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Comparison of PCR-DGGE and PCR-SSCP Analysis for Microflora of Kaburazushi and Daikonzushi, Traditional Fermented Foods Made from Fish and Vegetables

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Abstract: Kaburazushi and daikonzushi are traditional lactic acid fermented foods made in the Hokuriku area of Japan. In *kaburazushi* processing, salted turnip (*Brassica rapa* var. *glabra*) and salted yellowtail (*Seriola quinqueradiata*) are fermented with malted rice for several days to 2 weeks in winter (0-10°C). Daikonzushi is made from Japanese white radish (*Raphanus sativus* var. *raphanistroides*), dried and leached Pacific herring (*Clupea pallasi*) and malted rice processed the same as for kaburazushi. To clarify the microbial property for the processing and human health, researchers employed a molecular approach to analyze the bacterial flora of kaburazushi and daikonzushi and their ingredients, combining PCR amplification of the V3 region of the 16S rDNA gene and Denaturing Gradient Gel Electrophoresis (DGGE) analysis. Furthermore we identified the isolated strains using PCR-Single Strand Conformation Polymorphism (SSCP) analysis. In the PCR-DGGE analysis, *Lactobacillus sakei* was detected as the predominant bacteria in both products. On the other hand, the bacterial DGGE band was not detected in salted turnip and salted yellowtail. *L. sakei* and *Leuconostoc mesenteroides* were isolated and identified as the predominant lactic acid bacteria in the products by PCR-SSCP analysis. The halo-tolerant bacteria *Staphylococcus gallinarum* was also isolated and identified.

Key words: Kaburazushi, daikonzushi, Denaturing Gradient Gel Electrophoresis (DGGE), Single Strand Conformation Polymorphism (SSCP), *Lactobacillus sakei*, *Leuconostoc mesenteroides*

INTRODUCTION

In modern Japanese cuisine, sushi is made from vinegar flavored rice combined with seafood. It is thought that sushi originates from the salted and long-fermented fish called narezushi in Japan. The earliest reference to sushi appeared in a code named the Yoro-Ritsuryo issued in AD 718 and this earliest sushi is postulated to have been narezushi (Ichishima, 2004). Since that time, narezushi products have been made from various fishes in several areas located inland rather than in coastal regions. Funazushi, a fermented crucian carp with cooked rice made near Lake Biwa, located in central Japan is the most famous narezushi, characterized by its strong flavors and odors. Fujii *et al.* (2008) reported that funazushi is a typical lactic acid fermented product.

Kaburazushi and daikonzushi are traditional fermented foods made in the Hokuriku area of Japan (Nakazawa, 1983). Historically, lactic acid fermented foods were thought to have been derived from narezushi since the Edo era (AD 1600-1860). In kaburazushi processing, fish and cylindrical formed turnips (*Brassica rapa* var. glabra) are salted and individual salted fish slices are placed within the salted turnip channels. The prepared materials are then fermented with rice malted with *Aspergillus oryzae* (koji) in a barrel for a period of several days to 2 weeks in winter (0-10°C). While daikonzushi is made from Japanese white radish, dried and leached herring and koji, it is processed the same as for kaburazushi.

It is well known that Lactic Acid Bacteria (LAB) in fermented foods affect not only product quality 20 and preservation (Riebroy *et al*., 2008; Lore *et al*., 2005) but also food functionality, such as improving 21 the intestinal environment in a manner similar to probiotics (Vinderola *et al*., 2008; Guo *et al*., 2009; Pennacchia *et al*., 2006), immune and antiallergic activities (Masuda *et al*., 2008; Vinderola *et al*., 2007) and anti-hypertensive effects (Kilpi *et al*., 2007). Recently, a high content of Gamma Aminobutyric Acid (GABA) was detected in kaburazushi (Aida and Sumino, 2007). Production of GABA by type strains of LAB in cluding isolates from traditional

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fermented foods was reported (Komatsuzaki *et al*., 2005). Determination of the microflora of kaburazushi and daikonzushi using culture methods has been reported (Aida and Sumino, 2007; Kuda *et al*., 1998). During fermentation there is an increase in LAB and lactic acid and a decrease in pH (4.2 and lower). However, the microflora has not yet been clearly identified at the level of genus and species.

Due to the known limitations of cultivation methods many recent studies have used culture independent 16S rDNA-based PCR techniques (Singh *et al*., 2009) including PCR-Denaturing Gradient Gel Electrophoresis (DGGE) and PCR-Single Strand Conformation Polymorphism (PCR-SSCP) to determine the microflora of various traditional fermented foods (Chamkha *et al*., 2008; Chen *et al*., 2008; Kim *et al*., 2009). In this study, to clarify the microbial property for processing and human health we investigated the bacterial flora of kaburazushi and daikonzushi and their ingredients using a molecular approach, combining PCR amplification of the V3 region of the 16S rDNA gene and DGGE. Furthermore, researchers identified the isolated strains using PCR-SSCP.

MATERIALS AND METHODS

Kaburazushi and daikonzushi samples: The fermented products kaburazushi and daikonzushi (Fig. 1) and their materials, were kindly provided by Shijimatya-Honpo Co. (Kanazawa, Ishikawa, Japan) in December 2008. Prior to fermentation processing (Fig. 2), turnip (*Brassica rapa* var. *glabra*) and Japanese white radish (*Raphanus sativus* var. *raphanistroides*) were salted for 3 days (shitazuke) and the yellowtail (*Seriola quinqueradiata*) was salted for 1 year. Dried pacific herring (*Clupea pallasi*) was leached in clean water overnight. Rice malted with *Aspergillus oryzae* (koji) was emulsified with cooked rice and water (amazake, saccharified rice gruel) before use. The fermentation was carried out at ambient temperature (0-10°C) for 1 week (honzuke).

Chemical analysis: Salinity, pH and organic acids of the samples were determined using methods cited in the previous reports (Kuda *et al*., 2009, 2010).

Microbiological analysis and collection of isolates and cell mixtures: Samples (25 g) were emulsified in 225 mL of sterile phosphate buffer (PBS, 20 mM KH_2PO_4-0.01 M K_2HPO_4, pH 7.2) and blended for 60 sec (Stomacher 400, Seward, London, UK). The sample suspensions were diluted in PBS and appropriate dilutions were spread in duplicate on Tryptone Soy (TS) agar (Oxoid, Basingstoke,

Fig. 1: Fermented products, (a) kaburazushi and (b) daikonzushi

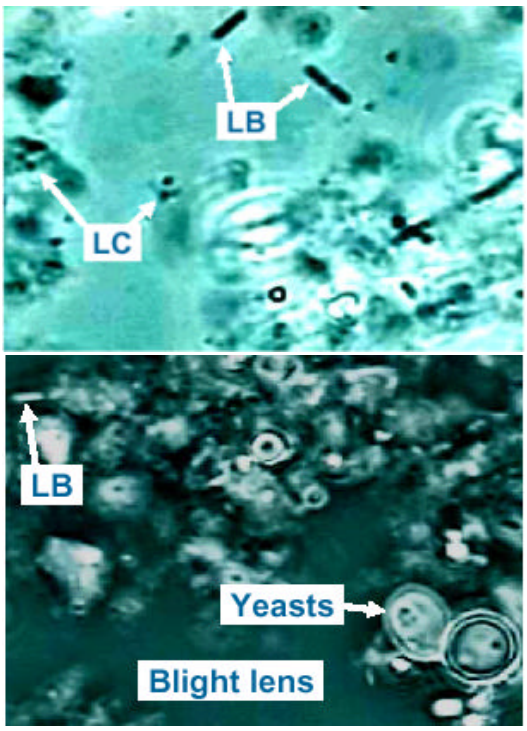

Fig. 2: Production processes of kaburazushi and daikonzushi

UK), TS agar containing 10% (w/v) NaCl and de Man, Rogosa and Sharpe (MRS) agar plates (Oxoid). TS agar plates were aerobically incubated at 30°C for 2-4 days. MRS agar plates were anaerobically incubated at 30°C using an AnaeroPack system (Mitsubishi Gas Chemical, Tokyo, Japan) for 5-7 days. About 25 colonies from each sample were picked and re-streaked for purification prior to PCR-SSCP analysis.

Direct extraction of DNA and PCR amplification: DNA from 1 mL of homogenate per sample and the isolates was extracted using FastPure DNA Kit (Takara, Otsu, Japan). Purified DNA was dissolved in TE buffer and used as the DNA template in PCR.

The following primer pair was chosen for the amplification of the V3 region (-220 bp) of the 16S rRNA gene: forward primer with GC clamp (Sheffiedld *et al*., 1998) GC-339f (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCT CCT ACG GGA GGC AGC AG-3') and reverse primer V3-53r (5'-GTA TTA CCG CGG CTG CTG G-3'). PCR amplification was performed in 100 μL reaction mixtures composed of 10 mM Tris-HCl (pH 8.3) 50 mM KCl, 1.5 mM $MgCl_2$, 50 pmol each of primer, 0.2 mM each of 4 dNTPs, 2.5 U of Takara Taq DNA polymerase (Takara Bio, Shiga, Japan) and 50 ng of template DNA. To minimize amplification of nonspecific products and to obtain large amounts of PCR products, touchdown PCR (Don *et al*., 1991) was performed where the initial annealing temperature was set at 8°C above the expected annealing temperature and decreased by 0.8°C every second cycle until the expected annealing temperature (62°C) was reached (total 20 cycles) and then 5 additional cycles were carried out. Amplification was carried out using the following cycle: denaturation at 94°C for 30 sec, annealing for 30 sec and primer extension at 72°C for 10 sec in a GeneAmp 9700 4 thermal cycler (Applied Biosystems, Foster City, CA, USA). Aliquots (5 μL) of the PCR products were analyzed first by electrophoresis in 2% (w/v) agarose gels.

DGGE analysis of PCR products: DGGE analysis of PCR amplification products was performed as previously described (Muyzer *et al*., 1993), using the DCode System apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Polyacrylamide gels (8% (w/v) acrylamide-bisacrylamide (37.5:1)) in 1×Tris-acetate-EDTA buffer with a denaturing gradient ranging from 30-60% denaturant (100% denaturation corresponds to M urea and 40% (v/v) formamide) was prepared with the Bio-Rad model 475 gradient delivery system. Polymerization was achieved by adding 0.9% (v/v) ammonium persulfate (10% solution) and 0.09% (v/v) N,N,N,N-tetra methyl ethylene diamine. The gels were electrophoresed at a constant voltage of 200 V at 60°C for 3 h. The DNA fragments were stained with ethidium bromide and washed with distilled water prior to UV transillumination.

The main DGGE fragments were selected for nucleotide sequence determination. Each band was excised with a sterile razor. The DNA of each fragment was eluted in 50 μL TE buffer at 100°C for 10 min. The extracts were re-amplified by PCR using the same primers and purified with SUPREC-PCR (Takara) according to the manufacturer's instructions. Purified DNA fragments were ligated in pT7blue-vectors (Novagen, Darmstadt, Germany) and transformed into *E. coli* JM109. The transformants were grown up on LB agar containing ampicillin and screened by galactosidase assay. Plasmid DNA of selected transformants was isolated using a Plasmid miniprep kit (Bio-Rad). The inserted DNA sequence, approximately 200 bp of 16S rDNA (*E. coli* position 389-530) (Neefs *et al*., 1990) was determined using a 3130 Genetic Analyzer (Applied Biosystems) with the Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems). To identify the inserted sequences, the BLAST 2.0 algorithm was used to compare the derived sequence to 16S rDNA sequences in the DNA Data Bank of Japan (DDBJ) 3 database. The DGGE analysis was done repeatedly 3 times.

PCR-SSCP analysis of 16S rDNA V3 region: In the PCR-SSCP analysis, we used precast polyacrylamide gels followed by silver staining because of the high sensitivity of silver staining. This method visualizes even a small amount of nonspecific amplification product; therefore, several PCR primers and thermal profiles were tested for specificity and differences in PCR efficiency. The primer set, SRV3-1 (5'- CGG YCC AGA CTC CTA CGG G-3') (Lee *et al*., 1996) as the forward primer and V3R53 (5'-GTA TTA CCG CGG CTG CTG GC-3') which was designed based on 536R (Weisburg *et al*., 1991) with minor modifications as the reverse primer gave acceptable results. PCR amplification was performed in 100 μL reaction mixtures composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 50 pmol each of primer, 0.2 mM each of 4 dNTPs, 2.5 U of Takara Taq DNA polymerase (Takara Bio, Shiga, Japan) and 50 ng of template DNA. In this analysis, touchdown PCR was also performed; the initial annealing temperature was set at 6°C above the target annealing temperature and decreased by 0.6°C every second cycle until the target annealing temperature (61°C) was reached and then additional cycles were carried out with the target annealing temperature. Amplifications were carried out in a GeneAmp 9700 thermal cycler (Applied Biosystems) using

the following cycle: denaturation at 94°C for 30 sec, annealing at the temperature regime described above for 30 sec and primer extension at 72°C for 10 sec for touchdown cycles and 72°C for 30 sec for the last 5 additional cycles.

SSCP analysis of PCR products was performed as described previously (Takahashi *et al*., 2004). Briefly, PCR products were mixed 1:2 with loading buffer (98% formamide-10 mM EDTA-0.5% bromophenol blue), denatured by heating for 10 min at 100°C, cooled on ice, loaded in a precast, ready-to-use gel (GeneGel Excel 12.5/24 kit; GE Healthcare) and electrophoresed on a GenePhor electrophoresis unit (GE Healthcare) at 650 V, 25 mA and 5°C until the bromophenol blue front reached the anode buffer strip (about 90 min). The gel was stained with PlusOne DNA silver staining kit (GE 3Healthcare). Scanned photographs of SSCP gels were stored as TIFF images.

RESULTS AND DISCUSSION

Salinity, pH value and organic acids: Table 1 shows salinity, pH and organic acid content of the samples. Salinity of the products was about 3 g/100 g. The predominant organic acid in the samples was lactic acid with other organic acids at very low levels; the lactic acid content in the fermented products was 4.3-4.6 mg g^{-1}. The pH value of the kaburazushi and daikonzushi products was 4.4 and 5.0, 11, respectively. These results agree with the previous reports (Kuda *et al*., 1998, 2010).

Viable plate count: In direct observation using phase-contrast microscope, lactobacilli, lactococci and yeasts were detected (Fig. 3). Viable plate counts of the kaburazushi and daikonzushi samples are shown in Table 1. The LAB count (on MRS agar) of both products was very high, approximately 8.8 and 8.9 log cfu g^{-1} for kaburazushi and daikonzushi, respectively. This result is in agreement with the previous report (Kuda *et al*., 1998). The LAB counts of amazake and herring were low, approximately 3.5 and 4.0 log cfu g^{-1}, respectively while viable counts on TS agar were high, approximately 6.3 and 7.3, respectively. Interestingly, the LAB count was already high in salted turnip (6.8 log cfu g^{-1}) while the count in salted radish was low (-2.8 log cfu g^{-1}). The viable count in the long salted yellowtail was very low and was not detected on TS agar plates.

Bacterial flora analyzed by PCR-DGGE method: For DGGE analysis, researchers selected the V3 region of 2416S rDNA as the target region. This region has been widely used in the analysis of bacterial community or the identification of isolated bacteria (Chen *et al*., 2008; Ercolini *et al*., 2003). PCR products originating from sample preparations were divided into one to four main fragments by DGGE analysis (Fig. 4) with the banding patterns differing by sample. Subsequently, to identify the main bands, each band was recovered from the DGGE-gel and sequenced. The results obtained from clone sequencing are 5 (Table 2).

In the case of amazake, three of the four bands detected were identified as *Bacillus subtilis* with the remaining band unidentified as rice *Oryza sative*. A single band was clearly detected in salted turnip and 8 was identified as turnip *Brassia rapa.* In the case of DGGE analysis for fermented plant foods, the chloroplast was detected as main band in early fermentation stage (Miambi *et al*., 2003; Nakayama *et al*., 2007; Ben-Omar and Ampe, 2000). No band was detected in salted yellowtail. The typical marine bacterium *Shewanella baltica* was detected in salted radish and dried-leached herring. *S. baltica* has been reported to be the most important H_2S-producing species during iced storage of Danish marine fish (Vogel *et al*., 2005). *Lactobacillus sakei* was predominantly detected in both kaburazushi and *daikonzushi*. *L. sakei* is moderately halo-tolerant and has been isolated from traditional fermented foods such as fermented sausage (Fontan *et al*., 2007; Ammor *et al*., 2005). There are several reports dealing with bacteriocins produced by *L. sakei* (Urso *et al*., 2006). Gao *et al.* (2010) reported on a novel bacteriocin with a broad inhibitory spectrum produced by *L. sakei*.

Table 1: Chemical compounds and viable counts of ingredients and finished products of kaburazushi and daikonzushi

		Kaburazushi			Daikonzushi		
Culture media	Amazake (koji)	Turnip*	Yellowtail*	Product	Radish*	Herring**	Products
NaCl (g/100 g)	0.2	4.2	11.6	2.80	6.0	0.4	3.30
pH	5.3	5.5	6.0	4.40	5.7	6.6	5.00
Organic acids (mg g^{-1})							
Lactic acid	NT	0.55	NT	4.69	ND	NT	4.33
Acetic acid	NT	0.49	NT	1.95	0.24	NT	0.71
Viable counts (log cfu g^{-1})							
TS agar	6.30	6.85	ND	6.70	3.90	7.30	8.85
TS+10%(w/v) NaCl agar	5.30	3.95	ND	5.30	3.00	5.95	3.30
MRS agar	3.48	6.78	2.60	8.78	2.78	4.00	8.90

Values are mean of duplicate measurement. ND: Not Detected. NT: Not Tested. TS agar: Non-selective medium; TS+10%(w/v) NaCl agar: For halophilic bacteria; MRS agar: For lacticic acid bacteria. *Salted:**Dried and leached

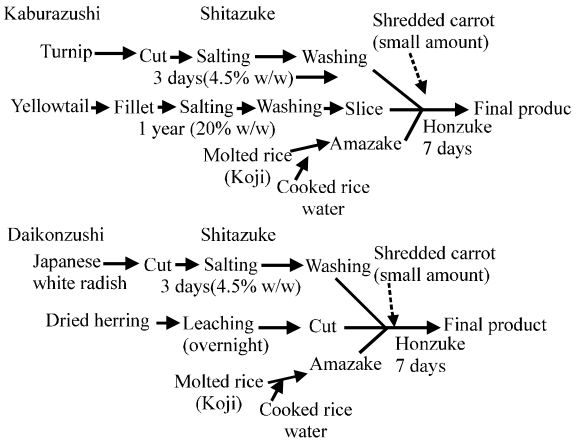

Fig. 3: Lactic acid bacteria and yeasts in decimal diluted kaburazushi observed by a phase-contrast microscope (ODEO Quatro, Iponacology). LB: Lactobacilli; LC: Lactococci

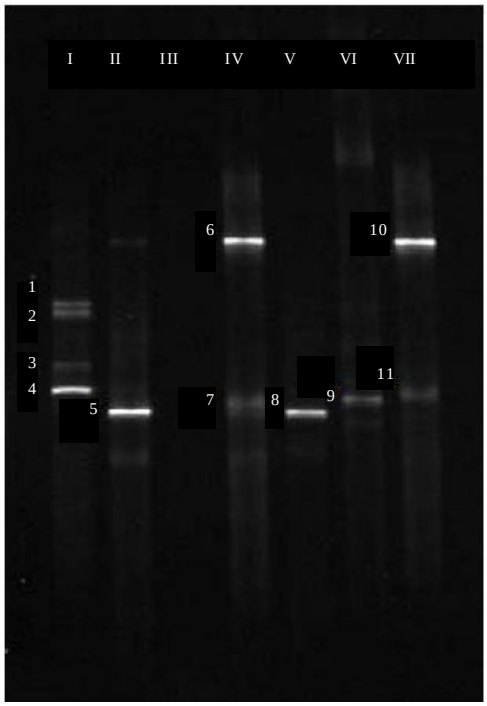

Fig. 4: DGGE analysis of PCR-amplified 16S rDNA fragments from kaburazushi and daikonzushi. Lanes re as follows. I: amazake; II: salted turnip, III: yellowtail, IV: kaburazushi; V: salted radish; VI: dried and leached herring and VII: daikonzushi

Bacterial flora analyzed by PCR-SSCP: PCR-SSCP analysis enables DNA fragments of similar sizes to be separated according to their configuration (secondary

Table 2: Identities of cloned fragment obtained from DGGE analysis of kaburazushi and daikonzushi

Sample	DGGE band number (Fig. 2)	Identification	Identity (%)
Amazake	1	*Bacillus subtilis*	99
	2	*Bacillus subtilis*	99
	3	*Oryza sativa* ***	98
	4	*Bacillus subtilis*	100
Turnip*	5	*Brassica rapa****	99
Kaburazushi	6	*Lactobacillus sakei*	99
	7	*Brassica rapa****	99
Radish*	8	*Shewanella baltica*	100
Herring**	9	*Shewanella baltica*	97
Daikonzushi	10	*Lactobacillus sakei*	100
	11	*Lactobacillus sakei*	100

*Salted: **Dried and leached; ***Botanical chloroplast DNA

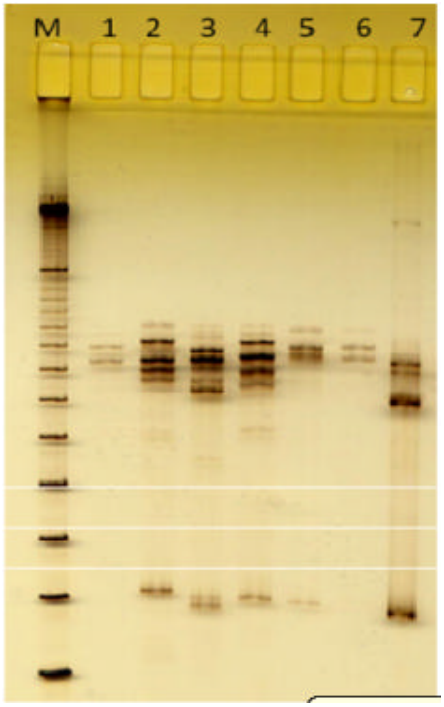

Fig. 5: SSCP analysis of PCR-amplified 16S rDNA fragment patterns of selected isolates. Lanes are as follows. 1 and 10: *Leuconostoc citreum*; 2 and 12: *Staphylococcus gallinarum*; 3: *Staph. kloosii*; 4, 8, 9, 13, 16 and 19: *Lactobacillus sakei*; 5: *Leu. gelidum*; 6,11,17 and 20: *Leu. mesenteroides* and 7: *Shewanella putrefaciens*; 14: *Stap. saprpphyticus*; 15: *Pseudomonas rhodesiae*; 18: *Kokuria*

structure) (Hebenbrock *et al*., 1995). Targeting the 16S rRNA V3 region which permits phylogenetic discrimination of microbial species allows for LAB monitoring in the fermented food microbial community by one profile of bands (Fig. 5) where each band corresponds to a different sequence of the 16S rRNA V3 region, i.e., one bacterium (Chamkha *et al*., 2008). As shown in Fig. 4

Table 3: Identitification of isolates from kaburazushi and daikonzushi by PCR-SSCP analysis

Samples	Culture media	No. of isolates	Identification	Identifity
Amazake	TS	11	*Staphylococcus gallinarum*	100
		1	*Staphylococcus kloosii*	100
	TS+10% NaCl	12	*Staphylococcus gallinarum*	100
	MRS	9	*Leuconostoc citreum*	100
Turnip*	TS	4	*Leuconostoc gelidum*	100
		2	*Leuconostoc mesenteroides*	100
		2	*Lactobacillus sakei*	100
		2	*Pseudomonas* sp.	90
		1	*Pseudomonas fluorescens*	100
		1	*Shewanella putrefaciens*	100
	TS+10% NaCl	5	*Psychrobacter adeliae*	99
		4	*Staphylococcus gallinarum*	100
		3	*Marinilactibacillus psychrotolerans*	100
	MRS	8	*Lactobacillus sakei*	100
		1	*Leuconostoc gelidum*	100
Yellowtail*	MRS	2	*Lactobacillus sakei*	100
Kaburazushi	TS	8	*Lactobacillus sakei*	100
		4	*Leuconostoc mesenteroides*	100
	TS+10% NaCl	12	*Staphylococcus gallinarum*	100
	MRS	8	*Lactobacillus sakei*	100
		3	*Leuconostoc mesenteroides*	100
		1	*Leuconostoc citreum*	100
Radish*	TS	4	*Plantibacter flavus*	100
		2	*Curtobacterium flaccumfaciens*	100
		1	*Curtobacterium citreum*	100
		2	*Psychrobacter faecalis*	100
		2	*Staphylococcus saprophyticus*	100
		1	*Pseudomonas rhodesiae*	99
	TS+10% NaCl	10	*Staphylococcus saprophyticus*	100
		1	*Staphylococcus equorum*	100
		1	*Psychroloccus glasimcola*	100
	MRS	3	*Lactobacillus sakei*	100
Herring**	TS	6	*Staphylococcus saprophyticus*	100
		2	*Psychrobacter cibarius*	100
		2	*Kocuria rhizophila*	100
		1	*Carnobacterium maltaromaticum*	100
		1	*Brachybacterium paraconglomeratum*	100
	TS+10% NaCl	11	*Psychroloccus glasimcola*	100
		1	*Staphylococcus equorum*	100
MRS		9	*Lactobacillus sakei*	100
		1	*Leuconostoc mesenteroides*	100
Daikonzushi	TS	7	*Lactobacillus sakei*	100
		4	*Leuconostoc mesenteroides*	100
		1	*Leuconostoc citreum*	100
	TS+10% NaCl	11	*Staphylococcus gallinarum*	100
		1	*Staphylococcus carmosus*	100
	MRS	9	*Lactobacillus sakei*	100

*Salted: **Dried and leached

and Table 3, a variety of bacteria were isolated. Several genera of LAB such as *Leuconostoc*, *Lactobacillus* and *Carnobacterium* were predominantly isolated by MRS and TS agar plates. On the other hand, *Staphylococcus* sp. and several marine and psychrotrophic bacteria, such as *Marinolacto bacillus*, Pseudomonas and Psychrobacter were isolated from TS 10% NaCl agar plates.

Although, *Leuconostoc citreum* was detected in amazeke from the colony counts of MRS and TS agar plates (Table 1), Staphylococcus was regarded as dominant. In salted turnip the dominant bacteria were *L. sakei. Marinolactobacillus psychrotolerans* was detected only in the salted turnip. Although various bacterial groups were detected in salted radish, the viable count was not high. The results of viable counts and PCR-SSCP analysis suggest that the predominant bacteria in dried and leached herring are *Staphylococcus saprophyticus*. *L. sakei* was the predominant isolate in both kaburazushi and daikonzushi which was confirmed by the DGGE analysis. *Leuconostoc mesenteroides* was also predominant in these products.

CONCLUSIONS

In this study, researchers studied the bacterial flora of the traditional fermented foods kaburazushi and daikonzushi using PCR-DGGE and PCR-SSCP. In the PCR-DGGE analysis, *L. sakei* was detected as the predominant bacteria in the products. The results differed from previously reports using cultivation methods. However, in the case of salted, the bacterial band of PCR-DGGE analysis could not be detected, though the various bacteria were isolated and identified by PCR-SSCP analysis. Therefore, PCR-DGGE 21 analysis using V3 and other regions may be necessary. Isolation and biochemical investigation of *L. sakei* for the fermentation process and its food functional properties are now in progress.

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