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# Analyses of Bacterial Communities in Meju, a Korean Traditional Fermented Soybean Bricks, by Cultivation-Based and Pyrosequencing Methods

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**Despite the importance of meju as a raw material used to make Korean soy sauce (ganjang) and soybean paste (doenjang), little is known about the bacterial diversity of Korean meju. In this study, the bacterial communities in meju were examined using both culture-dependent and independent methods in order to evaluate the diversity of the bacterial population. Analyses of the 16S rRNA gene sequences of the bacterial strains isolated from meju samples showed that the dominant species were related to members of the genera *Bacillus*, *Enterococcus*, and *Pediococcus*. The community DNAs extracted from nine different meju samples were analyzed by barcoded pyrosequencing method targeting of the V1 to V3 hypervariable regions of the 16S rRNA gene. In total, 132,374 sequences, with an average read length of 468 bp, were assigned to several phyla, with *Firmicutes* (93.6%) representing the predominant phylum, followed by *Proteobacteria* (4.5%) and *Bacteroidetes* (0.8%). Other phyla accounted for less than 1% of the total bacterial sequences. Most of the *Firmicutes* were *Bacillu*s and lactic acid bacteria, mainly represented by members of the genera *Enterococcus*, *Lactococcus*, and *Leuconostoc*, whose ratio varied among different samples. In conclusion, this study indicated that the bacterial communities in meju were very diverse and a complex microbial consortium containing various microorganisms got involved in meju fermentation than we expected before.**

***Keywords*:** bacterial communities, cultivation-based, fermented soybean bricks, meju, pyrosequencing

Meju is a major ingredient used to prepare many Korean traditional fermented soybean foods such as soybean paste (doenjang), soy souce (ganjang), and hot pepper paste (gochujang) (Kim *et al*., 2000; Park *et al*., 2000). Meju is produced through two types of processes in Korea: Korean homemade meju for a traditional product and Japanese refined meju for a commercial product (Lee, 1995). Generally, traditional meju is prepared by soaking, steaming, and molding the soybean, then aging it for one or two months under natural environmental conditions.

Many studies were carried out to characterize and evaluate the microorganisms from meju (Lee, 1995; Lee *et al*., 1997; Yoo and Kim, 1998), to investigate the enzymes (Lee, 1995), to optimize the fermentation condition (Park and Oh, 1995), to improve the manufacturing process (Lee *et al*., 1997) or to increase the quality of meju (Kim *et al*., 2001). According to previous studies, endospore forming bacteria such as *Bacillus subtilis* and its relatives were dominant species and frequently isolated from entire part of meju, whereas fungi were found only in the surface brick of meju (Park and Kim, 1970). However, most studies about microorganisms isolated from meju have employed mainly cultivation methods and few studies have assessed the total microbial flora in fermented meju.

The culture-dependent approach is not appropriate for analyzing the total microbial population in a sample since the obtained results covered only those microorganisms that could be cultivated (Claudia *et al*., 2001). Recently, molecular methods have been widely applied such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP), random amplification of polymorphic DNA (RAPD), multiplex PCR, and pyrosequencing for the detection, identification, and characterization of microorganisms in fermented food (ben Omar and Ampe, 2000; Muller *et al*., 2000; Ampe *et al*., 2001; Cocolin *et al*., 2001; Meroth *et al*., 2003; Lafarge *et al*., 2004; Randazzo *et al*., 2005; Di Cagno *et al*., 2007; Ferchichi *et al*., 2007; Garofalo *et al*., 2008; Scheirlinck *et al*., 2008; Humblot and Guyot, 2009).

Among these methods, a next-generation sequencing technology, developed recently and also called pyrosequencing, can generate hundreds of thousands of sequences in a single run (Ronaghi *et al*., 1998; Gharizadeh *et al*., 2002). Pyrosequencing which allows over 100-fold higher throughput DNA sequencing technique makes it possible to process large numbers of samples simultaneously (Sogin *et al*., 2006). This new technique has been used to examine microbial flora of various ecological environments such as animal and human oral cavities, chronic wounds, deep mines, deep sea, soil, and tidal flats (Edwards *et al*., 2006; Sogin *et al*., 2006; Roesch *et al*., 2007; Dowd *et al*., 2008; Keijser *et al*., 2008; Kim *et al*., 2008; Chun *et al*., 2010). Recently, Roh *et al*. (2010) applied 454-pyrosequencing approach for the analysis of microbial community in the

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**Table 1.** Collection of various meju samples from different locations in Korea

| Sample | Location[a] | Shape | Weight (kg) | Size (L×W×H, cm) |
|---|---|---|---|---|
| FM01 | Yeongwolgun, Gangwondo | Rectangle | 2.0 | 18×16×10 |
| FM02 | Yeongwolgun, Gangwondo | Rectangle | 2.1 | 18.5×15×6 |
| FM03 | Gyeongsansi, Gyeongsangbukdo | Rectangle | 1.1 | 18.5×15×6 |
| FM04 | Yangpyeonggun, Gyeonggido | Cylinder | 1.0 | (φ)8× (H)6 |
| FM05 | Hadonggun, Gyeongsangnamdo | Rectangle | 1.4 | 15×5×5 |
| FM06 | Buangun, Jeollabukdo | Rectangle | 1.0 | 20×11×7 |
| FM07 | Gwangju Gwangyeoksi, Jeollanamdo | Rectangle | 1.7 | 21×14.5×6.5 |
| FM08 | Jejusi, Jejudo | Rectangle | 1.3 | 13×10×8 |
| FM09 | Jeongeupsi, Jeollabukdo | Rectangle | 1.6 | 17×15.5×7.5 |
| FM10 | Gyeongsansi, Gyeongsangbukdo | Rectangle | 1.4 | 20×15.5×5.5 |
| FM11 | Gongjusi, Chungcheongnamdo | Rectangle | 1.5 | 17×11×5.7 |
| FM12 | Cheongwongun, Chungcheongbukdo | Rectangle | 1.6 | 15.5×14×7 |
| FM13 | Gwangjusi, Gyeonggido | Rectangle | 1.9 | 17.5×20×7.5 |
| FM15 | Haenamgun, Jeollanamdo | Rectangle | 0.9 | 15×8×6 |
| FM16 | Goesangun, Chungcheongbukdo | Cylinder | 1.5 | (φ)8× (H)9 |
| FM17 | Daegu Gwangyeoksi, Gyeongsangbukdo | Rectangle | 1.0 | 16×13×6 |
| FM18 | Hapchoongun, Gyeongsangnamdo | Rectangle | 1.3 | 15×14×8 |

[a] The homemade *meju* were collected nationwide from different manufacturers in Korea. All meju samples were made by Korean traditional methods.

Korean fermented seafood, jeotgal, and they showed that the bacterial populations in the jeotgal were diverse and barcoded pyrosequencing was very promising technique for rapid and preliminary microbial characterization in food samples.

In this study, we used both cultivation-based approach and uncultivation-based approach, namely pyrosequencing method to analyze the bacterial communities in meju. The results were also compared to previous studies which were performed mainly using culture-dependent approaches.

## Materials and Methods

### Sample collection

Meju samples were collected from different regions and manufacturers in Korea (Table 1). Seventeen samples were collected, namely, three from Chungcheong province, two from Gangwon province, two from Gyeonggi province, five from Gyeongsang province, one from Jeju province, and four from Jeolla province. The sizes and shapes of the meju samples varied among different samples. Most of the shapes of the meju products were rectangular but examples of cylindrical products also existed. The weights and sizes of the collected meju products were 0.9-2.1 kg and 301-2,880 $cm^3$, respectively (Table 1). The raw material for the collected meju is locally grown soybean from the region. Meju samples were divided into two parts, an exterior rigid and dry part that retains a light brown color similar to a boiled soybean, and a soft and sticky interior part with a dark brown color (Lee, 1995). Generally, the boundary between the exterior and interior part of the meju was 2-5 cm from the surface. The samples were ground, homogenized and sieved using a 2 mm sieve, and stored at 4°C for subsequent analyses.

### Isolation of bacteria

Ten grams of meju from each sample were transferred aseptically into a 90 ml volume of sterile saline solution (0.85 g/L NaCl) and mixed thoroughly in a flask for 30 min at 150 rpm. Serial dilutions ($10^{-1}$ to $10^{-7}$) were made for each sample and 0.1 ml of the each dilution was spread onto each selective media, namely MRS (de Man *et al*., 1960), R2A (Reasoner and Geldreich, 1985), Raka-Ray (Saha *et al*., 1974), and Rogosa (Rogosa *et al*., 1951) agars. After two to five days of incubation at 28°C, morphologically representative colonies were isolated from selective media and purified twice by streaking onto other plates. Purified colonies were suspended and preserved in 15% glycerol solution at -80°C.

### Identification of bacterial isolates

Bacterial cells were lysed from colonies on each culture medium as follows: colonies were picked from each medium, suspended in 50 μl sterile deionized water, and mixed thoroughly. Subsequently, five cycles of freezing in liquid nitrogen for 1 min and thawing in a water bath at 65°C for 1 min were conducted to release DNA from bacteria. The suspensions were used as templates for PCR amplification. 16S rRNA genes of isolates were amplified using universal primers, 27f and 1492r (Lane, 1991). PCR reactions were performed in a total volume of 20 μl containing 2 μl of 10× PCR buffer, 0.1 μl of dNTP (100 mM of each), 0.16 μl of each primer (100 pmol), 1 U of Taq DNA polymerase (SolGent Co., Korea), 16.4 μl of sterile deionized water, and 1 μl of DNA template. PCR reaction was performed in a thermal cycler (TGradient; Biometra GmbH, Germany) under the following conditions: initial denaturation (95°C; 5 min), 30 cycles of denaturation (95°C; 1 min), annealing (55°C; 1 min), extension (72°C; 2 min), and a final extension (72°C; 8 min). The amplified PCR products were purified using a Montage $PCR_{96}$ Cleanup kit (Millipore Corp., USA). Partial 16S rRNA gene sequences were determined using an ABI Prism 3100 sequencer (Applied Biosystems, USA) with the 510R sequencing primer. The identification of phylogenetic neighbors and the calculation of pairwise 16S rRNA gene similarities were performed using the EzTaxon server (http://www. eztaxon.org/) (Chun *et al*., 2007).

### Community DNA extraction from meju

Community DNA was extracted from each sample using a CTAB-based method modified from Doyle and Doyle (1987). Extracted DNA was dissolved in 50 μl of sterile deionized water and stored at -20°C. The yield and purity of DNA was measured using a spec-

trophotometer (ASP-2680, Celltagen).

### PCR reactions and pyrosequencing

The hypervariable regions of V1 to V3 in the bacterial 16S rRNA gene were amplified from extracted community DNA using the following universal primers: 27f (5′-B-AC-*CCTATCCCCTGTGTGCCTTGGCAGTCTCAGACGAGTTTGATCMTGGCTCAG*-3′) and 518r (5′-B-AC-*CCATCTCATCCCTGCGTGTCTCCGACTCAGATCAGCACACWTTACCGCGGCTGCTGG*-3′), where the sequences in italics are the broad-range universal regions of 27f and 518r. In the primer sequences, "B" indicates the unique 8 nucleotides barcode used to tag each PCR product. This barcode is followed by the common linker "AC". For each sample, a 50 μl volume of PCR mixture was prepared containing 5 μl of 10× PCR buffer, 1 μl of dNTP (100 mM of each), 1 μl of each primer (50 pmol), 1 U of Taq DNA polymerase (Roche Inc.), 40.8 μl of sterile deionized water, and 1 μl of the template DNA. PCR reactions were performed using a thermocycler (MJ Research, USA) under the following conditions: initial denaturation (94°C; 5 min), 25 cycles of denaturation (94°C; 30 sec), annealing (60°C; 30 sec), extension (72°C; 1 min 20 sec), and a final extension (72°C; 8 min). The amplified products were purified using resin columns. Equal concentrations of the extracted DNAs with different sample-specific barcode sequences were pooled and subjected to pyrosequencing. Pyrosequencing was performed by Macrogen Corporation (Korea) using the 454 GS FLX Titanium Sequencing System (Roche).

### Pyrosequencing data analysis

Sequences were separated based on their barcode sequences. The barcode sequence, linker, and both forward and reverse primers were removed from the original sequences. Sequences which showed no matches (expected value $>10^{-5}$) with the 16S rRNA gene database at GenBank (Altschul *et al*., 1997) were also removed. Each sequence was assigned by comparing it with the sequences in the EzTaxon-extended database (http://www.eztaxon-e.org), using the BLASTN searches and pairwise similarity comparisons (Chun *et al*., 2007).

Analysis of bacterial community structures was performed using operational taxonomic unit (OTU)-based approaches. We used the Mothur program (ver. 1.10.0) (Schloss *et al*., 2009) to screen sequences with the following parameter options: maxhomop=8 (maximum homopolymer length), minlength=300 (minimum sequence length), and maxlength=520 (maximum sequence length) (Weon, 2010). The

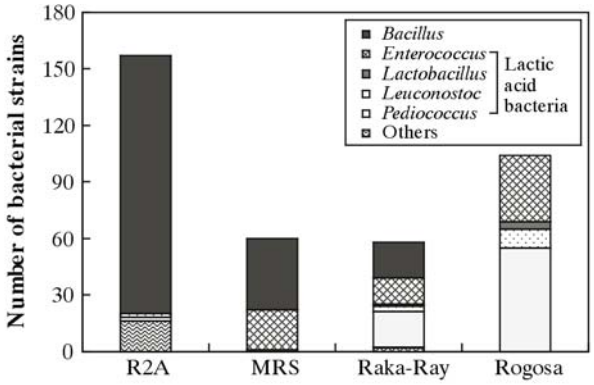


**Fig. 1.** Comparison of the bacterial flora of meju samples by different culture media revealed by 16S rRNA gene sequences. A total of 379 bacterial strains on MRS (53 isolates), R2A (178 isolates), Raka-Ray (79 isolates), and Rogosa (69 isolates) agar were isolated. Lactic acid bacteria included *Enterococcus*, *Lactobacillus*, *Leuconostoc*, and *Pediococcus* genera. Others included *Acinetobacter*, *Brevibacterium*, *Enterobacter*, *Hafnia*, *Leucobacter*, and *Staphylococcus* genera.

align.seqs command (with the parameter ksize=7) was used for aligning screened sequences using the core_set_aligned.imputed.fasta template sequences (DeSantis *et al*., 2006). Based on an alignment of multiple sequences, a distance matrix was generated using the dist.seqs command (with the options of cutoff=0.10 and cals=nogaps). The distance matrices were used to define OTUs for determining the abundance-based coverage estimator (ACE), Chao1 richness estimator (Chao and Bunge, 2002), Shannon and Simpson diversity indices, and for generating rarefaction curves and community trees. In this study, the distance cut-off in a single OTU was set to 0.03. Rarefaction curves were created by plotting the number of OTUs observed on the y-axis against the number of sequences sampled on the x-axis (Fig. 4) using the Mothur program (Magurran, 1998). The community trees showing similarities among the meju samples were also constructed using the Mothur program based on the Bray-Curtis index (Bray and Curtis, 1957). To identify OTUs shared by all samples, and between exterior and interior parts of the sample FM02, get.sharedseqs command of the Mothur program was used. The online ribosomal database project classifier (Cole *et al*., 2007) was used for taxonomic assignments of the shared OTUs in nine samples.

**Table 2.** The number and identification of bacterial strains isolated from meju samples

| Genus | FM 01 | FM 02 | FM 03 | FM 04 | FM 05 | FM 06 | FM 07 | FM 08 | FM 09 | FM 10 | FM 11 | FM 12 | FM 13 | FM 15 | FM 16 | FM 17 | FM 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Acinetobacter* | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - |
| *Bacillus* | 36 | 50 | 3 | 4 | 5 | 7 | 4 | 2 | 4 | 6 | 10 | 1 | 24 | 7 | 10 | 15 | 6 |
| *Brevibacterium* | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - |
| *Enterobacter* | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - |
| *Enterococcus* | 16 | 10 | 1 | 1 | 5 | 6 | 3 | 3 | 5 | 1 | 7 | 5 | 3 | 2 | 2 | - | - |
| *Hafnia* | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - |
| *Lactobacillus* | - | - | - | - | - | - | - | 2 | - | - | - | - | - | 2 | - | - | - |
| *Leucobacter* | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - |
| *Leuconostoc* | - | 9 | 1 | - | - | - | - | 1 | - | 1 | - | - | - | - | - | 1 | 3 |
| *Pediococcus* | - | - | 6 | 4 | 4 | 6 | 6 | - | 4 | 13 | 11 | 2 | 3 | - | 3 | 14 | - |
| *Staphylococcus* | - | 2 | - | 5 | - | - | - | 1 | - | - | 5 | - | - | - | - | - | - |
| Total strains | 52 | 71 | 11 | 14 | 14 | 21 | 13 | 11 | 14 | 22 | 33 | 8 | 30 | 11 | 15 | 30 | 9 |

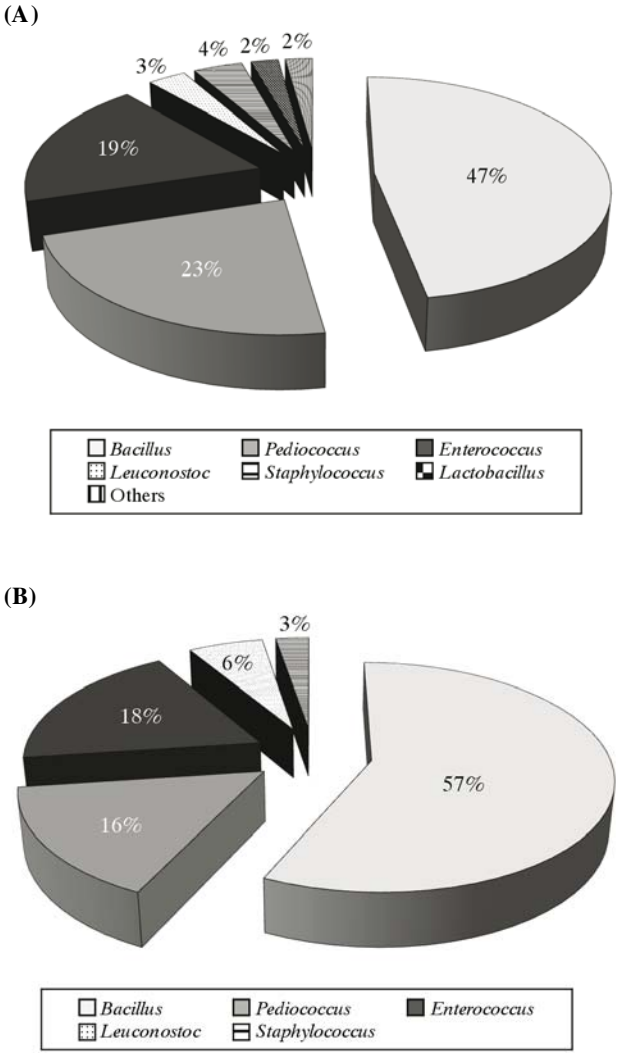


**Fig. 2.** Comparison of the bacterial flora in the exterior (A) and interior (B) parts of meju samples revealed by 16S rRNA gene sequences. A, Others included *Acinetobacter*, *Brevibacterium*, *Enterobacter*, *Hafnia*, *Leucobacter*, and *Staphylococcus* genera.

## Results

### Identification of bacterial strains isolated from meju

A total of 379 bacterial strains were isolated from the seventeen meju samples on MRS (53 isolates), Raka-Ray (79 isolates), Rogosa (69 isolates), and R2A (178 isolates) agars and were subjected to preliminary identification by using 16S rRNA gene sequencing (Fig. 1). These isolates were identified as *Bacillus* (194 isolates), *Pediococcus* (76 isolates), *Enterococcus* (70 isolates), *Leuconostoc* (16 isolates), *Staphylococcus* (13 isolates), *Lactobacillus* (5 isolates), and other genera (5 isolates) (Table 2). The total percentage of LAB genera was 44.1% (167 isolates), whereas 52.0% were from the genus *Bacillus* (194 isolates). The bacterial recovery rate and composition of isolates was similar between the exterior and interior parts of the meju (Fig. 2).

### Analysis of bacterial flora of meju by pyrosequencing

Because the community DNA extraction from meju and subsequent PCR amplification for pyrosequencing were not achieved in all seventeen meju samples, we processed nine samples with appropriate DNA size and concentration. In total, 132,374 sequences were obtained by pyrosequencing, where the average read length was 468 bp with range of 450 to 481 bp. The number of sequences per sample varied from 6,933 to 32,104 (Table 3).

The bacterial sequences with a mean relative abundance greater than 1% were assigned to four phyla, namely *Firmicutes* (93.6%), *Proteobacteria* (4.5%), *Bacteroidetes* (0.8%), and *Actinobacteria* (0.5%), with *Firmicutes* always being in highest abundance (range, 70.6-99.9%). In total, 36.6% of the sequences could be attributed to bacterial genera ($97> x \geq 94\%$, x; 16S rRNA gene sequence similarity). At the genus level, the ten most frequently found genera with a relative abundance greater than 7% were assigned in each sample (Fig. 4). The bacterial composition differed considerably depending on the sample. In sample FM01, 94.1% of the sequences were attributed to *Bacillus*, whereas only 1.5% were assigned to the same genus in sample FM05. In addition, 6,933 and 32,104 sequences of samples FM05 and FM06 were assigned to 218 and 561 genera, respectively, whereas 8,566 sequences of sample FM01 were assigned to only 23 genera.

**Table 3.** Data summary of pyrosequencing

| Sample | Total reads | Number of reads analyzed | Average length (bp) | OTUs[a] | Chao1 | ACE | Shannon | Simpson |
|---|---|---|---|---|---|---|---|---|
| FM01 | 8566 | 8196 | 460 | 2459 | 4275 | 5340 | 6.98 | 0.003 |
| FM02(E)[b] | 12858 | 12603 | 479 | 1639 | 3464 | 5241 | 4.89 | 0.041 |
| FM02(I)[c] | 12062 | 11814 | 479 | 1755 | 3665 | 5497 | 5.21 | 0.034 |
| FM04 | 7227 | 7033 | 481 | 1353 | 2806 | 4346 | 5.33 | 0.027 |
| FM05 | 6933 | 6859 | 479 | 1031 | 3015 | 5514 | 4.03 | 0.100 |
| FM06 | 32104 | 30176 | 450 | 6164 | 11281 | 15690 | 7.19 | 0.004 |
| FM13 | 10561 | 10007 | 461 | 2080 | 4284 | 6627 | 6.04 | 0.010 |
| FM15 | 19318 | 18146 | 455 | 3970 | 6557 | 8749 | 6.88 | 0.005 |
| FM16 | 22745 | 21743 | 464 | 4139 | 6816 | 8762 | 6.86 | 0.005 |

[a] The operational taxonomic units (OTUs) were defined with 3% sequence dissimilarity.
[b] FM02(E) is the exterior part of sample FM02.
[c] FM02(I) is the interior part of sample FM02.

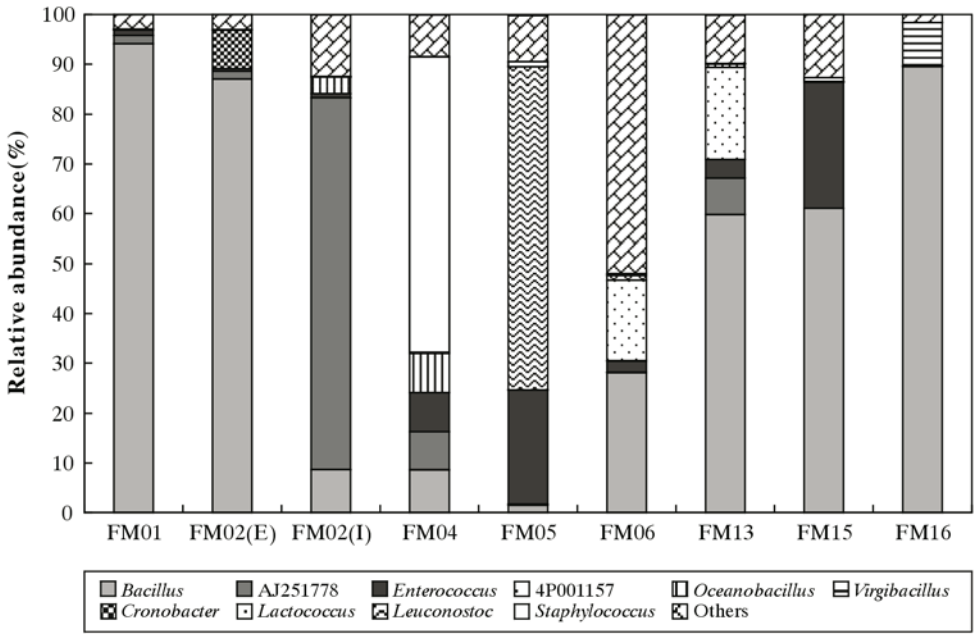


**Fig. 3.** Phylogenetic identification of bacterial pyrosequences in meju samples at the genus level. Others include the bacterial pyrosequences with a mean relative abundance less than 7%. FM02(E) and FM02(I) are the exterior and interior parts of sample FM02, respectively.

A completely different pattern of LAB population was observed depending on the sample, with three to seven LAB genera with different proportions. In particular, sample FM05 had a LAB population that was significantly different from those of other samples and 91.5% of the bacterial amplicons belonged to LAB genera. The relative abundance of LAB genera in sequences were 26.4% and 26.9% in samples FM06 and FM15, respectively, whereas LAB genera constituted 1.1% and 0.1% for the samples FM01 and FM16, respectively. We considered the sequences of FM05, FM06, and FM15 having the highest percentage of LAB group. In sample FM05, all of the LAB groups were divided into five families. Among these families, *Leuconostocaceae* (represented by the genus *Leuconostoc*) and *Enterococcaceae* (represented by the

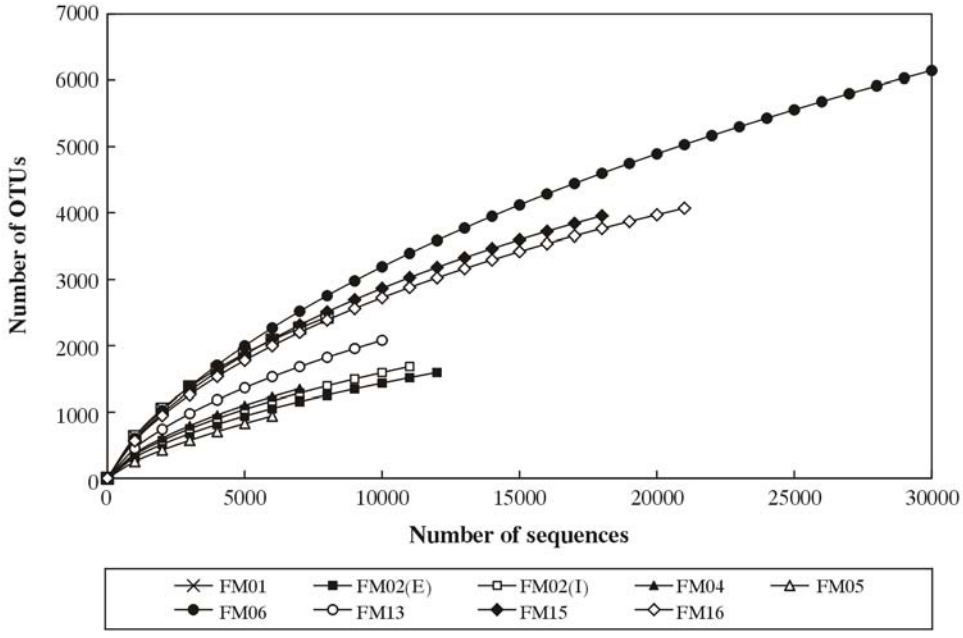


**Fig. 4.** Rarefaction analysis at the 97% sequence similarity level performed by the Mothur program.

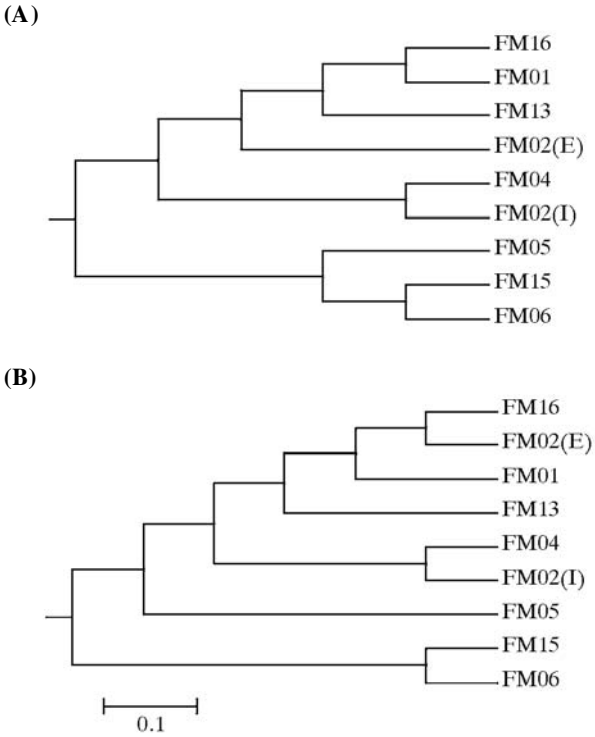


**Fig. 5.** Community trees showing the similarity of the meju samples, using distance matrices for the Yue and Clayton theta (A) and the Bray-Curtis index (B). FM02(E) and FM02(I) are the exterior and interior parts of sample FM02, respectively.

genus *Enterococcus*) accounted for 73.2% and 26.4% of the amplicons, respectively, and the remaining three families represented less than 1% of the amplicons. LAB groups of sample FM06 were divided into seven families, and 61.6% of the sequences of LAB groups could be attributed to the family *Streptococcaceae* (represented by the genera *Lactococcus* and *Streptococcus*), with only 13.0% of the family *Leuconostocaceae* (represented by the genus *Leuconostoc*). LAB groups of sample

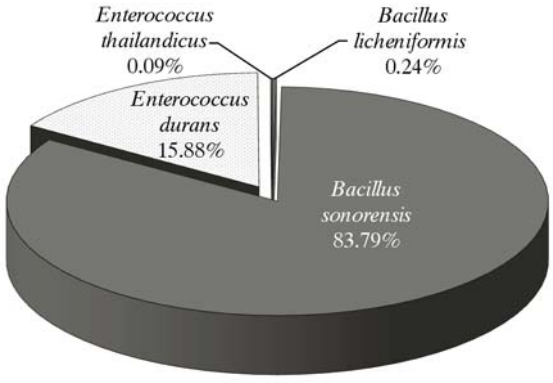


**Fig. 6.** Phylogenetic identification of shared OTUs in the nine meju samples performed by the RDP. The nine meju samples shared 26.1% (4,578 OTUs) of the total OTUs (17,555 OTUs) contained in the samples.

FM15 included seven families, and the family *Enterococcaceae* (94.8%, represented by the genus *Enterococcus*) had the largest proportion.

### Comparison of bacterial flora from different meju samples by pyrosequencing

The sequences were then analyzed using the Mothur program to identify OTUs, to generate community trees, rarefaction curves, and Venn diagrams, and to calculate various richness estimators. The rarefaction curve for sample FM06 rose more steeply than those of the other samples (Fig. 4). On the other hand, the rarefaction curve for sample FM05 gently rose and displayed more curvature toward the horizontal than the curves for other samples. The rarefaction curves of all samples did not reach an asymptote, increasing even at the highest numbers of sequences sampled. This result implied that not all phylotypes present in the bacterial communities were detected. Bacterial diversity was also the highest in sample FM06 based on the diversity indices and richness estimators, whereas samples FM04 and FM05 were less diverse (Table 3). The cluster analyses with the community trees performed using the Mothur program showed similar results between Bray-Curtis index and Yue and Clayton theta structural diversity measure (Fig. 5). Samples FM06 and FM15 occupied separated positions in the clustering analysis, as expected from their unique taxonomic composition and richness estimators. In order to elucidate the factors affecting the community clustering, surveys of manufacturing sites and fermentaion processes as well as analyses of density, pH, and water content were investigated (data not shown). No correlation was found between these factors and the community clustering.

Importantly, the nine samples shared 26.1% (4,578 OTUs) of the total OTUs (17,555 OTUs) in the samples at 3% of the dissimilarity level. The predominantly shared OTUs were for *Bacillus sonorensis* (83.8%), followed by *Enterococcus durans* (15.9%), *Bacillus licheniformis* (0.24%), and *Enterococcus thailandicus* (0.09%) (Fig. 6). Considering the proportions of these four species in each sample, *Bacillus licheniformis* and *Enterococcus thailandicus* were not considered to be important for the fermentation of meju because of their low proportions in the total number of shared OTUs. To determine how many OTUs were shared between the exterior and interior parts of sample FM02, get.sharedseqs command of the Mothur program was used. The results showed that only 9.8% OTUs (634 OTUs) of the total number of the shared OTUs (6,495 OTUs) were common to the exterior and interior parts of this sample.

## Discussion

The study of the microbial ecology of naturally fermented meju has been conducted to date mainly using cultivation-based methods, including plate counts, isolation, and biochemical identification. This approach has been criticized since it can only isolate easily culturable microorganisms, whereas members that require selective enrichment are typically not isolated. In this study, our objective was to analyze and compare the bacterial community structures of seventeen meju products made in various provinces in Korea using both culture-dependent and -independent methods.

Data obtained by cultivation-based methods showed that LAB had higher percentage (total ranged from 20% to 87.5%) than *Bacillus* species in ten of seventeen samples (Table 2). This result may be due to the fact that selective media including MRS agar were effective to isolate LAB strains.

In previous studies of the bacterial distribution in the exterior and interior parts of meju, the exterior was limited to the surface 2 to 3 cm, with the interior portion below that (Lee and Cho, 1970; Hur and Ha, 1991; Kang *et al*., 2000). In this study, however, the degree of fermentation of meju was considered when the samples were divided into two parts. The data related to the ecological aspects of bacteria between these two parts were similar (Fig. 2), which has also been seen in other samples and described by several authors (Hur and Ha, 1991; Yoo, 1998). The only difference in the bacterial distribution was the presence of a few additional genera, including *Acinetobacter*, *Brevibacterium*, *Enterobacter*, *Hafnia*, *Lactobacillus*, and *Leucobacter* in the exterior parts of meju. Their presence might be due to differences in habitat, where the exterior parts may have facilitated more diverse growth of bacteria under aerobic conditions. However, the bacterial composition differed between the exterior and interior parts of the meju, as determined by pyrosequencing. Thus, in-depth comparisons of various samples will be required for additional analyses of microbial diversity, which varies greatly among samples.

Four media used for isolation and enumeration of LAB from meju products were compared (Fig. 1). Among the media, Rogosa agar showed the best results for *Pediococcus* selection and Raka-ray agar showed the best results for *Enterococcus* growth. LAB are generally used in the food industry for several reasons. Their growth lowers the pH enough to inhibit the growth of the common microorganisms, thus providing prolonged shelf life for LAB-containing food. The acidity also changes the biochemical conversions involved in enhancing flavor. More recently, numerous LAB have been identified to have several probiotic (Goldin, 1998) roles that improve general health and well-being (Klaenhammer *et al*., 2005). Because of the important role of LAB, there have been several attempts to isolate LAB from fermented food (Hur and Ha, 1991; Lee *et al*., 2005; Lim *et al*., 2005; Lim and Im, 2009). However, there have been no studies on the isolation of various types of LAB from meju. Therefore, we propose that Raka-Ray and Rogosa agar could be used as effective media for isolating LAB from various fermented foods.

Recently, microbial communities in doenjang, a Korean fermented soybean paste made from meju, were assessed using nested PCR-DGGE (Kim *et al*., 2009, 2010). According to the studies, microbial community structures differed among samples. The bacterial DGGE profile indicated that LAB such as *Enterococcus*, *Leuconostoc*, and *Tetragenococcus* species were predominant. Meanwhile *Bacillus* species, known to be the principal microorganisms in doenjang were not dominant members in this study.

We also used barcoded pyrosequencing to analyze the microbial diversity of meju. In this study, the genus *Bacillus* was observed in every sample as expected (Fig. 3). Interestingly, we found a completely different pattern compared with that reported in previous study (Choi *et al*., 2003) in which genus *Bacillus* is the main microorganisms related to fermentation in Korean fermented soybean. In sample FM01, 94.1% of the sequences were attributed to *Bacillus*, whereas only 1.5% were assigned to the same genus in sample FM05. Given the processing conditions of traditional facilities involved in open-air fermentation, it is not surprising to find that bacterial flora varied considerably in a sample-dependent manner.

Phylogenetic identification of the pyrosequences indicated that the microbial composition of the meju samples differed considerably at the genus level, i.e., the number of genera found in each sample varied from 23 to 561 in nine different samples (Fig. 3). Although various studies of the physicochemical properties of traditional fermented food have been performed, few reports have described microbial properties and their correlation with sensory properties (taste and flavor) (Ahn *et al*., 2003; Lee *et al*., 2003; Park *et al*., 2003). Based on the study of another traditional fermented soybean product, gochujang, sensory properties had a positive correlation with the number of species, whereas no significant correlation was identified with regard to other microbial properties such as the total number of bacteria, the dominant bacteria, or the subdominant bacteria (Yoo and Kim, 1998). Therefore, the sensory scores of samples FM06 and FM15 suggested that diversity may vary considerably in the other samples.

To identify the core bacteria in meju fermentation, pyrosequences shared among all meju samples were analysed. The results showed that overlapping OTUs among the nine samples were mainly represented by *Bacillus sonorensis* and *Enterococcus darans* (Fig. 6). *Bacillus sonorensis* is Gram-positive, motile, and has a growth temperature of 15 and 55°C. A comparison of taxonomic characteristics indicates that *B. sonorensis* appears to be related most closely to *B. licheniformis*. These two species can be differentiated according to their salt tolerance. *Bacillus subtilis* grows in 5-10% NaCl, whereas *B. sonorensis* cannot grow under the same conditions (Palmisano *et al*., 2001).

*Enterococcus darans* was another major species in meju fermentation based on the shared OTUs. *E. durans* is Gram-positive, non-motile, and has a growth temperature of 10 to 45°C (Collins *et al*., 1984). Although *Enterococcus* can adapt to high saline conditions, the maximum NaCl concentration for growth was reported to be 6.5% (Fisher and Phillips, 2009). Therefore, it would be unlikely for the genus *Enterococcus* to survive in jang products which have high salt concentration more than 18% during fermentation process. Further studies on the activities of *B. sororensis* and *E. durans* in meju could be required to find out their roles during fermentation.

Consequently, this study indicated that the microbial community structures in meju were very complicated and diverse than we expected and more detailed analyses should be investigated since our results suggest that the genus *Bacillus* is not the only genus important to meju fermentation. In addition, considering abundant data from pyrosequencing analysis in meju, this technique was confirmed to be an practical tool for rapid preliminary characterization of the microbial community structures in fermented food.

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