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Identification and Enumeration Method of Both Eukaryotic and Prokaryotic Microorganisms in Food Sample

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Abstract

The method to analyze both eukaryotic and prokaryotic microorganisms without preliminary microbial information of sample seemed to be useful not only for research and investigation of microorganisms but also for industry using microorganisms. In the present manuscript, preparation of a new DNA primers, new reference database for 18S rDNA for our newly developed method [1]-[3], and analyses of eukaryotic and prokaryotic microorganisms in fermentation products were presented. In komekouji, *Aspergillus* spp., was enumerated to be 46.5 × 10^6 MPN g^{-1}, and *Penicillium* spp., was enumerated to be 1.5 × 10^6 MPN g^{-1}. In dry yeast, *Saccharomyces* group, were enumerated to be 8600 × 10^6 MPN g^{-1}. In komekouji-miso, no eukaryotic microorganism was detected, while the other *Bacillus* spp., was numerically dominant (21.5 × 10^6 MPN g^{-1}) as prokaryotic microorganisms, followed by *B. subtilis* group (4.65 × 10^6 MPN g^{-1}), and the other Firmicutes (3.7 × 10^6 MPN g^{-1}). The komekouji-miso included lower number of Actinobacteria (0.15 × 10^6 MPN g^{-1}), *Burkhokderia* sp. (1.5 × 10^6 MPN g^{-1}), and the other *α,β,γ*-proteobacteria (0.12 × 10^6 MPN g^{-1}). In sake-kasu, both prokaryote and eukaryote were not detected by the method. Present results indicated that using both universal primers for eukaryotic and prokaryotic microorganisms, each groups of prokaryotic and eukaryotic microorganisms were enumerated without any preliminary information nor setting up standard curve, which were required for real time PCR.

Keywords

Eukaryotic Microorganisms, Prokaryotic Microorganisms, Multiple Enzyme Restriction Fragment Length Polymorphism Analysis, the Most Probable Number Method, Microchip Electrophoresis

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1. Introduction

By PCR-based analysis methods such as real time quantitative PCR (qPCR) or multiplex PCR, eukaryotic and prokaryotic microorganisms [4]-[7] could be detected or quantified by the same procedure by changing used DNA primers. By qPCR using universal primer for the both microorganisms, total numbers of eukaryote and prokaryote in sample could be enumerated, however we could not know what kinds of microorganisms were mainly included without analyzing amplified DNA [4]. By the multiplex PCR or qPCR using several selective primers, the exact numbers of each specific microorganisms could be detected or enumerated, however we could not know what kinds of microorganisms were mainly included, because selective primer targeted the specific microorganisms and afford no-information as to microorganisms which were not covered by the used primers [4] [8]-[10]. Although denaturing gradient gel electrophoresis (DGGE) [11]-[14] provided taxonomical information of each microbial group and had been used for exploration of microbial diversity. We could not know relative abundance of each microbial group exactly, because of the PCR bias, which altered the ratio of each microbial groups in the amplified DNA from that of original DNA in sample [15]-[18]. Moreover we could not compare a number of eukaryote to that of prokaryote obtained by different PCR primers, respectively [11]-[14], because the amplification rate and PCR bias were varied with the used DNA primers.

The method to compare the both eukaryote and prokaryote directly without preliminary microbial information of sample seemed to be useful not only for research and investigation of microorganisms but also for industry using microorganisms.

Until now, we had developed a new affiliation system based on multiple enzyme restriction fragment polymorphism analysis (MERFLP), by which bacteria was systematically affiliated [1], and the method to provide numbers of each taxonomically different bacterial groups in the former papers [2] [3]. Although the same primers for 16S rDNA (41f/1066r) had been used until now, the other DNA primer could additionally be used for the analysis. In the present manuscript, preparation of a new DNA primers, PCR condition, and new reference DNA database for 18S rDNA, and microbial analysis of fermentation products by the both primers for the 16S rDNA and the 18S rDNA were presented.

2. Materials and Methods

2.1. Samples and Isolation of Microorganisms

Precision of the affiliation and enumeration of microorganisms in sample was found to be depend on a microbial diversity in the sample. Kome-kouji (K), which included kouji proliferated on steamed rice and used as seed for fermentation, was selected as a representative sample including higher ratio of *Aspergillus oryzae*. Dry yeast (P) was selected as a representative sample including higher ratio of *Saccaromyces* sp. Komekouji-miso (M), which was a traditional fermentation product of soybean by addition of kome-kouji, was selected as a representative sample including lower ratio of eukaryotic microorganisms and higher ratio of prokaryotic microorganisms. Sake-kasu (S), which was a residual material after filtering rice wine from moromi where rice was fermented using kouji and yeast, was selected as a representative sample including lower ratio of both eukaryotic and prokaryotic microorganisms.

For MPN, serial 10-fold dilutions (10^{-3} to 10^{-9}) prepared from sample (1 g fresh wt.) were inoculated to test vials (3 replicates) including potato dextrose medium (potato extract 4 g, dextrose 20 $g \cdot L^{-1}$). Microbial DNAs were extracted after 3 days of the incubation. Reference strains of *Penicillium roqueforti* (NBRC5459), *Gibe-rellazeae* (NBRC7189), *Aspergillus oryzae* (NBRC30113) and *Saccharomyces uvarum* (NBRC10968^{T}) were purchased from NBRC (NITE Chiba Japan).

Microorganisms were newly isolated from komekouji (K; 3 isolates), sake-kasu (K; 5 isolates), dry yeast (P; 3 isolates), and komekouji-miso (M; 5 isolates) using an agar plate containing potato dextrose medium. As eukaryotic microorganisms were not isolated from sake-kasu (K) and komekouji-miso (M), prokaryotic microorganisms were isolated from the both samples.

2.2. PCR Amplification of 18S rDNA and 16S rDNA and Their Restriction Digestion

Chromosomal DNAs of each MPN vials and isolates were extracted by bead beating (3000 rpm 2 min; shake-Man 2, Biomedical Science Co., Tokyo, Japan) using zirconia beads (diameter 2.3 mm) according to the manufacture's instruction, and purified by conventional methods, which was used for the both 16S rDNA and 18S

rDNA analyses.

DNA primers for 18S rDNA (forward primer EF41; 5'GGAAKGGRTGYAYTTAYYAG3', reverse primer EF31; 3'GYTTDSACCRRTAAATCTCCT5') were newly designed based on those of the reported DNA primers (EF4/EF3), and PCR condition was also little modified from those of the reported PCR condition [19] [20] because some of isolated fungi were not amplified by the EF4/EF3. After denaturation at 94˚C for 1 min, 18S rDNA was amplified with the following parameters; 40 cycles of denaturation at 94˚C for 1 min, primer annealing at 51˚C for 1 min, and primer extension of 72˚C for 3 min. After amplification cycles, the final elongation step lasted for 10min at 72˚C.

Amplification of 16S rDNA was according to the former study [21] [22] using the 41f forward primer (V2), and the 1066r reverse primer (V6) [23]. After restriction digestion of the PCR product (10 µl) by each restriction enzyme, *Hae*III or *Hha*I or *Rsa* I or *Alu*I (10 units, Takara Bio Co. Ltd. Shiga Japan) in buffer solution (10xLow salt buffer, Takara Bio Co. Ltd.) and 5 folds dilution by de-ionized water, restriction fragment lengths were measured by microchip electrophoresis system (MCE-202 MultiNA; Shimadzu Co., Ltd. Kyoto Japan).

2.3. Reference MERFL Database for EF41/EF31 and 41f/1066r Used for the Phylogenetic Estimation

The database for 18S rDNA was newly edited using the method of Watanabe and Okuda [1] as described previously [21]. For EF41/EF31 primers, 2154 post-amplification sequence files, which were consisted from 897 eukaryotic genera, including uncultured and unidentified microorganisms, were mainly re-edited using small subunit rRNA files in RDP II release 9_61 [24] under 5 - bases mismatches in the both in primer annealing sites. By checking the database it was re-confirmed that the primers selectively amplified fungi and yeast with no cross reactivity to bacteria, algae, plants and nematodes as reported for the original EF4/EF3 primer [19]. Compared to the database for 41f/1066r primers, which had also been edited from small subunit rRNA files in RDP II release 9_61 [24], total number of MERFLs for EF41/EF31 primers (2154 MERFLs) were much lower (30,844 MERFLs), which was caused from lower number of 18S rDNA registered in RDP II release 9_61 [24], and lower ratio of the registered 18S rDNA with whole length of this gene in spite that MERFL database for EF41/EF31 primers required almost whole length of the 18S rDNA (1555 bp DNA for *S. cerevisiae* (SayCe101), 1558 bp DNA for *A. oryza* (Aspe. Oryza), and 1557 bp DNA for *P. roqueforti* (GQ458035).

For the phylogenetic analysis fragment smaller than 100 bp were eliminated from the both theoretical MERFL database and measured MERFL as described previously [21] [22].

2.4. Selection of MERFL Originated from Homogeneous rDNA Sequence from Mixed MERFLs and Phylogenetic Estimation

As the reference MERFL databases were for the homogeneous 16S rDNA and 18S rDNA, the measured MERFL originated from the homogeneous 16S rDNA and 18S rDNA had to be selected among the mixed MERFLs for phylogenetic estimation. The major RFs (H) having the highest relative mole concentration (ratio of fluorescent intensity to fragment size) were selected among the mixed RFs as described previously [2]. The 2nd major RFs (M) were selected among the remained FRs after subtraction of the major RFs. The 3rd major RFs (L) were selected among the remained FRs after subtraction of the 2nd major RFs.

For similarity search, the theoretical MERFL (B) having the lowest pairwise distance (D_{ABME}) to the measured MERFL (A), which was an average of all the pairwise distance for used restriction enzymes (D_{ABs}) calculated according to Nei and Li [25], were searched in the reference database as described previously [21]. The equation $(1 - D_{ABME}) \times 100)$ was used as similarity (%).

In similarity search, identical reference MERFL (100%) was searched by using all the MERFL preferentially. In case when the 100% identical reference MERFL was not found, combinations of fewer RFs was used for the next searches (**Table 1**). In case when the 100% identical reference MERFL (100%) was not found using 2 RFs, the reference MERFL with the highest similarity was indicated in **Table 1** [2] [3].

2.5. Estimation of Each Microbiological Numbers by MPN

Most probable numbers of each groups (A - M) were estimated by 3 tube, 3 decimal dilution experiment [26] (**Table 2**). Confidence limits were obtained using FDA's Bacterial Analytical Manual [26] (**Table 2**).

Table 1. Affiliation of microorganisms by MERFLP[a] of 16S rDNA(A-H) and 18S rDNA(I-M).

	Vial No.[b]	Restriction enzymes[c]	Similarity (%)	Name (Accession number)[d]
A	MuP10^{-3}1H	A, Ha, Hh	86%	*Bacillus subtilis* (AB115958, AB192294, AF447803, AF549498, AF058766, AY962473, AY995568, AY995571, AY995572, D26185, D84213, DQ207730, B. subtili 3, B. subtrrn A, B. subtrrn B, B. subtrrn E, B. subtrr G2, B. subtrrn J, B. subtrrn W, B. subtrr H2), B. amyloliquefaciens (CP000560, B. amylique), B. licheniformis (DQ020262, AY971527), Paenibacillus popilliae (Pae. popil 2)
	MuP10^{-3}3H	A, Hh	100%	
	MuP10^{-4}1H	A, Ha, R, Hh	92%	
	MuP10^{-4}2H	A, Ha, Hh	100%	
	MuP10^{-4}3H	A, Ha, Hh	100%	
	MuP10^{-5}1H	A, Ha, Hh	100%	
	MuP10^{-5}2H	A, Ha, Hh	95%	
	MiP4	A, Ha, Hh	100%	
	MiP6	A, Hh	100%	
	SiP5	Ha, Hh	100%	
	MuP10^{-6}3H	A, Ha, Hh	100%	*Bacillus pumilus* (CP000813, DQ112245, DQ209209, DQ232736, DQ275671)
	SiP1	A, Ha, Hh	92%	
B	MuP10^{-3}2H	A, Ha, Hh	92%	*Bacillus cereus* (AY986507, DQ173159), uncultured bacterium (AY858402, AY858392, AB185692)
	MuP10^{-5}2M	A, Hh	100%	
	MuP10^{-5}3H	A, Ha, Hh	87%	*B. simplex* (B. simplex3), *B. megaterium* (DQ207561), *Bacillus* sp. (AY590138, AY965250, AY965252, DQ201643), *Caulobacter* sp. (Cau. RWC17)
	MiP3	A, Ha, Hh	93%	
	MiP2	Ha, Hh	100%	*B. alveyuensis* (AY605232)
	MuP10^{-5}2H	A, Ha, Hh	95%	*Bacillus* sp. (AJ864277, AY590138, DQ201643), uncultured bacterium (AB179530, AY534095)
	MiP5	A, Ha, Hh	100%	
	SiP2	Ha, Hh	100%	Clostridium barati (C. barati 2, C. barati 3), Eubacterium multiforme (Eub. multiforme), E. nitritogenes (Eub. nitrit)
	SiP4	Ha, R, Hh	93%	Clostridium botulinum (C. botulin D)
C	MuP10^{-3}2L	A, Hh	90%	*Desulfotomaculum geothermicum* (X80789), *Peptostreptococcus anaerobius* (L04168)
	MuP10^{-5}1L	Ha, Hh	83%	*Desulfotomaculum orientis* (M34417)
	MuP10^{-6}1H	Ha, R	100%	*Sporolactobacillus inulinus* (AB362770, M58838), *S.terrae* (AJ634662)
	Mu10^{-4}2L	A, Hh	100%	Selenomonas-like sp.oral strain (AF287789, AF287790, AF287791)
D	MuP10^{-4}2M	R, Hh	90%	*Actinomadura* sp.E6 (AY766302)
E	MuP10^{-3}1M[e]	R, Hh	100%	*Oxobacter fennigii* (Ox. phennig), *Candidatus* Neohrichiami *kurensis* (AY135531)
F	MuP10^{-3}3M	A, Hh	100%	*Thiomonas* sp. ML1-98 (DQ146066)
	MuP10^{-6}2L	A, Hh	100%	*Burkholderia solanacearum* (U27986, X607040, X607041), *Spiroplasma mirum* (*Spp. mirum*)
G	MuP10^{-3}1M[e]	A, Hh	100%	*Haemophilus influenza* (H. inflrrn C, H. inflrrn D, H. inflrrn E, H. inflrrn D)
	MuP10^{-3}1M[e]	A, R	100%	*Moritella* sp. (AB059262)
	MuP10^{-3}3L	A, Ha	100%	*Buchnera aphidicola* (AJ296757)
	SiP3	A, Hh	93%	*Pseudomonas* sp. AH2 (AF451275), *P. fluorescens* (D84013)

Continued

H	MuP10^{-3}2M	A, Hh	93%	unidentified and uncultured bacterium (AB179495, AF317770, AF371874, AY177783, DQ129379)
	MuP10^{-4}1M	Ha, Hh	93%	
	MuP10^{-4}3M	A, Hh	93%	
	MuP10^{-5}2L	R, Hh	90%	
	MuP10^{-6}2M	Ha, Hh	93%	
I	RefESU	R, Hh, A	100%	*Saccharomyces cerevisiae* (SayCe 101), *S. dairenensis* (SayDair8), *Zygosaccharomyces microellipsoides* (Zygs. micro), *Z. mrakii* (Zygs. mraki), *Z. mellis* (Zygs. melli), *Torulospora delbrueckii* (TorDe113), *T. globosa* (Trls. globo), *T. pretoriensis* (Trls. preto), *Kluyveromyces delphensis* (KluDeip4), *K. polysporus* (KluPoly8), *Kazachstania vitcola* (X99526), *Candida colliculosa* (Can. colli)
	PiE1	Ha, R, Hh, A	100%	
	PiE2	Ha, R, Hh, A	100%	
	PiE3	Ha, R, Hh, A	100%	
	PuE10^{-8}1H	Ha, R, Hh, A	100%	
	PuE10^{-8}2H	Ha, R, Hh, A	100%	
	PuE10^{-8}3H	Ha, R, Hh	100%	
	PuE10^{-9}2H	Ha, R, Hh, A	100%	
J	RefEGZ	Ha, R	92%	*Gibberella zeae* (AB250414), *G. intermedia* (JN236216)
K	RefEPC	Ha, R, Hh, A	100%	*P. chrysogenum* (AFF411201, AF548086), *P. expansum* (DQ912698), *P. camemberti* (GQ458020)
	KiE2	Ha, Hh, A	93%	
	KuE10^{-6}1H	, Hh, R, A	97%	
L	RefEAO	Ha, R, Hh, A	100%	*Aspergillus clavatus* (Asp. Clav9), *A.awamori* (Aspe. awamo), *A. fumigatus* (Aspe. fumig, Asp. fumi 2, Asp. fumi 9), *A. niger* (Aspe. niger), *A. parasiticus* (Aspe. psiti), *A. restrictus* (AspRes10), *A. terreus* (Asp. Ter15), *A.sparsusr* (AspSpar2), *P. tardum* (AF245256), *P. purpurogenum* (AF245261, AF245264), *P. glabrum* (AF245270), *P. decumbens* (EU136028), *Eremascus albus* (Erms. albus), *Elaphomyces maculatus* (EloMacul), *Eurotium herbariorum* (EutHerba)
	KiE3	Ha, Hh, A	100%	
	KiE4	Ha, Hh, A	100%	
	KuE10^{-5}1H	Ha, Hh, A	100%	
	KuE10^{-5}2H	Ha, Hh, R, A	95%	
	KuE10^{-5}3H	HaHh, A	100%	
	KuE10^{-6}2H	Ha, Hh, A	97%	
	KuE10^{-6}3H	Ha, Hh, A	97%	

[a]Grouping was based on affiliation by MERFL; *Bacillus subtilis* group (Group A), the other *Bacillus* spp. (Group B), the other Firmicutes (Group C), *α*-Proteobacteria (Group D), *β*-Proteobacteria (Group E), *γ*-Proteobacteria (Group F), *δ*-Proteobacteria (Group G), unidentified and uncultured bacterial group (Group H), Hemiascomycetes (Group I), Sordariomycetes (Group J), Eurotiomycetes (*Penicillium* spp.; Group K), and Eurotiomycetes (*Aspergillus* spp.; Group L). [b]The 1st letter in vial indicates origins of microorganisms; "Ref" stands for reference strains (SU; *Saccharomyces uvarum* (NBRC10968^{T}), GZ; *Gibberella zeae* (NBRC7189), PC; *Penicillium roqueforti* (NBRC5459), AO; *Aspergillus oryzae* (NBRC30113)), "M" stands for the sample from komekouji-miso, "S" stands for the sample from sake-kasu, "P" stands for the sample from dry yeast, and "K" stands for the sample from komekouji The next letter in the samples means origins of DNAs; "i" means DNA from microbial isolate, and "u" means DNA from culture medium. The next letter indicates used DNA primers; "P" stand for 41f/1066r primers for 16S DNA, and "E" stand for EF4/EF3 primers for 18S rDNA. Vial number in the isolate represents strain number. Exponential of vial number in un isolated DNA (u) represents the decimal dilution of the vial. The 2nd number of vial number (1-3) represents number in 3 replicates for the each decimal dilution. H of last letter represents MERFL originating from the major 16S rDNA, M represents from the 2nd major 16S rDNA, and L represents from the 3rd major 16S rDNA. [c]Restriction enzymes used for similarity search; "Ha", "R", "Hh", and "A" stand for *Hae* III, *Rsa*I, *Hha* I, and*Alu*I. For the measured MERFLP which had no completely identical theoretical MERFLP, the theoretical MERFLP having the highest similarity using all the RFLPs was presented with the similarity as described in the materials and method. [d]Species name (accession number) of the theoretical MERFL having the highest similarity with the measured MERFL. [e]Additional name (accession number) of the theoretical MERFL using the different restriction enzymes.

3. Results and Discussions

3.1. Affiliation of Isolated and Non-Isolated Microorganisms by MERFLP

Affiliations of 27 MERFLs for 18S rDNA of reference strains (4 strains), isolates (13 strains), and each MPN vials (10 genomes), and those of 36 MERFLs for 16S rDNA of isolates (10 isolates), and each MPN vials (26 genomes) were summarized in **Table 1**. MERFLs for 16S rDNA were divided into 8 groups for the MPN calculation as the followings; *B.subtilis* group (Group A, 10 MERFLPs), *Bacillus* spp. (Group B, 9 MERFLs), the other Firmicutes (Group C, 6 MERFLs), Actinobacteria (Group D, 1 MERFL), *α*-Proteobacteria (Group E, 1

Table 2. Most probable numbers of each groups (A - L) and 5% confidence limits obtained using FDA's Bacterial Analytical Manual [26]. In sake-kasu (S), both prokaryote and eukaryote were not detected by the method (<0.3 × 10^5 MPN g^{-1}). T*; Total numbers of microorganisms.

Groups	komekouji-miso				dry yeast				komekouji,			
	Three dilutions	Score	×10^5 MPN g^{-1} matter	5% limits Low/High	Three dilutions	Score	×10^5 MPN g^{-1} matter	5% limits Low/High	Three dilutions	Score	×10^5 MPN g^{-1} matter	5% limits Low/High
A	$10^{-4}10^{-5}10^{-6}$	3-2-0	4.65	0.9/21	nd		<0. 3	-	nd		<0.3	-
B	$10^{-5}10^{-6}10^{-7}$	3-1-0	21.5	4.5/90	nd		<0. 3	-	nd		<0.3	-
C	$10^{-5}10^{-6}10^{-7}$	1-1-0	3.7	0.65/10	nd		<0. 3	-	nd		<0.3	-
D	$10^{-4}10^{-5}10^{-6}$	0-1-0	0.15	0.01/0.6	nd		<0. 3	-	nd		<0.3	-
E + F + G	$10^{-3}10^{-4}10^{-5}$	3-0-0	0.12	0.02/0.5	nd		<0. 3	-	nd		<0.3	-
E	$10^{-5}10^{-6}10^{-7}$	0-1-0	1.5	0.08/5.5	nd		<0. 3	-	nd		<0.3	-
H	$10^{-5}10^{-6}10^{-7}$	1-1-0	3.7	0.65/10	nd		<0. 3	-	nd		<0.3	-
I	nd		<0.3	-	$10^{-7}10^{-8}10^{-9}$	3-1-0	8600	1800/ 360000	nd		<0.3-	-
K	nd		<0.3	-	nd		<0. 3	-	$10^{-5}10^{-6}10^{-7}$	0-1-0	1.5	0.075/5.5
L	nd		<0.3-	-	nd		<0. 3	-	$10^{-5}10^{-6}10^{-7}$	3-2-0	46.5	9/210
T*	$10^{-5}10^{-6}10^{-7}$	3-3-0	120	21/500	$10^{-7}10^{-8}10^{-9}$	3-1-0	8600	1800/ 360000	$10^{-5}10^{-6}10^{-7}$	3-3-0	120	21/500

MERFLs), *β*-Proteobacteria (Group F, 2 MERFLs), *γ*-Proteobacteria (Group G, 2 MERFLs), and uncultured and unnamed bacterium (Group H, 5 MERFLs) (**Table 1**). MERFLs for 18S rDNA were divided into 5 groups as the followings; Hemiascomycetes (Group I, 8 MERFLs), Sordariomycetes (Group J, one MEPFL), Eurotiomycetes (*Penicillium* spp.; Group K, 3 MERFLs), and Eurotiomycetes (*Aspergillus* spp.; Group L, 8 MERFLs) (**Table 1**).

Most of MERFLs of isolates were placed in the same OUT as the MPN vials of the original samples as the following (**Table 1**); in Group A, two isolates (MiP4, MiP6) and the 7 vials (MuP10^{-3}1H, MuP10^{-3}3H, MuP10^{-4}1H, MuP10^{-4}2H, MuP10^{-4}3H, MuP10^{-5}1H, MuP10^{-5}2H) were placed in the same OUT (**Table 1**); in Group B, two isolates (MiP3,MiP5) and two vials (MuP10^{-5}3H，MuP10^{-5}2H) were placed in the same OUT (**Table 1**); in Group I, two isolates (PiE1, PiE2) and four vials (PuE10^{-8}1H, PuE10^{-8}2H, PuE10^{-8}3H, PuE10^{-9}2H) were placed in the same OUT (**Table 1**); in Group K, one isolate (KiE2) and one vial (KuE10^{-6}1H) were placed in the same OUT (**Table 1**); in Group L, two isolates (KiE3, KiE4) and five vials (KuE10^{-5}1H, KuE10^{-5}2H, KuE10^{-5}3H, KuE10^{-6}2H, KuE10^{-6}3H) were placed in the same OUT (**Table 1**). These results indicated that microorganism included in food sample were properly affiliated using the MPN vials without isolation. As total MERFLs of most of the MPN vials in komekouji (K) and dry yeast (P) were the same as those of isolates and reference strains, the both samples were suggested to include almost pure single microorganism.

*Bacillu*s spp. (Group A and B) were amplified preferentially to the other gram positive bacterial group (Group C and D) and gram negative bacterial group (Group E, F and G) by 41f/1066r primers as the followings; all the *Bacillu*s spp. (Group A and B) were the major MERFLs, indicated as "H", in the MPN vials, while 20% of the other gram positive bacterial group (Group C and D) was the major MERFLs, and no gram negative bacterial group (Group E, F and G) were the major MERFLs (**Table 1**), which were coincident with the former manuscripts [2] [3].

For 18S rDNA, difference between the theoretical MERFL and the measured MERFL, which caused to decrease precision of pyrogenetic estimation as described in the former papers [21] [22], was inevitably caused by used restriction enzyme as the followings; contiguous fragments of 354 bp and 356 bp in theoretical *Alu* I RF of *Asperigillus* spp. (Group L) were misread as one fragment in the respective measured *Alu* I RF; those of 353 bp and 356bp in theoretical *Alu* I RF of most *Penicillium* spp. (Group K) were misread as one fragment in the respective measured *Alu* I RF; those of 119 bp and 120 bp in theoretical *Rsa* I RF and those of 151 bp and 160 bp

of *Hha* I RFs of *G.zaea* (Group J) were misread as one fragment in the respective measured RFs; contiguous fragments of 177 bp and 174 bp and those of 155 bp and 151 bp in theoretical *Hae* III RFs of *Saccharomyces* spp. (Group K) were misread as one fragment in the respective measured RFs, the fragments of 99 bp in theoretical *Rsa* I RF of *Saccharomyces* spp. (Group K) were sometimes measured over 100 bp fragment due to the measuring error. As the relative mole ratios of these contiguous fragments were almost twice as those of the other fragments in the same RF, they were manually divided into 2 fragments in this study. Fragments from 100 bp to 103 bp of the measured *Rsa* I RF of *Saccharomyces* spp. (Group K) were manually eliminated in this study. Development of a program, which achieved these data processing automatically, may be useful to reduce a time for analysis.

Until now, following 5 restriction enzymes, *Hae*III, *Hh*I, *Rsa* I, *Scr*F1, and *Alu* I, have been used because they had more restriction sites in 16S rDNA than the others, in order to affiliate bacterial isolates precisely by using a fewer restriction enzymes [21] [22]. However such the restriction enzyme increased the opportunity of overlapped fragment, when they were used for longer DNA or sample having huge microbial diversity without isolation. Another combination of restriction enzymes, which had fewer restriction sites in 16S rDNA or 18S rDNA, was found suitable for such the analyses (unpublished results). Analysis using the other combination of restriction enzymes will be presented in the following manuscript.

3.2. Enumeration of Each Prokaryotic and Eukaryotic Microbial Groups by MPN

In komekouji (K), Group L, including *Aspergillus* spp., was enumerated to be 46.5×10^6 MPM g^{-1}, and Group K, including *Penicillium* spp., was enumerated to be 1.5×10^6 MPM g^{-1}, and no other eukaryotic nor prokaryotic microorganisms were detected (**Table 2**, **Figure 1**). As the sample (K) included almost pure *Aspergillus* spp., the sample might be analyzed without cultivation as same as lactic acid bacterial groups in yoghurt [27]. In dry yeast (P), Group I, including *Saccharomyces* group, was enumerated to be 8600×10^6 MPM g^{-1}, which was higher than that estimated by dilution plate count (220×10^6 CFU g^{-1}; unpublished result), and no other eukaryotic nor prokaryotic microorganisms was detected (**Table 2**). As the sample (P) included almost pure *Saccharomyces* group, the sample might be analyzed without cultivation as same as lactic acid bacterial groups in yoghurt [27].

In komekouji-miso (M), eukaryotic microorganism was not detected. As prokaryotic microorganisms, Group B (the other *Bacillus* spp.) was numerically dominant, which was enumerated to be 21.5×10^6 MPM g^{-1}, followed by Group A (*B. subtilis* group.; 4.65×10^6 MPM g^{-1}), and Group C (the other Firmicutes; 3.7×10^6 MPM g^{-1}) (**Table 2**, **Figure 1**). Number of Group A was similar level as those reports, in which *B. subtilis* originated from soybean was present at $10^4 \sim 10^6$ CFU g^{-1} during miso fermentation, and decreased to under $10^4 \sim 10^5$ CFU g^{-1} [28] [29]. Because of the difference of used incubation medium, total bacterial number in komekouji-miso (K) in this study was higher than that of the former study using MRS medium for lactic acid bacteria [27], and lactic acid bacteria was not detected in this study (**Table 2**). No *Aspergillus* spp. added with komekoji, nor

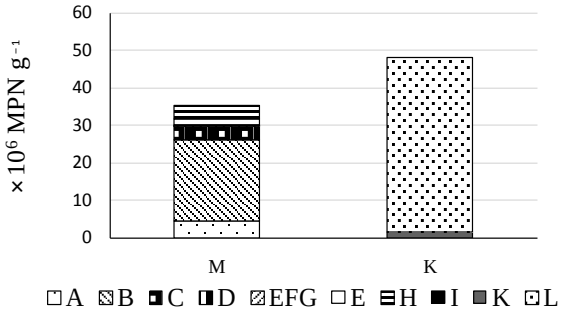

Figure 1. Numbers of microbial groups estimated by MPN and MERFLP in komekouji-miso (M), and komekouji (K). Number of the other *Bacillus* spp. (Group A;), *B. subtilis* (Group B;), the other Firmicutes (Group C;), Actinobacteria (Group D;), *α*,*β*,*γ*-Proteobacteria (Group E, F, G;), *Burkholderia* sp. (Group E;), unidentified and uncultured bacteria (Group H;), *Saccharomyces* sp. (Group I;), *Penicillium* spp. (Group K;), and *Aspergillus* spp. (Group L;) were presented.

yeast, which progressed alcohol fermentation, were also detected in komekouji-miso (<0.3 × 10^5 MPN g^{-1}) (**Table 2**). The sample M also included lower number of Group D (Actinobacteria; 0.15 × 10^6 MPM g^{-1}), Group E (*Burkhokderia* sp.; 1.5 × 10^6 MPM g^{-1}), and Group E, F, G (the other *α*, *β*, *γ* proteobacteria; 0.12 × 10^6 MPM g^{-1}) (**Table 2**, **Figure 1**). In sake-kasu (S), both prokaryote and eukaryote were not detected by the method, while bacteria affiliated to be Goup A (SiP5), Group B (SiP1), Group C (SiP2, SiP4), and Group G (SiP3) were isolated from the lowest dilution agar plate. As skekasu was residue filtered rice wine from moromi after finish of alcohol fermentation and sterilization by heating at 60 to 65℃, eukaryotic microorganisms, such as *Aspergillus* spp. and *Saccharomyces* spp., which progressed alcohol fermentation, nor prokaryotic bacteria were not founded in MPN vials, but spore forming bacteria such as *Bacillus subtilis* (group A; SiP5), *B. pumilus* (group B; SiP1), *Clostridium barati* (group C; SiP2), and *C. botulinum* (group C; SiP4) were isolated in lowest dilution agar plate as heat tolerant bacteria (**Table 1**).

As PCR amplification were not tested under 10^{-5} dilution vials and lower detection limit was high (<0.3 × 10^5 MPN g^{-1}) in the present experiment except for prokaryotic microorganisms in komekouji-miso (K) (**Table 1**, **Table 2**), the lower detection limit could be lowered by using more effective DNA purification method.

4. Conclusions

By DGGE [8]-[11], which used directly extracted DNA, exact ratio of each microbial groups was not obtained due to PCR bias and it was no use to compare the numbers and composition of prokaryotic microorganisms to those of eukaryotic microorganisms because of a difference in the PCR amplification rates [15]-[18]. As numbers of each microbial groups were estimated by MPN not by an amount of the amplified DNA in this method as described in the former manuscripts [1] [2], the difference in the primers did not affect the numbers and composition of numerically dominant microorganisms but their effect was limited in numerically minor microorganisms. Present results indicated that using both universal primers for eukaryotic and prokaryotic microorganisms, each group of prokaryotic and eukaryotic microorganisms were simultaneously enumerated without any preliminary information nor setting up standard curve, which were required for real time PCR. The method seemed useful not only to evaluate whole microbial compositional change during traditional fermentation process, and manure composting process, but also the variety of other purpose such as to ensure food safety by evaluating unintended or unexpected contamination or proliferation of opportunistic pathogens or foodborne pathogens or non-pathogenic spoilage microorganisms during food distribution system.

In this study, universal PCR primers for eukaryotic and prokaryotic microorganisms were used to evaluate what kinds of microorganisms was mainly included in the sample. Another combination of universal primers and selective primers will afforded more detailed information of each microbial group, and functional or toxicological information of each microbial group will similarly be obtained by changing DNA primers.

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