were amplified by PCR with a set of universal primers (530f and 1392r) for 3 domains of organisms (Eubacteria, Archaea, and Eukaryote). PCR was performed using TaKaRa Ex Taq (TaKaRa, Otsu, Japan) with the PCR Thermal Cycler Dice TP600 (TaKaRa, Otsu, Japan) as described in Matsui et al. (2008). The PCR products were cloned with a TA Cloning Kit (Invitrogen, Carlsbad, CA) as described in the manufacturer's protocol. Positive clones were randomly selected. The cloned DNA fragments were commercially sequenced by Shimadzu Corporation (Kyoto, Japan).

Homology search, chimera formation, and phylogenetic analysis of cloned sequences were carried out as described in Matsui et al. (2008). OTU assignment and calculation of the Shannon-Wiener index were carried out with the FastGroupII online program (Yu et al., 2006). The Shannon-Wiener index (H') is calculated by the following equation:

$$H' = - \sum_{i=1}^s P_i \ln P_i (P_i = n_i / N)$$

where s is the number of OTUs, P_i is the proportion of the i th OTU, n_i is the number of the clone in i th OTU, and N is total number of clones in the library. Cloned sequences were assigned into an OTU based on a 97% identity threshold. The statistical significance of differences between the resulting 2 16S rRNA gene clone libraries was calculated with the webLIBSHUFF

program (<http://libshuff.mib.uga.edu>) (Singleton et al., 2001). DNA sequences were deposited in the DDBJ database under the accession numbers AB326710-AB326810 for *ayu-narezushi* 1 and AB326811-AB326916 for *ayu-narezushi* 2.

A total of 101 and 106 clones were randomly selected from the 16S rRNA gene clone libraries of *ayu-narezushi* 1 (Library 1) and *ayu-narezushi* 2 (Library 2), respectively (Table 1). Nucleotide sequence length analyzed was approximately 890 bp. These clones were subjected to DNA sequence analysis; no chimeric sequence was found using the CHECK_CHIMERA program. In this study, we used a primer set that covered 3 domains of organisms. However, no sequence affiliated to archaea or eukaryote was recovered from either library. On the basis of the 97% sequence-identity threshold, 25 OTUs were found in Library 1 and 16 OTUs in Library 2 (Table 1). The bacterial diversity of the 2 libraries, expressed as the Shannon-Wiener index (H'), was higher for Library 1 than for Library 2. The values of the index were greater than our published results for *samma-narezushi* (Table 1).

For each OTU, the abundance of clones and sequence similarity to known bacterial species is shown in Table 2, and a phylogenetic tree is shown in Figure 1. Lactobacillales dominated the *ayu-narezushi* 1 sample, making up 83.2% of the total number of analyzed sequences and 16 out of 25 OTUs (Table 2). *Lactobacillus fuchuensis*, *Lactococcus piscium*, and *Leuconostoc gelidium* were the predominant bacterial species in Library 1. Two OTUs (AYU1 OTU-01 and -02), making up 28.7% of the total number of sequences in the library, were classified as *L. fuchuensis*. Four OTUs (AYU1 OTU-07, -08, -09, and -10), making up 27.7% of the library, were classified as *L. piscium*. AYU1 OTU-13 was classified as *L. gelidium*; clones affiliated with this OTU made up 15.8% of the library. AYU1 OTU-11 showed 96% similarity to *L. piscium*, and its phylogenetic place-

TABLE 1. Number of clones, operational taxonomy units (OTUs), and Shannon-Wiener indexes of diversity (H') for 2 clone libraries derived from *ayu-narezushi* produced by 2 different manufacturers.

Library	No. of clones	No. of OTUs	Shannon-Wiener index (H')	Reference
<i>Ayu-narezushi</i> 1	101	25	2.235	This study
<i>Ayu-narezushi</i> 2	106	16	1.313	This study
<i>Samma-narezushi</i>	89	12	1.225	Matsui et al., 2008.

$$H' = - \sum_{i=1}^s P_i \ln P_i (P_i = n_i / N)$$

s = number of OTUs

P_i = proportion of the i th OTU

n_i = number of clone in i th OTU

N = total number of clones in the library

TABLE 2. Abundance and similarity of OTUs to known species of bacteria from 2 *ayu-narezushi* samples produced by different manufacturers, using the 16S rDNA clone library.

OTU (number of clones)	Nearest known species	Similarity (%)	Composition (%)
<i>Ayu-narezushi 1</i>			
AYU1 OTU-01 (28), AYU1 OTU-02 (1)	<i>Lactobacillus fuchuensis</i>	99	28.7
AYU1 OTU-03 (1)	<i>L. fuchuensis</i>	94	1.0
AYU1 OTU-04 (2), AYU1 OTU-05 (1)	<i>Lactobacillus sakei</i>	99	3.0
AYU1 OTU-06 (1)	<i>Lactobacillus curvatus</i>	99	1.0
AYU1 OTU-07 (23), AYU1 OTU-08 (3), AYU1 OTU-09 (1), AYU1 OTU-10 (1)	<i>Lactococcus piscium</i>	99-100	27.7
AYU1 OTU-11 (1)	<i>L. piscium</i>	96	1.0
AYU1 OTU-12 (1)	<i>Lactococcus lactis</i>	99	1.0
AYU1 OTU-13 (16)	<i>Leuconostoc gelidum</i>	99	15.8
AYU1 OTU-14 (1)	<i>Leuconostoc gasicomitatum</i>	99	1.0
AYU1 OTU-15 (2)	<i>Vagococcus carniphilus</i>	98	2.0
AYU1 OTU-16 (1)	<i>Carnobacterium</i> sp. MKJ37	100	1.0
AYU1 OTU-17 (1)	<i>Brochothrix thermosphacta</i>	100	1.0
AYU1 OTU-18 (3),	<i>Psychrobacter alimentarius</i>	99	3.0
AYU1 OTU-19 (6), AYU1 OTU-20 (1)	<i>Hafnia alvei</i>	99-100	6.9
AYU1 OTU-21 (1)	rape rhizosphere bacterium tsb085	96	1.0
AYU1 OTU-22 (2)	<i>Enterobacter amnigenus</i>	100	2.0
AYU1 OTU-23 (1)	<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i>	99	1.0
AYU1 OTU-24 (1)	<i>Comamonas</i> sp. 12022	98	1.0
AYU1 OTU-25 (1)	<i>Myroides odoratus</i>	94	1.0
			100
<i>Ayu-narezushi 2</i>			
AYU2 OTU-01 (73), AYU2 OTU-02 (1), AYU2 OTU-03 (3), AYU2 OTU-04 (4), AYU2 OTU-05 (1), AYU2 OTU-06 (1), AYU2 OTU-07 (1), AYU2 OTU-08 (1), AYU2 OTU-09 (1), AYU2 OTU-10 (1)	<i>L. sakei</i>	99	82.1
AYU2 OTU-11 (12), AYU2 OTU-12 (1), AYU2 OTU-13 (1), AYU2 OTU-14 (1)	<i>Enterococcus faecium</i>	99	14.2
AYU2 OTU-15 (1)	<i>Leuconostoc</i> <i>pseudomesenteroides</i>	99	0.9
AYU2 OTU-16 (3)	bacterium SV6XVII	99	2.8
			100

ment was distant from *L. piscium* and *L. lactis*. AYU1 OTU-03 had only 94% sequence similarity to *L. fuchuensis*. This OTU was also distantly related to the cluster of Lactobacillaceae, Carnobacteriaceae, and Enterococcaceae, indicating that the OTU is an unknown species of Lactobacillales (Figure 1). Other OTUs of Lactobacillales showed high similarity (greater than 98%) to *Lactobacillus sakei* (AYU1 OTU-04 and -05), *Lactobacillus curvatus* (AYU1 OTU-06), *Lactococcus lactis* (AYU1 OTU-12), *Leuconostoc gasicomitatum* (AYU1 OTU-14), *Vagococcus carniphilus* (AYU1 OTU-15), and *Carnobacterium* sp. MKJ37 (AYU1 OTU-16). The rest of the OTUs in Library 1 were classified as Bacillales, gammaproteobacteria, betaproteobacteria,

or Flavobacter.

In Library 2, 15 OTUs, constituting 97.2% of total number of sequences, were affiliated with Lactobacillales (Table 2). Ten out of 16 OTUs, making up 82.1% of the total number of sequences, were classified as *L. sakei*. AYU2 OTU-11, -12, -13, and -14 were classified as *Enterococcus faecium*, making up 14.2% of the library. AYU2 OTU-15 showed 99% similarity to *Leuconostoc pseudomesenteroides*. Only AYU2 OTU-16 showed 99% similarity to bacterium SV6XVII, which is grouped with the gammaproteobacteria, and constituted only 2.8% of the total number of sequences. The results of LIBSHUFF indicated a significant difference between Library 1 and Library 2 ($P = 0.001$, data not shown).

FIG. 1. Phylogenetic tree of 16S ribosomal RNA gene fragments recovered from 2 samples of *ayu-narezushi* (sweetfish *narezushi*). The scale bar represents 0.1 substitutions per nucleotide position. Bootstrap values for 1,000 trees are shown at branch points. Only values of 70% or above are shown. Numbers in brackets are GenBank accession numbers.

Previously, we analyzed the microbial community structure in *samma-narezushi* (Matsui et al., 2008). In the present paper, we used the 16S rRNA gene clone library method to analyze the bacterial communities in 2 *ayu-narezushi* samples produced by different manufacturers. These libraries were statistically compared with the LIBSHUFF program.

The diversity of bacteria in Library 1 was higher than that of Library 2 (Table 1). Although Lactobacillales dominated both libraries, more diverse bacterial species were found in Library 1 (Table 2). LIBSHUFF results indicated that Library 1 was significantly different from Library 2 (Table 3). *Ayu-narezushi* 1 was salted for a longer period than *ayu-narezushi* 2 (6 months vs. 2 months) but had the same fermentation period (3 weeks). One reason for the differences in microbial diversity would be this longer salting period. The materials used in the production of *ayu-narezushi* would serve as bacterial inocula for its fermentation. In the present study, *ayu-narezushi* was obtained from 2 different manufacturers who purchased the materials from different suppliers. The materials purchased from different suppliers could have different bacterial compositions.

The predominant bacterial species in *ayu-narezushi* 1 were psychrotrophic lactic acid bacteria, such as *L. fuchuensis*, *L. piscium*, and *L. gelidum* (Matamoros et al., 2009; Williams et al., 1990), whereas *L. sakei* was the predominant species in *ayu-narezushi* 2. These lactic acid bacteria would play important roles in the fermentation of *ayu-narezushi*. It is difficult to specify which bacteria plays major roles in the production of flavors or tastes in *ayu-narezushi* 1. In our previous report on *samma-narezushi*, 72% of the cloned sequences were classified as *L. sakei* (Matsui et al., 2008). *L. sakei* was detected in both samples of *ayu-narezushi* in the present study. The proportion of *L. sakei* may vary between different kinds of *narezushi* but this bacterium seems to be a common constituent of *narezushi*. The roles of *L. sakei* on the production of flavors in fermented fish were studied in detail (Nordvi et al., 2007). When the *L. sakei* was used as starter culture, volatile compounds such as alcohols, alkanes, esters, aldehydes, and ketones were released from fermented fish product during cold storage (Nordvi et al., 2007). Fadda et al. (1999) demonstrated that *L. sakei* CECT 4808 produces protease and aminopeptidase and they contributed to releasing free amino acids from pork muscle sarcoplasmic proteins. Although compounds that related to taste and flavors have not been determined in the present study, it is highly possible that *L. sakei* plays important roles in the formation of free amino acids or

volatile compounds in *ayu-narezushi* 2. OTUs classified as *L. fuchuensis*, *L. sakei*, *L. carvatus*, *L. piscium*, *L. lactis*, and *L. gelidum* were obtained from *ayu-narezushi* (Table 2). Some strains of these lactic acid bacteria are known as beneficial microbes with probiotic (*L. fuchuensis*) or bacteriocin production/-antibacterial activity (*L. sakei*, *L. carvatus*, *L. piscium*, *L. lactis*, and *L. gelidum*) (Diop et al., 2009; Hagi and Hoshino, 2009; Matamoros et al., 2009; Schillinger et al., 1991; van Belkum and Stiles, 1995; Verluyten et al., 2004).

Two OTUs (AYU1 OTU-03 and AYU1 OTU-11) within Lactobacillales showed sequence similarity of less than 97% to any known bacterial species, suggesting that these OTUs were derived from unknown bacteria. This implies that *narezushi* can be a source for novel lactic acid bacteria.

ACKNOWLEDGEMENTS

In the present study, only genotypic information was obtained. Efforts to isolate bacterial strains in *ayu-narezushi* should be done in order to obtain novel or beneficial species or strains.

The present study was financially supported by Grants-in-Aid for Scientific Research, Japan Society for the Promotion of Science (15500536).

REFERENCES

- Diop, M. B., Dubois-Dauphin, R., Destain, J., Tine, E., and Thonart, P. (2009) Use of a nisin-producing starter culture of *Lactococcus lactis* subsp. *lactis* to improve traditional fish fermentation in Senegal. *J. Food Prot.*, **72**, 1930-1934.
- Fadda, S., Sanz, Y., Vignolo, G., Aristoy M. C., Oliver, G., and Tolda F. (1999) Hydrolysis of pork muscle sarcoplasmic proteins by *Lactobacillus curvatus* and *Lactobacillus sakei*. *Appl. Environ. Microbiol.* **65**, 578-584.
- Fukatsu, T. (1999) Acetone preservation: a practical technique for molecular analysis. *Mol. Ecol.*, **8**, 1935-1945.
- Godon, J., Zumstein, E., Dabert, D., Habouzit, F., and Moletta, R. (1997) Molecular microbial diversity of an anaerobic digester as determined by small-subunit rDNA sequence analysis. *Appl. Environ. Microbiol.*, **63**, 2802-2813.
- Hagi, T., and Hoshino, T. (2009) Screening and characterization of potential probiotic lactic acid bacteria from cultured common carp intestine. *Biosci. Biotechnol. Biochem.*, **73**, 1479-83.
- Ishige, N. (1993) Cultural aspects of fermented fish products in Asia. In *Fish fermentation technology* (Lee, C. H., Steinkraus, K. H., and Reilly, A. P. J., eds.), pp. 13-32, United Nations University Press., Tokyo, Japan.
- Kim, B. J., Lee, H. J., Park, S. Y., Kim, J., and Han, H. U. (2000) Identification and characterization of *Leuconostoc gelidum*, isolated from kimchi, a fermented cabbage product. *J. Microbiol.*, **38**, 132-136.
- Matamoros, S., Pilet, M. F., Gigout, F., Prévost, H., and Leroi, F. (2009) Selection and evaluation of seafood-

- borne psychrotrophic lactic acid bacteria as inhibitors of pathogenic and spoilage bacteria. *Food Microbiol.*, **26**, 638-44.
- Matsui, H., Tsuchiya, R., Isobe, Y., Maeda, H., and Narita, M. (2008) Diversity of the bacterial community found in *samma-narezushi* (Saury *Narezushi*) revealed by the 16S rRNA gene clone library. *Biocontrol Sci.*, **13**, 97-102.
- Nordvi, B., Langsrud, Ø., Egeland, B., Slinde, E., Vogt, G., Gutierrez, M., Olsen, E. (2007) Characterization of volatile compounds in a fermented and dried fish product during cold storage. *J. Food Sci.*, **72**, S373-380.
- Schillinger, U., Kaya, M., and Lücke, F. K. (1991) Behaviour of *Listeria monocytogenes* in meat and its control by a bacteriocin-producing strain of *Lactobacillus sake*. *J. Appl. Bacteriol.*, **70**, 473-478.
- Singleton, D. R., Furlong, M. A., Rathbun, S. L., and Whitman, W. B. (2001) Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. *Appl. Environ. Microbiol.*, **67**, 4374-4376.
- van Belkum, M. J. and Stiles, M. E. (1995) Molecular characterization of genes involved in the production of the bacteriocin leucocin A from *Leuconostoc gelidum*. *Appl. Environ. Microbiol.*, **61**, 3573-3579.
- Verluyten, J., Leroy, F., and de Vuyst, L. (2004) Influence of complex nutrient source on growth of and curvacin A production by sausage isolate *Lactobacillus curvatus* LTH 1174. *Appl. Environ. Microbiol.*, **70**, 5081-5088.
- Yu, Y., Breitbar, M., McNairnie, P., and Rohwer, F. (2006) FastGroupII: a web-based bioinformatics platform for analyses of large 16S rDNA libraries. *BMC Bioinformatics*, **7**, 57.
- Williams, A. M., Fryer, J. L., and Collins, M.D. (1990) *Lactococcus piscium* sp. nov. a new *Lactococcus* species from salmonid fish. *FEMS Microbiol. Lett.*, **56**, 109-113.