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Evaluation of the Method Based on Restriction Fragment Length Polymorphism Analysis as Simple Analysis Method of Lactic Acid Bacteria in Foods

Kunimasa Matsumoto[1], Kouya Shimada[2], Naoto Horinishi[1], Katsuji Watanabe[1,2*]

[1]Department of Life, Environment and Materials Science, Graduate School of Engineering, Fukuoka Institute of Technology, Fukuoka, Japan
[2]Department of Life, Environment and Materials Science, Faculty of Engineering, Fukuoka Institute of Technology, Fukuoka, Japan
Email: *k-watanabe@fit.ac.jp

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Abstract

Lactic acid bacteria have not only been used to produce various kinds of fermented food, but also used as probiotic products. As lactic acid bacterial group was consisted from diverse genera, a simple inspection method by which numbers and contained microorganisms could be automatically analyzed without any preliminary information was required to use them more effectively. In this manuscript, lactic acid bacterial groups in commercial products of kimuchi, komekouji-miso, and yoghurt were identified and enumerated by our newly developed method [1]-[3], to evaluate whether the method could be used as an inspection method of various food samples. In kimuchi, numerically dominant bacteria were *Lactobacillus sakei*, and *L. casei* (1.4×10^4 MPN g^{-1}) and *Leuconostoc* spp. (1.4×10^4 MPN). In kouji-miso, numerically dominant bacteria was *Bacillus* spp. (3×10^3 MPN), which mainly included *B. subtilis* group and *B. cereus* group. Lactic acid bacteria such as *Lactobacillus* spp., or *Lactococcus* spp., included in the komekouji-miso, could be enumerated after 3 days incubation (1.24×10^4 MPN), but not detected after 7 days incubation. In yoghurt A and C, *Lactococcus lactis* was detected as numerically dominant lactic acid bacteria (3.0×10^5 MPN). In yoghurt B, *Lactobacillus* spp., or *Lactococcus* spp., was detected not only by a culture-based method but also by an unculture-based method, although there was a difference between the both estimated numbers. The present results suggested that the method might become useful as a simple inspection method of food microorganisms, because time and labor of the analysis could be reduced by using an unculture-based method and MCE-202 MultiNA. In this study, *Bifi-*

*Corresponding author.

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***dobacteriium* spp. was not detected in B and C yoghurt, in spite of indicating their existence, and numbers of lactic acid bacteria were lower than the level of the daily product regulation, because 16S rDNA of *Bifidobacteriium* spp. might not be amplified by the used PCR condition. The PCR condition must be changed so as to amplify *Bifidobacterium* spp., before the method will be used as an inspection method for lactic acid bacteria.**

Keywords

Multiple Enzyme Restriction Fragment Length Polymorphism Analysis, Most Probable Number Method, Lactic Acid Bacteria, Komekouji-Miso, Kimuchi, Yoghurt

1. Introduction

Lactic acid bacteria have not only been used to produce various kinds of fermented food, but also used as probiotic products to exert a health benefit by eating living cells. There has recently been an increasing demand by the consumers and producers to use them more effectively. In the traditional fermentation process, various kinds of lactic acid bacteria were concerned with the fermentation process, which effected taste, texture, and flavor of the final products. As probiotics, their effect was suggested to modulate mucosal and systematic immunity [4], and improve the nutritional and microbial balance in the intestinal tract [5].

As lactic acid bacteria comprises the following genera, *Streptcoccus* spp., *Bifidobacterium* spp., *Lactobacillus* spp., *Enterococcus* spp., *Lactococcus* spp., and *Leuconostoc* spp., variety of culture-based and unculture-based techniques have been used not only to analyze their compositions in products [6], starter [7], and their natural source [8], but also to control fermentation process until now. As a probiotic product, they are often used as multispecies containing different probiotic species that belong to one or preferentially more genera. These methods were used to demonstrate the effect as probiotics [9] and investigate a mechanism of its functional effect [4].

Although most widely used unculture-based techniques, such as denaturing gradient gel electrophoresis (DGGE) [8]-[11] [12], or clone library sequencing [7] [13], provided relative abundance of each microbial groups, numbers of each microbial group remained unclear [14], and it took a lot of time and labor to provide the information of each lactic acid bacterial group. These faults seemed to disturb these methods to come into wide use. Especially as an inspection method, there was no method available by which numbers and contained microorganisms could be automatically analyzed without any preliminary information of sample and included microorganisms.

Until now, we had found a new affiliation method of microorganisms based on restriction fragment polymorphism analysis, and developed a system and method by which bacterial affiliations could systematically be completed [1]. By using isolated environmental bacteria, precision of bacterial affiliation had been evaluated [15]-[17]. Its combined use of the most probable number method (MPN) was found effective to provide numbers and taxonomies of each bacterial group without isolation in the former papers [2] [3]. In order to evaluate whether the method may become useful in a field of food microbiology, we have started to analyze microbial groups in various food samples by using this method. In this study, affiliation and enumeration of lactic acid bacterial group in commercial products, kimuchi, komekouji-miso, and yoghurt, by the method were presented.

2. Materials and Methods

2.1. Samples

We assumed that precision of the affiliation and enumeration of lactic acid bacteria in sample were depend on a ratio of lactic acid bacteria to the other bacterial groups in the sample. Komekouji-miso was selected as a representative sample including lower ratio of lactic acid bacterial group, kimuchi was selected as a representative sample including both lactic acid bacterial group and the other bacterial group, and yoghurt was selected as a representative sample including higher ratio of lactic acid bacterial group.

Commercial products of yoghurt A, yoghurt B, yoghurt C, komekouji-miso, and kimuchi directly imported from Korea were used. For MPN, serial 10-fold dilutions (10^{-2} to 10^{-7}) prepared from sample (1 g fresh wt.) were inoculated to test vials (3 replicates) including MRS medium (Difco, Sparks MD). As to komekouji-miso

(A), microbial DNA in each vial was extracted after 3 days (MC3) and 7 days (MC7) of incubation at 30˚C. Microbial DNAs of the other samples (yoghurt A; YAC, yoghurt B; YBC, yoghurt YC; CC, kimuchi (KC)) were extracted after 3 days of the incubation. As yoghurt B, microbial DNA was directly extracted without cultivation (YBU) after neutralization of pH, and removal of casein. Each bacterial group was counted by MPN after the phylogenetic estimations.

2.2. MERFLP of the Amplified 16S rDNA

Chromosomal DNAs of each MPN vials were prepared as described previously and purified by conventional methods. Amplification of 16S rDNA was according to the former study [15]-[17] using the V2 forward primer (41f), and the V6 reverse primer (1066r) [18] [19]. PCR product (10 μl) was separately digested by each of 10 units of the restriction enzyme, *Hae* III or *Hha* I or *Rsa* I or *Alu* I (Takara Bio Co. Ltd. Shiga Japan) in Low salt buffer solution (10× Low salt buffer, Takara Bio Co. Ltd.). Fragment lengths were measured by microchip electrophoresis system (MCE-202 MultiNA; Shimadzu Co., Ltd. Kyoto Japan). The sample was diluted by de-ionized water (5 folds for Low salt buffer) before measuring by MCE-202.

2.3. Theoretical Multiple Enzyme Restriction Fragment Length (MERFL) Database Used for the Estimation

The newly constructed database was used for this research, which was edited using the method of Watanabe and Okuda [1] described previously [15]. For 41f/1066r primers, 30,844 post-amplification sequence files, which were consisted from 1379 bacterial genera, including uncultured and unidentified bacteria, were mainly re-edited using small subunit rRNA files in RDP II release 9_61 [20] under 5-bases mismatches in the both in primer annealing sites. The number and diversity of the registered MERFLs in the database greatly increased from the former database, 4370 MERFLs, and 576 bacterial genera, using small subunit rRNA files in RDP II release 7.01 [21], and included the following lactic acid bacteria, 307 MERFLs of *Streptcoccus* spp., 68 MERFLs of *Bifidobacterium* spp., 416 MERFLs of *Lactobacillus* spp., 105 MERFLs of *Enterococcus* spp., 46 MERFLs of *Lactococcus* spp., and 31 MERFLs of *Leuconostoc* spp.

2.4. Data Processing for Phylogenetic Estimation Using Multi-Template DNA and Phylogenetic Estimation

As each MPN vials included multi-template DNAs originated from heterogeneous bacteria, most of the measured MERFL was the mixed MERFLs digested from the heterogeneous 16S rDNA. Whereas all the theoretical MERFLs were originated from the homogeneous 16S rDNA sequence, the measured MERFL digested from the homogeneous 16S rDNA was selected as described previously [2] [3].

The restriction fragments (RFs) with the highest relative mole concentration (ratio of fluorescent intensity to fragment size) were selected and used as the major RFs (represented as H in **Table 1**). After subtraction of the above the major RFs from the mixed heterogeneous RFs, RFs originated from the 2nd major gene were similarly selected and used for similarity search (represented as M in **Table 1**).

The similarity between the measured RFLP (A) and the theoretical RFLP (B) was calculated as described previously [1] [15]-[17] based on the pairwise distance (D_{AB}) by the following equation; $D_{AB} = 1 - 2N_{AB}/(N_A + N_B)$, where N_A and N_B were the numbers of fragments of each RFLs and N_{AB} was the number of shared fragments that indicated same sizes within an allowance limit for measuring error according to Nei and Li [22]. The pairwise distance of the MERFLs (D_{ABME}) was an average of all the D_{ABs} for used restriction enzymes. Similarity (%) was $(1 - D_{ABME}) \times 100$ (**Table 1**).

If the completely identical theoretical MERFL was not found by using all of the measured MERFL data, combinations of restriction enzymes used for the analysis was changed (**Table 1**) [15]-[17]. As to the measured MERFL which had no completely identical theoretical MERFL, the theoretical MERFL having the highest similarity to the measured MERFL was indicated in **Table 1** [2] [3].

2.5. Estimation of Numbers of Each Taxonomically Different Groups by the Most Probable Number Method

Most probable numbers of each groups (A-F) were estimated for three-tube, three-decimal-dilution experiment

Table 1. Affiliation of bacteria grown in serially diluted MRS medium by MERFLP[a].

	Vial No.[b]	Restriction enzymes[c]	Similarity (%)	Name (accession number)[d]
A	YBC10^{-4}1H	Ha,R	100	*Lactobacillus plantarum* (M58827), *L. sakei* (M58829), *L. hilgardii* (M58821), *L. brevis* (M58810), *L. buchneri* (M58811), *Lactococcus plantarum* (X54259), *L. piscium* (X53905), *L. raffinolactis* (X54261), *Streptococcus salivarius* (M58839)
	YBC10^{-4}2H	Ha,R,Hh	89	
	YBC10^{-5}1H	Ha,R	100	
	YBU10^{-3}1H	Ha,A	93	
	YBU10^{-3}2H	Ha,Hh	100	
	YBU10^{-3}3H	Ha,Hh	100	
	YBU10^{-4}1H	Ha,Hh	100	
	YBU10^{-4}2H	Ha,Hh	100	
	YBU10^{-4}3H	Ha,Hh	100	
	YBU10^{-5}1H	Ha,Hh	100	
	YBU10^{-5}2H	Ha,Hh	100	
	YBU10^{-5}3H	Ha,Hh	100	
	YCC10^{-3}1H	Ha,R,Hh	100	
	YCC10^{-3}2H	Ha,R,Hh	100	
	MC310^{-5}1H	Ha,Hh	75	
	MC310^{-5}2H	Ha,Hh	75	
	KC10^{-4}1H	Ha,R, Hh,A	100	*Lactobacillus casei* (AJ272201)
	KC10^{-5}2H	Ha,Hh,A	100	*Lactobacillus* sakei (AB362607, AB362609, CR936503, M58829)
B	YAC10^{-4}3H	Ha,R,A,	92	*Lactococcus lactis* (AY971748, X54260, AF515226, AB008215)
	YAC10^{-5}2H	Ha,R,A	92	
	YAC10^{-5}3H	Ha,R,A	88	
	YAC10^{-6}1H	Ha,R,A	88	
	YCC10^{-4}2H	Ha,R	100	
	YCC10^{-5}1H	Ha,R	100	
	YCC10^{-5}2H	Ha,R,A	100	
	YCC10^{-6}2H	Ha,R	100	
C	KC10^{-4}2H	Ha,R, Hh,A	100	*Leuconostoc gelidum* (AF175402), *L. mesenteroides* (AB362704, AB362705, AB326298, CP000414), *L. pseudomesenteroides* (AB326299)
	KC10^{-5}2H	Ha,R, Hh,A	100	
D	MC710^{-2}1H	Ha,Hh,A	89	*Bacillus subtilis* (X60646), *B. popilliae* (X60633), *B. amyloliquefaciens* (X60605),
	MC710^{-2}2H	Ha,Hh,A	89	
	MC710^{-3}3H	Ha,Hh,A	89	
	MC710^{-4}3H	Ha,Hh,A	89	
	MC310^{-2}2H	Ha,Hh,A	89	
	MC310^{-2}3H	Ha,Hh,A	89	
	MC310^{-3}2H	Ha,Hh	100	
	MC310^{-4}1H	Ha,Hh	100	
	MC710^{-3}1H	Ha,Hh,A	100	*B. mycoides* (X55061), *B. thuringiensis* (X55062), *B. cereus* (X55063), *B. medusa* (60628)
	MC310^{-3}1H	Ha,Hh,A	100	
	MC310^{-1}1H	Ha,Hh,A	100	
E	MC710^{-2}3H	Hh,A	100	*Sarcina ventriculi* (AF110272)
	MC710^{-3}2H	Hh,A	90	
	MC710^{-2}1M	Ha,Hh	83	*Desulfotomaculum putei* (AF053932)
	MC310^{-3}2M	Hh,A	100	*Clostridium tyrobutyricum* (M59113)
	MC310^{-3}2H	Ha,A	100	*Enterococcus faecalis* (Y18293)
	KC10^{-4}1M	Ha,R, Hh,A	87	*Micrococcus brunensis* (AY119686), *M. lamae* (AY119687), *M. carouselicus* (Y15713), *M. equipericicus* (Y15712, AJ576067)

Continued

	MC710^{-2}1M	Hh,A	83	*Ralstonia gilardii* (AF076645)
	MC710^{-4}1H	Hh,A	90	*Comamonas acidovorans* (AB021417)
	MC310^{-2}2M	Hh,A	93	*Aquaspirillum gracile* (AF078753)
F	MC310^{-1}3H	Hh,A	100	*Coxiella burnetii* (D89791)
	YCC10^{-5}2M	Ha,Hh	100	*Desulfovibrio fairfieldensis* (U42221)
	YAC10^{-3}1H YAC10^{-5}1H	Ha,Hh,R Ha,R	83 100	*Capnocytophaga sputigena* (X67609), *Porphyomonas gingivalis* (L16429)

[a]Grouping was based on affiliation by MERFL; lactic acid bacteria (Group A), *Lactococcus lactis* (Group B), *Leuconostoc* spp. (Group C), *Bacillus* spp. (Group D), the other Firmicutes (Group E), and gram negative bacterial group (Group F). [b]The 1st letter in vial indicates samples; "Y" stands for yoghurt followed by product items "A", "B", and "C", "M" stands for kouji-miso, and "K" stand for kimuchi. The next letter indicates extraction method; "C" stand for culture-based followed by incubation period "3" stand for 3 days, and "7" stand for 7 days, and "U" stand for unculture-based as described in materials and method. Exponential of vial number represents the decimal dilution of the vial. The 2nd number of vial number (1 - 5) represents number in 5 replicates for the each decimal dilution. H of last letter represents MERFL originating from the major 16S rDNA, and M represents from the 2nd major 16S rDNA. [c]Restriction enzymes used for similarity search; "Ha", "Hh", "R", and "A" stand for *Hae* III, *Hha* I, *Rsa* 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.

(**Table 2**). MPN numbers and confidence limits shown in **Table 2**, which were calculated by the modified of de Man [23], were obtained using FDA's Bacterial Analytical Manual [24].

3. Results

3.1. Affiliation of Bacteria by MERFLP without Isolation

Fifty two MERFLs in each MPN vials were differentiation into 8 groups based on the results of the affiliation and summarized in **Table 1**. The major group (Group A; 18 MERFLPs) contained *Lactobacillus* spp., such as *L. plantarum*, *L. sakei*, *L. hilgardii*, *L. brevis*, and *L. buchneri*, *Lactococcus* spp., such as *L. plantarum*, *L. piscium*, and *L. raffinolactis*, and *Streptococcus salivarius* (**Table 1**). Although, MERFL of KC10^{-3}1H was completely identical with that of *Lactobacillus casei* (AJ272201) and that of KC10^{-4}2H was completely identical with that of *Lactobacillus sakei* (AB362607, AB362609, CR936503, M58829), they were included in this group (**Table 1**). The other lactic acid bacterial group were affiliated to be *Lactococcus lactis* (Group B; 8 MERFLs), and *Leuconostoc* spp., such as *L. gelidum*, *L.mesenteroides*, and *Lpesudomesenteride*s (Group C; 2 MERFLs) (**Table 1**). The 2nd major group (Group D; 11 MERFLPs) contained *Bacillus* spp., which were further differentiated into the following 3 subgroups, *B. subtilis* group, *B. licheniformis/sphaericus*, and *B. cereus* group (**Table 1**). The other Firmicutes (Group E; 6 MERFLs) and gram negative bacterial group (Group F; 8MERFLPs) were also existed (**Table 1**).

In the major MERFL, represented as "H" of last letter, ratio of the MERFLs having 100% similarity to the corresponding theoretical MERFLs (62.5%) was almost as same as that of the former study (62.9%) [3] and lower than that of the previous study (90.5%) [2]. This might be caused from a difference of used incubation media as the following; As TSA medium used in this study was not a selective medium, more diverse microorganisms were proliferated in the each MPN vials than that used in the former study, which made it more difficult to select the MERFLP digested from the homogeneous 16S rDNA among the mixed MERFL.

3.2. Enumeration of Each Bacterial Groups by MPN

The numerically dominant bacteria in komekouji-miso after 3 days (AC3) and 7 days (AC7) cultivation were *Bacillus* spp. (Group D), which included *B. subtilis* group and *B. cereus* group (3×10^3 MPN in the both AC7 and AC3 (**Table 2**, **Figure 1**). Although their similarity to the corresponding theoretical MERFLP (75%) was lower than the others (**Table 1**), Group A lactic acid bacteria was only detected in AC3 (1.2×10^3 MPN) (**Table 2**, **Figure 1**). There was not so large difference in numbers of the group E between AC7 and AC3 (0.15×10^3 MPN in AC7 and 0.22×10^3 MPN in AC3), and numbers of group D were 0.14×10^3 MPN in AC7 and 0.014×10^3 MPN in AC3 (**Table 2**, **Figure 1**).

Table 2. Most probable numbers of each groups (A-F) in komekouji-miso (AC3, AC7) and kimuchi (KC) and 5% confidence limits obtained using FDA's Bacterial Analytical Manual [24]. Group B was not detected.

	Group A		Group C		Group D		Group E		Group F	
	Three dilution	$\times 10^4$ MPN	Three dilution	$\times 10^4$ MPN	Three dilution	$\times 10^4$ MPN	Three dilution	$\times 10^4$ MPN	Three dilution	$\times 10^4$ MPN
	Score	5% limits	Score	5% limits	Score	5% limits	Score	5% limits	Score	5% limits
AC7	nd		nd		$10^{-3}10^{-4}10^{-5}$ 2-1-0	0.30 0.074/0.82	$10^{-2}10^{-3}10^{-4}$ 1-1-0	0.0148 0.0026/0.04	$10^{-2}10^{-3}10^{-4}$ 1-0-1	0.0144 0.0026/0.036
AC3	$10^{-4}10^{-5}10^{-6}$ 0-2-0	1.24 0.24/3.6	nd		$10^{-3}10^{-4}10^{-5}$ 2-1-0	0.3 0.0074/0.84	$10^{-2}10^{-3}10^{-4}$ 1-2-0	0.022 0.007/0.084	$10^{-1}10^{-2}10^{-3}$ 1-1-0	0.00144 0.00026/0.004
KC	$10^{-4}10^{-5}10^{-6}$ 1-1-0	1.44 0.26/4.0	$10^{-4}10^{-5}10^{-6}$ 1-1-0	1.44 0.26/4.0	nd		$10^{-4}10^{-5}10^{-6}$ 0-1-0	0.06 0.003/0.22	nd	

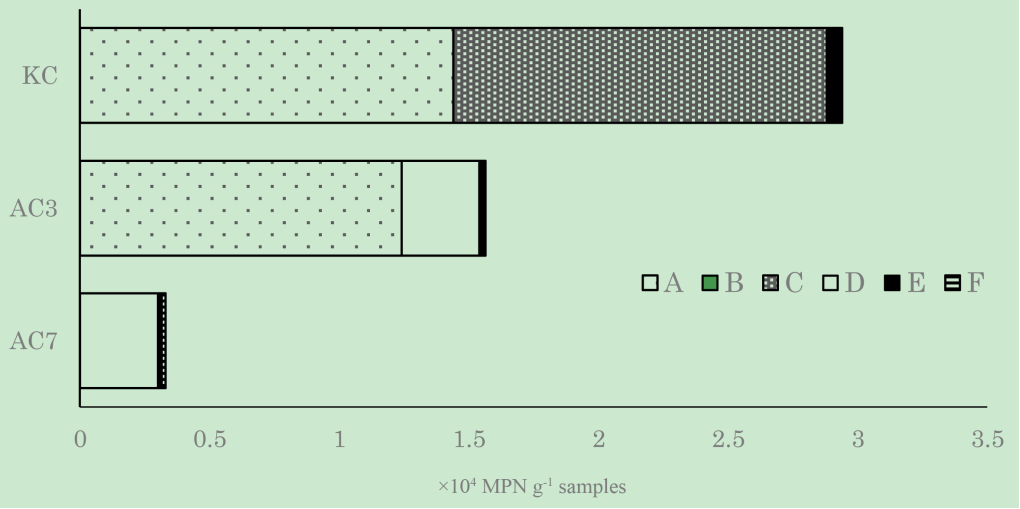

Figure 1. Numbers of bacterial groups estimated by MPN and MERFLP in kimuchi (KC), and kouji-miso after 3days incubation (AC3), and 7 days incubation (AC7). Number of *Lactobacillus* spp. and *Lactococcus* spp. (Group A;), *Lactococcus lactis* (Group B;), *Leuconostoc* spp., (Group C;), *Bacillus* spp. (Group D;), the other Firmicutes (Group E;), and gram negative bacteria (Group F;) were presented.

The numerically dominant lactic acid bacteria in kimuchi (KC) after 3 day cultivation were Group A (1.4×10^4 MPN, *Lactobacillus sakei*, and *L. casei*,) and Group C (1.4×10^4 MPN, *Leuconostoc* spp.), and lower number of the other Firmicutes (6×10^2 MPN, *Macrococcus* spp.) was included (**Table 2**, **Figure 1**).

Lactococcus lactis (Group B) was the numerically dominant lactic acid bacteria (3×10^5 MPN/g product) in yoghurt A (YAC) and yoghurt C (YCC), which also contained lower numbers of gram negative bacterial group (Group F; 6×10^3 MPN (2%) in YCC and 1.44×10^3 MPN (0.48%) in YAC), and YCC contained a trace number of the other lactic acid bacteria (Group A; 1.24×10^2 MPN 0.04%) (**Table 3**, **Figure 2**). In the both cultured (YBC) and uncultured (YBU) yoghurt B, the lactic acid bacteria of group A was solely detected (**Table 3**, **Figure 2**). These result indicated that yoghurt of commercial product included mainly lactic acid bacterial group. The number of YBU, which used the DNA directly extracted from the yoghurt B, was 8 times higher than that of YBC, used the DNA extracted after incubation in TSA medium (**Table 3**, **Figure 2**).

4. Discussion

During miso fermentation, *B. subtilis* was reported to present at 10^4 - 10^6 CFU g^{-1}, and decreased to under 10^4 - 10^5 CFU g^{-1} [19] [20]. The number of *Bacillus* spp., in the both AC7 and AC3 (3×10^3 MPN g^{-1}, **Table 2**),

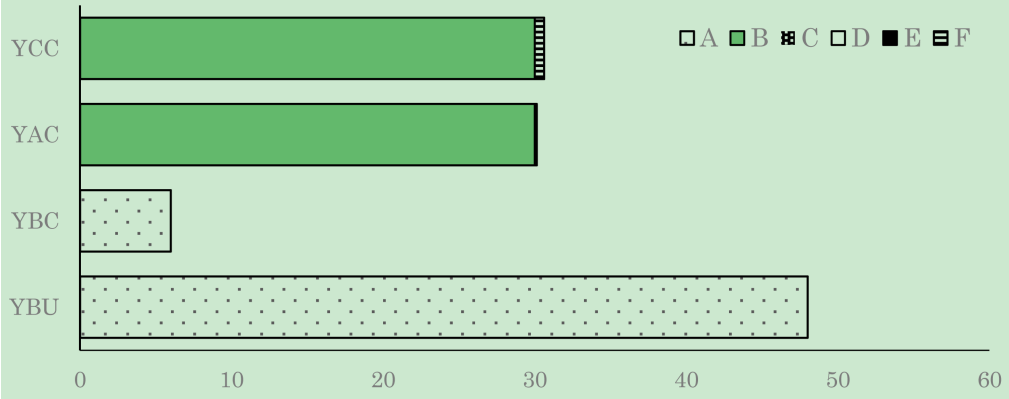

Figure 2. Numbers of bacterial groups estimated by MPN and MERFLP in yogurt C (YCC), yogurt A (YAC), and yogurt B (YBC) by culture-based method, and that of yogurt B (YBU) by unculture-based mehod. kouji-miso after 3days incubation (AC3), and 7 days incubation (AC7). Number of *Lactobacillus* spp. and *Lactococcus* spp. (Group A;), *Lactococcus lactis* (Group B;), *Leuconostoc* spp., (Group C;), *Bacillus* spp. (Group D;), the other Firmicutes (Group E;), and gram negative bacteria (GroupF;) were presented.

Table 3. Most probable numbers of each groups (A-F) in yoghurt (YA, YB, YC) and 5% confidence limits obtained using FDA's Bacterial Analytical Manual [24]. Group C, D, and E were not detected.

Samples	Group A		Group B		Group F	
	Three dilution	$\times 10^4$ MPN	Three dilution	$\times 10^4$ MPN	Three dilution	$\times 10^4$ MPN
	Score	5% limits	Score	5% limits	Score	5% limits
YCC	$10^{-2}10^{-3}10^{-4}$	0.0124	$10^{-5}10^{-6}10^{-7}$	30	$10^{-4}10^{-5}10^{-6}$	0.6
	0-2-0	0.0024/0.036	2-1-0	7.4/84	0-1-0	0.03/2.2
YAC			$10^{-5}10^{-6}10^{-7}$	30	$10^{-3}10^{-4}10^{-5}$	0.144
	nd		2-1-0	7.4/84	1-0-1	0.026/0.36
YBC	$10^{-4}10^{-5}10^{-6}$	6.0				
	2-1-0	0.074/8.4	nd		nd	
YBU	$10^{-4}10^{-5}10^{-6}$	48				
	3-3-0	8.2/200	nd		nd	

which included *B. subtilis* group (**Table 1**), were within an average of commercial products. Lactic acid bacteria of group A, which included miso fermentation bacteria of *Lactobacillus plantarum* (MC310^{-5}1H, MC310^{-5}2H), and *Enterococcus faecalis* (MC310^{-3}2H) [20] [21], were only detected in AC3 but not in AC7 (**Table 1**). PCR amplification of lactic acid bacteria in AC7 was inhibited by the *Bacillus* spp. proliferated during the longer incubation period of AC7, which also inhibited the precise affiliation (75%) of lactic acid bacteria of group A (MC310^{-5}1H and MC310^{-5}2H) in AC3 (**Table 1**).

Leuconostoc spp., and *Lactobacillus sakei* were reported to be dominant lactic acid bacteria during kimuchi fermentation [21]. As *Bacillus* spp. was not included in the sample, lactic acid bacteria (KC10^{-4}1H, KC10^{-5}2H, KC10^{-4}2H, KC10^{-5}2H) was affiliated precisely (100%) (**Table 1**).

Most of bacteria included in the yoghurts (YCC, YAC, YBC, and YBU) were lactic acid bacteria and no-*Bacillus* spp. detected (**Table 3**, **Figure 2**), which resulted in more precise affiliations as the following; with respect to yoghurts (YCC, YAC, YBC, and YBU), a ratio of 100% similarity to those of the corresponding theoretical MERFL increased to 75% from 62.9% (total samples) (**Table 1**).

5. Conclusions

The total number of included bacterial group in komekouji-miso (MC7 and 3) and kimuchi (KC) was lower than

that of Japanese food sanitary regulations where numbers of microorganisms included in commercial product must be lower than 10^5 cells/g (**Table 2**), and most of the detected bacteria were originated from their fermentation process (**Table 2**).

However, the total number of lactic acid bacteria, YCC; 3.06×10^5 MPN g^{-1}, YAC; 3.01×10^5 MPN, YBC; 0.6×10^5 MPN, YBU; 4.8×10^5 MPN, was lower than that of Japanese daily product regulation, which indicated that daily product including lactic acid bacteria or yeast over 10^7 cell/mL could be treated as fermented daily product (**Table 3**, **Figure 2**). *Bifidobacteriium* spp. was not detected in all the yoghurt tested, in spite that *Bifidobacterium longum* was indicated to be included in yoghurt B as the major lactic acid bacteria, and the following lactic acid bacteria, *Bifidobacterium lactis*, *Lactobacillus delbrueckii*, *L. helveticus*, and *Streptococcus thermophilus* were indicated to be included in yoghurt C. Absence of *Bifidobacterium* and lower number of lactic acid bacteria were caused from the used PCR condition; recent research indicated that 16S rDNAs of some of the Actinobacteria, especially *Bifidobacteriium* spp., and Firmicutes were not amplified by the used PCR condition, because the annealing site of some 16Sr DNAs of such the Actinobacteria and Firmicutes included over 3 miss-matched bases to this primer by using computer simulation (unpublished results). We concluded a difference between estimated number using directly extracted DNA and that using DNA extracted after incubation as follows; during incubation, *Bifidobacterium* spp., which could not be amplified by the PCR condition, might proliferated preferentially to the group A of lactic acid bacterial group, which might decrease relative DNA mole ratio of Group A and inhibited PCR amplification. The detection of *Bifidobacteriium* spp. by a new PCR condition including newly designed PCR primer for these bacteria will be presented in the following manuscript.

Until now the method had been used as a culture-based method because accurate affiliation was impossible for sample having huge microbial diversity and large amount of PCR inhibiting substance such as soil sample [2] [25] [26] and manure [3]. As precise affiliation mainly depended on whether the measured MERFLP digested from the homogeneous 16S rDNA could precisely be selected among the mixed MERFLs digested from the heterogeneous 16S rDNA, microbial diversity and concentration of PCR inhibiting substance had to be decreased by selective incubation for such the samples [2]. The present result indicated that a sample having lower microbial diversity and having no PCR inhibition substance, such as yoghurt, was found to be analyzed without cultivation.

Compared to the next-generation method such as pyro-sequencing, by which each bacterial number was estimated by quantitative PCR from relative abundance of dominant microorganism after affiliations of all the microorganisms [27] [28]. Our method provided information of the most dominant microorganisms preferentially to the minor one more simply and rapidly. As reliable affiliations of all the bacteria might be difficult by our method, our method might not be suitable for pure research purpose, but suitable as an inspection method due to its lower running cost and simplicity. Microchip electrophoresis system (MCE-202 MultiNA) was found suitable for this usage because 95 samples were automatically analyzed within a short time, and unculture-based method added an advantage to reduce a time required for analysis. Compared to the traditional culture based method, this method provided information of both affiliations and numbers of bacterial group at the same time; *i.e.*, we could know what kinds and how many lactic acids bacteria were included in the sample by using the method alone.

The availability as evaluation method for the other microbial groups, such as multi-drug resistant bacteria, bacteria causing food poisoning, bacteria having special functions, eukaryote, a precision of the affiliation, and validation of enumeration of each microbial group will be described in the following manuscripts.

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