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Bacterial community analysis during fermentation of ten representative kinds of kimchi with barcoded pyrosequencing

Eun-Jin Park [a,b,c], Jongsik Chun [d], Chang-Jun Cha [e], Wan-Soo Park [f], Che Ok Jeon [g,**], Jin-Woo Bae [a,b,*]

[a] Department of Life and Nanopharmaceutical Sciences, Kyung Hee University, Seoul 130-701, Republic of Korea
[b] Department of Biology, Kyung Hee University, Seoul 130-701, Republic of Korea
[c] Department of Food Bioengineering, Jeju National University, Jeju 690-756, Republic of Korea
[d] School of Biological Sciences and Institute of Microbiology, Seoul National University, Seoul 151-742, Republic of Korea
[e] Department of Biotechnology (BK21 Program), Chung-Ang University, Anseong 456-756, Republic of Korea
[f] World Institute of Kimchi, Seongnam 463-746, Republic of Korea
[g] Department of Life Science, Chung-Ang University, Seoul 156-756, Republic of Korea

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ABSTRACT

Kimchi, a food made of fermented vegetables, is densely populated by indigenous microorganisms that originate from the raw ingredients under normal conditions. Most microbiological studies on kimchi have been on the most popular dish, baechu-kimchi (Chinese cabbage kimchi). Therefore, relatively little is known about the various other kinds of kimchi (depending on the region, season, main ingredient, starter culture inoculation and recipe). In this study, we collected 100 samples periodically during the fermentation of ten representative kinds of kimchi (including starter-inoculated kimchi) that were stored in the refrigerator (4 °C) during the 30–35 days fermentation period. The multiplex barcoded pyrosequencing of a hypervariable V1–V3 region of the 16S ribosomal RNA (rRNA) gene tagged with sample-specific barcodes for multiplex identifiers was employed for bacterial community profiling. We found that bacterial communities differed between starter-inoculated and non-inoculated kimchi at the early stages of fermentation, but overall there were no significant differences in the late phases. Also, the diversity and richness of bacterial communities varied depending on the various types of kimchi, and these differences could largely be explained by the major ingredients and the manufacture processes of each types of kimchi. This study provides the comprehensive understanding of the factors influencing the biodiversity of the kimchi ecosystem.

1. Introduction

Fermented foods are an important constituent of the human daily diet and are consumed worldwide (Sieuwerts et al., 2008). Food fermentation can be driven by wild indigenous microorganisms (e.g., bacteria, archaea and yeast) in the raw ingredients and this influences the nutritional availability, organoleptic quality and safety of the final products (Sieuwerts et al., 2008). Since the lactic acid bacteria (LAB) play a prominent role in the food fermentation process, scientific and industrial interests in food microbiology have been focused on their ecological roles and functionalities.

Kimchi is a traditional fermented vegetable food of Korea and is now widely consumed as a side dish around the world. Although every kimchi includes various vegetables and fermented seafood products, the varieties of kimchi are determined by the main vegetable ingredients (e.g., Chinese cabbage [baechu-kimchi], radish [ggakdugi-kimchi], young radish [chonggak- or yulmoo-kimchi], green onion, mustard leaf, cucumber, garlic chive and perilla leaf) and the ratio of seasonings used (e.g., red pepper powder, salt, sugar, fermented and salted seafood [jeotgal] and starch paste [made of rice or wheat starch]). White kimchi (baek-kimchi made from Chinese cabbage) and watery kimchi (mul-kimchi made from Chinese cabbage and radish, and dongchimi made from radish) are spiced without red pepper powder. Overall, there are hundreds of varieties of kimchi, depending on seasonal ingredients and regional recipes.

A variety of studies have previously provided an opportunity for the identification of prokaryotic and eukaryotic community structures in kimchi using traditional culture-dependent and -independent methods, such as polymerase chain reaction (PCR)-

* Corresponding author. Department of Life and Nanopharmaceutical Sciences, Kyung Hee University, Seoul 130-701, Republic of Korea. Tel.: +82 2 961 2312; fax: +82 2 961 0244.
** Corresponding author. Tel.: +82 2 820 5864; fax: +82 2 821 8132.
E-mail addresses: cojeon@cau.ac.kr (C.O. Jeon), baejw@khu.ac.kr (J.-W. Bae).

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denaturing gradient gel electrophoresis (DGGE) (Chang et al., 2008), genome probing microarrays (GPM) (Nam et al., 2009) and amplified ribosomal DNA restriction analysis (ARDRA) (Kim and Chun, 2005). However, these approaches are time consuming and expensive, and do not capture unidentified organisms or offer comprehensive analysis of microbial community structures within multiple samples at one time (Ercolini, 2004). Although the previous studies have monitored the microbial communities in kimchi, there is still a scarcity of information describing uncultivable and less abundant microbial groups, as well as their relative abundance in a given environment. As already described, there are various types of kimchi with distinct tastes and microbial communities, but most of the studies have focused on the microbial community ecology of baechu-kimchi. Along with the production of commercial kimchi inoculated with starter bacteria, the role of inoculum on kimchi ecosystems and the differences with non-inoculated kimchi are also interesting.

By using more advanced sequencing techniques, such as massively parallel pyrosequencing, researchers are now elucidating microbial community structures at a much higher resolution than was previously possible. In particular, the large dataset generated by pyrosequencing without traditional cloning may help to address questions about rare microbes in the environment and to compare many microbial samples based on the phylogenetic classification of the microbes living in the environment (Kirchman et al., 2010; Liu et al., 2007). So far, this sequencing method has been used to analyze microbial communities and their dynamics from soil (Roesch et al., 2007), the deep sea (Sogin et al., 2006), deep mines (Edwards et al., 2006), tidal flats (Kim et al., 2008), human samples such as oral microflora (Keijser et al., 2008), skin (Fierer et al., 2010) and distal intestine (Claesson et al., 2009), and some fermented foods (Humblot and Guyot, 2009; Roh et al., 2010). Also, multiplex barcoded pyrosequencing that links 4–8 nucleotide barcodes to PCR primers allows the analysis of multiple samples from different microbial communities in a single run (Hamady et al., 2008; Liu et al., 2008), which reduces cost, labor and analysis time.

Generally, a batch of kimchi product processed in a container has a distinct microbial community that is influenced by environmental factors, such as the major vegetable ingredients, temperature, seasonal changes and inoculated starter microorganisms, and is considered as an individual closed ecosystem (Cho et al., 2009). Here, we report the metagenomic analysis of bacterial community dynamics with time-series samples of ten representative kimchi during the fermentation process. We characterized the bacterial diversity and community composition of ten representative kimchi and compared bacterial community structures to determine differences among the communities depending on the major ingredient and the starter inoculation during fermentation. We employed pyrosequencing of the ubiquitously hypervariable V1–V3 region of the bacterial 16S ribosomal RNA (rRNA) gene in the combination with sample-specific barcodes for the in-depth analysis of 100 samples.

2. Materials and methods

2.1. Kimchi sample preparation

Ten industrial-scale batches of kimchi samples, designated K1 to K10, were selected for the study and purchased from commercial distributors in Korea. Six types of kimchi (K1 to K6) were prepared by the Daesang F&F Corp., and four (K7 to K10) were made by CJ CheilJedang Co., Ltd., Hansung Food Co., Ltd., Sindeok Food Co., Ltd., Luchia Food Co., Ltd., respectively. Thirty bags of each kimchi types (1 kg each bag) were transported to the laboratory immediately after they were made in the factory and stored at 4 °C during the sampling period. Each of the kimchi samples comprised a main vegetable, seasonings and additional ingredients, and details were listed in Table 1.

2.2. Fermentation monitoring and sampling of kimchi

For monitoring of bacterial dynamics during kimchi fermentation, kimchi juice was periodically sampled at 0–40 days at 1–3 day intervals. At each sampling point, 200 mL of kimchi juice was sampled under aseptic condition (total of 10–20 samples per kimchi). Measurement of pH was carried out by inserting the electrode of a pH meter directly into the kimchi juice. The kimchi juice was centrifuged at 7000 rpm for 20 min at 4 °C to pellet the bacteria. The resulting pellet was transferred into a cryotube and then stored at −80 °C until further processing. For the analysis of the bacterial community composition, ten samples from each kimchi during the total fermentation periods were chosen (total 100 samples for ten types of kimchi).

2.3. Bacterial 16S rRNA gene amplification and barcoded pyrosequencing

Total bacterial genomic DNA was extracted from 1 g of each of the pellet samples using a previously described bead-beating method (Yeates et al., 1998). Extracted DNA was further purified as previously described (Bae et al., 2005). Hypervariable regions (V1–V3) of the bacterial 16S rRNA gene were amplified from bacterial DNA extracts by PCR (Ashelford et al., 2005). For each sample ($n = 100$), the composite forward primer was 5′–XXXXXXXX*ACGAGTTTGATCMTGGCTCAG*–3′, where the underlined sequence contains the barcodes consisting of 4–8 nucleotides and the sequence in italics contains the universal primer 8F. The reverse primer was 5′–XXXXXXXX*ACWTTACCGCGGCTGCTGG*–3′, where the underlined sequence contains the barcodes consisting of 4–8 nucleotides and the sequence in italics contains the universal primer 518R. The barcode sequences for the forward and reverse primers were identical. Negative controls, not containing any template, were amplified for all barcode-primer sets. The PCR conditions were as follows: 1 min at 94 °C; 18 cycles of denaturation (1 min at 94 °C), annealing (30 s at 60 °C) and extension (30 s at 72 °C); followed by the final elongation (10 min at 72 °C). The PCR products with approximately 510 nucleotides were confirmed by gel electrophoresis using 1 μL of the PCR reaction mixture in a 1.0% agarose gel (0.5X tris-acetate-ethylenediaminetetraacetic acid [TAE] buffer) and purified using the QIAquick® PCR Purification kit (Qiagen, Valencia, CA, USA). The concentration of DNA was quantified using a spectrophotometer (Nanodrop Technologies,

Table 1
Ingredients of kimchi samples (K1 to K10) analyzed in this study.

	Chinese cabbage	Young radish	Common ingredients[a]	Red pepper powder	Pear puree	Pine nut	Starter inoculum[b]
K1	•		•	•			•
K2	•		•	•			
K3	•		•		•	•	•
K4	•		•		•	•	
K5		•	•	•			•
K6		•	•	•			
K7	•		•	•			
K8	•		•	•			
K9	•		•	•			
K10		•	•	•			

[a] Common ingredients in all kimchi samples are garlic, ginger, green onion, radish, onion, starch paste, fermented seafood sauce, salt and sugar.
[b] *Leuconostoc mesenteroides* DRC 0211 [0.1% (w/w)] was inoculated as a liquid culture.

Rockland, DE, USA). The 100 samples were divided into two pools, each containing 50 samples amplified with different sample-specific barcoded primer sequences. Equal amounts of each of the 50 PCR products (0.05 μg) were pooled (total 2.5 μg) and subsequently amplified by emulsion PCR before sequencing in 1/8th of a region of the PicoTiterPlate device, as described above. Pyrosequencing was performed with the Genome Sequencer (GS) FLX Titanium (Roche-454 Life Science) using the massively parallel pyrosequencing protocol (Margulies et al., 2005) by a sequencing provider (Macrogen, Korea).

2.4. Analysis of bacterial 16S rRNA gene sequences

Sequencing reads from the different kimchi samples were separated by unique barcode sequences. Then, barcode, linker and PCR primer sequences at the 5′ end of the read were removed from the original pyrosequencing reads. All remaining sequences were subjected to a filtering process where only reads resulting PCR chimera, containing 0–1 ambiguous base (Ns) and longer than 300 base pairs on the basis of the length histogram were selected for the final bioinformatic analyses. Non-specific PCR amplicons that showed no match with the 16S rRNA gene database and chimera sequences using Hidden Markov Model (HMMER 3.0; http://hmmer.janelia.org/) and BLASTN (*E*-value $> 10^{-5}$) were also excluded from further analyses.

For taxonomic assignment of each pyrosequencing read, the sequences were compared with an extension of the EzTaxon database (http://www.eztaxon.org) (Chun et al., 2007), which provides 16S rRNA gene sequences of type strains of validly published name. In addition to the sequences of type strains, the EzTaxon-extended database (http://www.eztaxon-e.org) contains representative phylotypes of either cultured or uncultured entries in the GenBank public database with complete hierarchical taxonomic classification from phylum to species. Representative phylotypes were designated as tentative species with artificially-given specific epithets. For example, the specific epithet *Streptococcus* EU453973_s was given for the GenBank sequence entry EU453973, which plays a role as the type strain of a tentative species belonging to the genus *Streptococcus*. Similarly, tentative names for taxonomic ranks that were higher than species were also assigned where appropriate. Using this approach, the presence of species that have not yet been described can be compared across multiple bacterial community datasets (Chun et al., 2010).

Each pyrosequencing read was taxonomically assigned by comparisons against the database using a combination of initial BLASTN-based searches and pairwise similarity comparisons as described by Chun et al. (Chun et al., 2007). The following criteria were used for taxonomic assignment of each read (x = similarity): species ($x \geq 97\%$), genus ($97\% > x \geq 94\%$), family ($94\% > x \geq 90\%$), order ($90\% > x \geq 85\%$), class ($85\% > x \geq 80\%$) and phylum ($80\% > x \geq 75\%$). If the similarity was below the cutoff value, the read was assigned to an “unclassified” group.

To assess bacterial species richness estimators (Chao1, ACE and Jackknife) and diversity indices (Shannon and Simpson) and rarefaction curves, the Ribosomal RNA database project’s pyrosequencing pipeline (http://pyro.cme.msu.edu) was applied (Cole et al., 2009). The assignment of a sequence to the same group (phylotype) was determined with similarities $\geq$ 97%.

2.5. Database submission

Bacterial metagenomic sequences from ten representative kimchi are accessible in the NCBI Short Read Archive under accession number SRA029140.1.

3. Results

3.1. Sequence analysis by pyrosequencing

A total of 263493 sequence reads of 16S rRNA gene amplicons were obtained from the two 1/8 regions of the PicoTiterPlate using GS FLX Titanium chemistry on a 454 Genome sequencer. After barcode sorting, the number of sequences from each phase and kind of kimchi sample varied from 172 to 10276. For some of the samples, the sequences classified as domain *Eukarya* were identified mainly as genera *Yucca*, *Brassica* and *Solanum* belonging to phylum *Magnoliophyta*. The detection of eukaryotic sequences in kimchi was expected due to the plant ingredients used, such as Chinese cabbage, radish, red pepper powder and garlic. Since the eukaryotic 18S rRNA gene can be amplified by using bacterial 16S rRNA gene-targeting universal PCR primers, and the eukaryotic contamination might contribute to an overestimation of bacterial diversity (Huys et al., 2008), the sequences classified as domains *Archaea* and *Eukarya* were excluded from further analysis. Specifically, a total of 191194 sequences (72.6%) were identified as bacterial sequences. After pre-processing, average read length was approximately 470 bases for domain *Bacteria*. The number of bacterial sequences from each phase and kind of kimchi sample varied from 189 to 6923. The bacterial sequences identified were phylogenetically classified into phylum and genus level.

3.2. The pH changes of kimchi samples during fermentation

The changing pH patterns during fermentation (up to 35 days) of the ten types of kimchi examined are shown in Table 2. The pH levels measured immediately after production of all kimchi samples were 5.23–5.91. To more clearly compare the pH levels, not only between starter-inoculated and non-inoculated samples, but also by major vegetable ingredient, kimchi samples K1 to K6 (produced simultaneously at the same J brand) were investigated separately. We

Table 2
The pH profiles in kimchi samples during fermentation time.

K1 (days)	K2 (days)	K3 (days)	K4 (days)	K5 (days)	K6 (days)	K7 (days)	K8 (days)	K9 (days)	K10 (days)
5.50 (0)	5.23 (0)	5.90 (0)	5.89 (0)	5.91 (0)	5.90 (0)	5.64 (0)	5.70 (0)	5.65 (0)	5.48 (0)
5.50 (6)	5.43 (6)	5.85 (3)	5.80 (3)	5.87 (3)	5.87 (3)	5.43 (3)	4.75 (3)	4.45 (3)	4.40 (3)
5.43 (9)	5.37 (10)	5.65 (6)	5.40 (6)	5.85 (6)	5.80 (6)	5.35 (6)	4.40 (6)	4.35 (6)	4.35 (6)
5.38 (12)	5.44 (14)	4.80 (9)	4.36 (9)	5.65 (9)	5.00 (9)	5.21 (9)	4.47 (9)	4.37 (9)	4.38 (9)
5.20 (15)	5.45 (17)	4.55 (12)	4.33 (12)	5.35 (12)	4.80 (12)	5.09 (12)	4.55 (12)	4.38 (12)	4.38 (12)
4.95 (17)	5.40 (20)	4.45 (15)	4.30 (15)	5.00 (15)	4.70 (15)	4.81 (15)	4.50 (15)	4.23 (15)	4.47 (15)
4.88 (20)	4.80 (22)	4.25 (18)	4.25 (18)	4.85 (18)	4.66 (18)	4.68 (16)	4.35 (18)	4.27 (18)	4.32 (18)
4.58 (24)	4.44 (25)	4.24 (21)	4.24 (21)	4.51 (21)	4.52 (21)	4.59 (17)	4.30 (21)	4.24 (21)	4.30 (21)
4.51 (26)	4.47 (29)	4.27 (24)	4.25 (24)	4.50 (24)	4.50 (24)	4.50 (19)	4.45 (24)	4.28 (24)	4.31 (24)
4.48 (28)	4.40 (35)	4.24 (27)	4.24 (27)	4.50 (27)	4.47 (27)	4.37 (26)	4.47 (27)	4.30 (27)	4.40 (27)

observed a more rapid decrease of pH in the non-inoculated baek-kimchi (K4) and chonggak-kimchi (K6) than in the starter-inoculated equivalents, K3 and K5, respectively. By contrast, in the baechu-kimchi samples, the pH of the starter-inoculated sample (K1) decreased more rapidly than the non-inoculated K2. After 9 days of fermentation, the pH level in baek-kimchi was lower (4.38–4.80) than that of baechu- and chonggak-kimchi (5.00–5.65). Therefore, the fermentation process of baek-kimchi (spiced without red pepper powder or fermented seafood sauce) was faster than the other types of kimchi.

Another baechu-kimchi (K7) had a similar pH profile to the baechu-kimchi (K1 and K2) presented above. However, samples K8 (baechu-kimchi), K9 (cut baechu-kimchi) and K10 (chonggak-kimchi) exhibited sharp decreases in pH immediately after production. After 3 days of fermentation, the pH levels had decreased to around 4.5 in these three samples, and this was maintained until the late stages of the fermentation. There were no apparent effects by the shape (whole or cut) of Chinese cabbage on pH profiles. In all the kimchi samples, the decreased pH levels (4.2–4.5) were maintained until the end of the fermentation period.

3.3. Diversity of bacterial communities in ten types of kimchi

To analyze the community richness in the ten types of kimchi, we calculated rarefaction curves at 97% similarity levels (Figure S1), and diversity indices (Shannon and Simpson) and richness estimators (Chao1, ACE and Jackknife) (Tables S1–S5). All rarefaction curves showed that the numbers of phylotypes (operational taxonomic units [OTUs]) decreased during fermentation (Figure S1). Phylotypes ranging from 10 to 104 OTUs were identified in all samples. The species richness according to the rarefaction curves was supported by statistical diversity estimates, such as Shannon, ACE, Chao1, Jacknife and Simpson. The Chao1 richness and Shannon diversity indices for clusters sharing >97% similarity ranged mainly from 13 to 183 and from 1.74 to 4.11, respectively. Significant differences in the richness levels between the kimchi fermented with starter culture (K1, K3 and K5) and indigenous microflora (K2, K4 and K6) were not found.

According to the Shannon index quantifying biodiversity on the basis of richness and evenness, there were no significant differences in the bacterial communities between starter-inoculated (K3) and non-inoculated baek-kimchi (K4) during the overall fermentation process. In contrast, the overall bacterial communities in starter-inoculated baechu- (K1) and chonggak-kimchi (K5) were less diverse than those of the non-inoculated kimchi samples, K2 and K6, respectively. In the K1 to K6 samples, the bacterial diversity, which decreased gradually during the fermentation process, was analogous to the changing pH value. However, in another baechu-kimchi sample (K7), with a comparable pH pattern to samples K1 to K6, the Shannon index did not change significantly during the overall fermentation process. The samples K8 (baechu-kimchi), K9 (cut baechu-kimchi) and K10 (chonggak-kimchi), which displayed rapid decreases in pH during the initial 3 days following production but then maintained these levels.

3.4. Phylogenetic composition of bacterial communities in ten types of kimchi

The phylogenetic classification of bacterial sequences from the ten kinds of kimchi is presented in Figs. 1 and 2. Figs. 1 and 2 shows

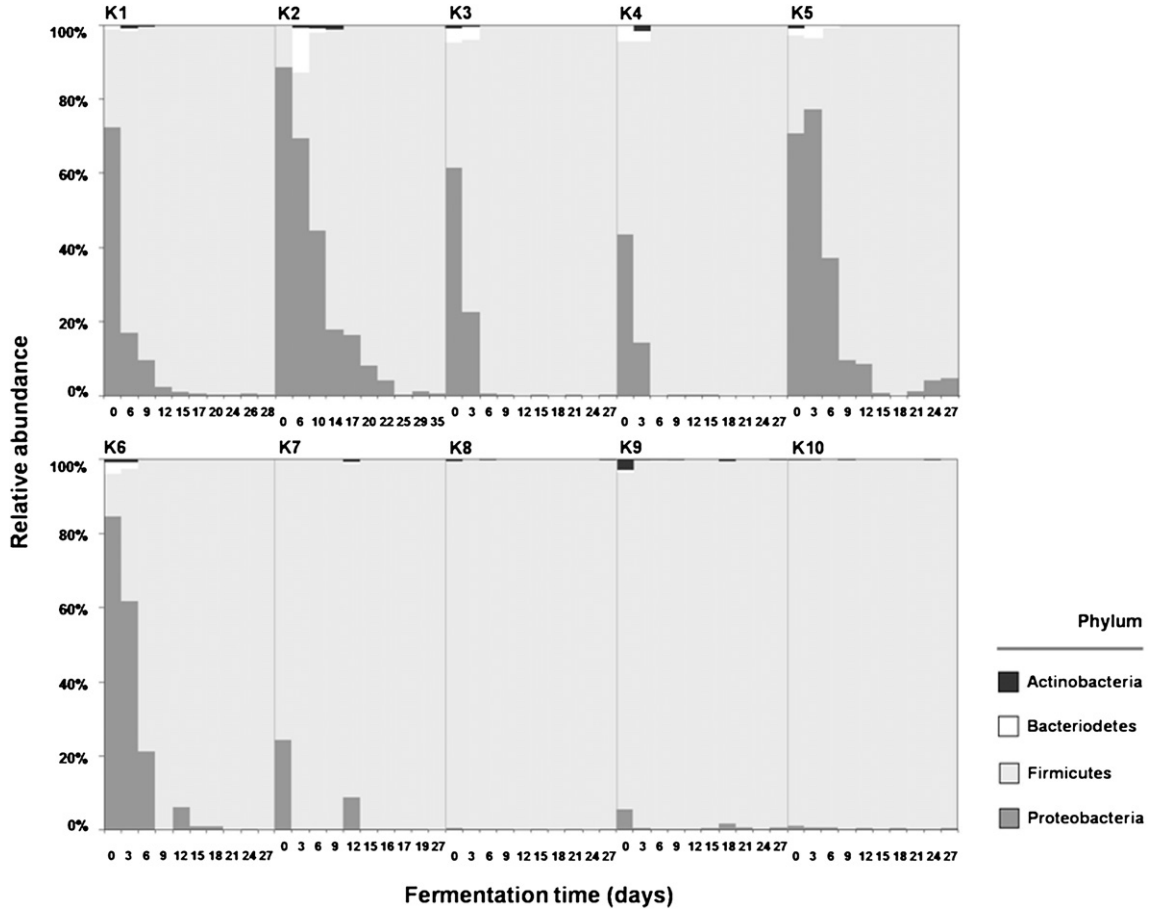

Fig. 1. Relative abundance of bacterial phyla in the kimchi samples (K1 to K10) during the fermentation periods.

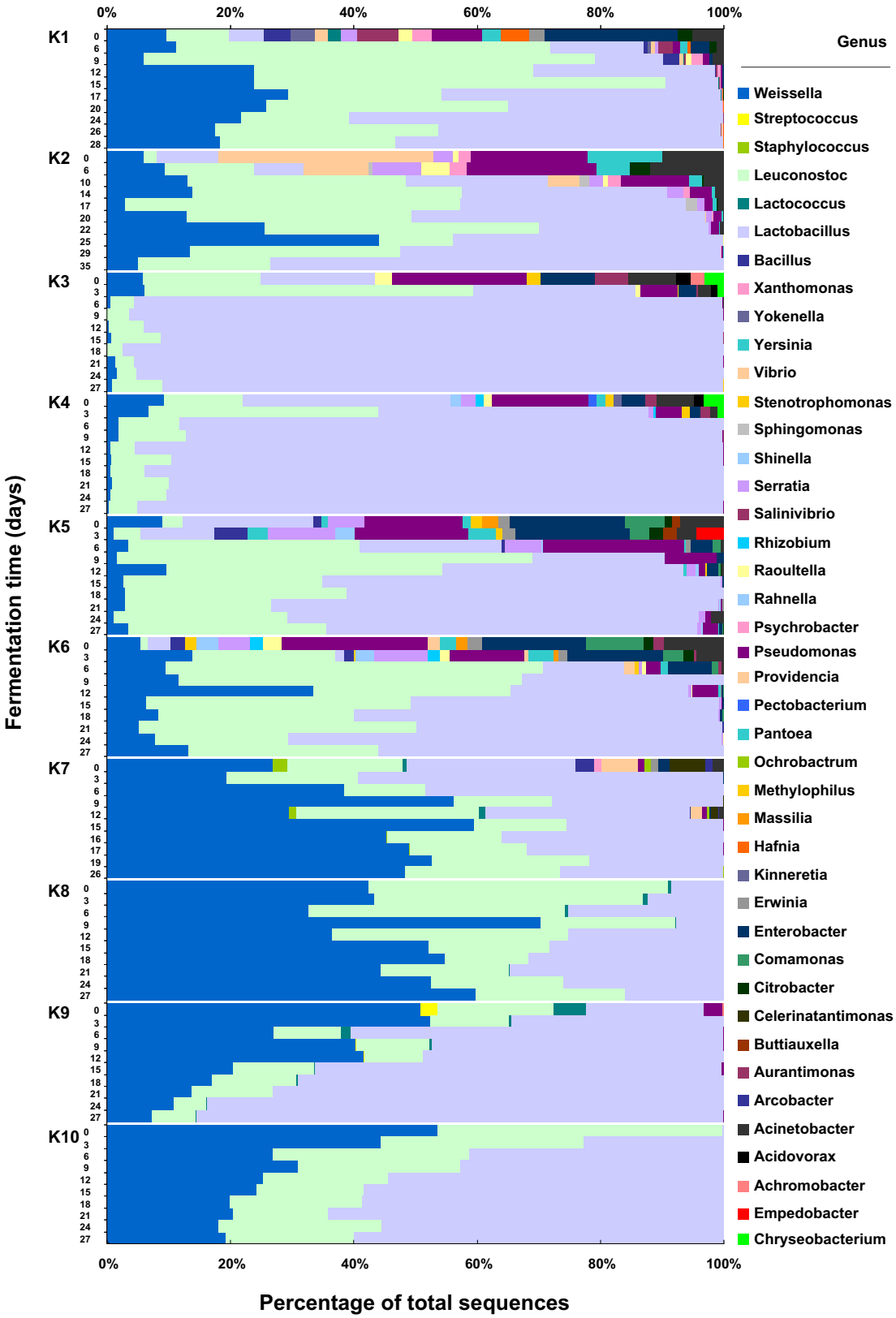

Fig. 2. Relative abundance of bacterial genera (>1% of the total sequences) obtained from pyrosequencing analysis in kimchi samples K1 to K10.

the relative phylum and genus abundances (>1.0%), respectively. In all the kinds of kimchi samples including starter-inoculated and non-inoculated, the vast majority (>99%) of the annotated reads were assigned to four bacterial phyla (*Actinobacteria*, *Bacteriodetes*, *Firmicutes* and *Proteobacteria*) (Fig. 1). At the early phase of fermentation (0 or 1 days), the phylum *Proteobacteria* was dominant in K1 (72.5%), K2 (88.7%), K3 (61.3%), K5 (70.1%) and K6 (84.7%), and the relative abundance was maintained until 6 days.

Thereafter, the *Firmicutes* became the predominant phylum until the end of the fermentation period (27–28 days). However, in samples K4 and K7 to K10, phylum *Firmicutes* was dominant in all fermentation processing.

Of the samples fermented with *L. mesenteroides* DRC 0211 as starter inoculum (K1, K3 and K5), the relative abundance of the genus *Leuconostoc* in K1 (starter-inoculated baechu-kimchi) and K3 (starter-inoculated baek-kimchi) was significantly higher than in the K2 and K4 samples (non-inoculated kimchi), respectively (Fig. 2). However, there was no significant difference in the relative abundance of the genus *Leuconostoc* between K5 (starter-inoculated chonggak-kimchi) and K6 (non-inoculated chonggak-kimchi).

At the early phase of fermentation (time 0–5 days), bacterial communities in samples K1 to K6 were more diverse than in later samples, which were composed mainly of Lactobacillales, related to the LAB. Genera *Enterobacter*, *Vibrio* and *Pseudomonas* belonging to the phylum *Proteobacteria* were also identified in K1 to K6 samples during the early phase of fermentation (Fig. 2). Genus *Enterobacter* (17.8%) was predominant in sample K1 at time 0, and genera *Vibrio* (16.5%) and *Pseudomonas* (10.5%) were predominant in K2 samples at 1 day and 5 days, respectively. Genus *Pseudomonas* comprised 16.6% and 14.3% of samples K3 and K4 at 0 days of fermentation, respectively.

In sample K5, genera *Lactobacillus* (15.2% and 10.0%), *Enterobacter* (13.4% and 15.5%) and *Pseudomonas* (11.5% and 15.5%) were the major components at time 0 and 3 days, respectively. At 6 days, genus *Pseudomonas* was detected at 21.9% relative abundance. Similarly, the sequences of 20.2% and 14.3% were identified as genera *Pseudomonas* (20.2%) and *Enterobacter* (14.3%) at time 0 of fermentation in sample K6. However, the dominant genera in samples K7 to K10 throughout the fermentation period were *Lactobacillus*, *Leuconostoc* and *Weisella* which are belonging to the order Lactobacillales (68.7–98.1%). The relative abundance of genera *Leuconostoc* and *Lactobacillus* was remarkably similar to sample K1 to K6. Genera *Leuconostoc* and *Lactobacillus* were predominant at the early and late stages of fermentation, respectively.

In samples K1 (starter-inoculated baechu-kimchi) and K2 (non-inoculated baechu-kimchi), genus *Leuconostoc* increased until 9–12 days and then decreased. Genus *Weisella* was detected evenly during the fermentation periods but predominantly at 17 days (K1) and 15 days (K2). Genus *Lactobacillus* was represented toward the end of periods in K1 (60.0% at 24 days) and K2 (73.3% at 18 days). Genus *Leuconostoc* was representative at the beginning of fermentation (3 days) in the K3 (starter-inoculated baek-kimchi) (45.3%) and K4 (non-inoculated baek-kimchi) (34.4%) samples. At the same time, genus *Lactobacillus* increased, representing 95.1% (K3) and 87.1% (K4) at 6 days, and accounting for up to 97.0% until the end of fermentation.

The relative abundance of genera *Leuconostoc* and *Lactobacillus* was similar in samples K5 (starter-inoculated chonggak-kimchi) and K6 (non-inoculated chonggaki-kimchi). The genus *Leuconostoc* dominated at 9 days in K5 (66.8%) and at 6 days in K6 (56.1%), but then genus *Lactobacillus* increased, to 72.4% in K5 (at 21 days) and 70.3% in K6 (at 24 days). In sample K7 (baechu-kimchi), genera *Weisella* and *Lactobacillus* were identified at the early phases of fermentation, whist genus *Lactobacillus* was dominant at the end of fermentation. The highest abundances of genera *Lactobacillus* and *Leuconostoc* were 58.9% (3 days) and 25.4% (19 days), respectively, in sample K7. The genus *Weisella* was predominant throughout the fermentation time in K8 (baechu-kimchi), and the genera *Leuconostoc* and *Lactobacillus* represented 47.7% and 34.5% at time 0 and 21 days, respectively. In samples K9 (cut baechu-kimchi) and K10 (chonggak-kimchi), the genera *Weisella* and *Leuconostoc* were dominant at the early stages of fermentation (time 0–3 days), but these then decreased. The genus *Lactobacillus* was predominant throughout the late stage of fermentation. At the end of the sampling time, the genus *Lactobacillus* represented 83.1% (27 days) and 63.8% (21 days) in the K9 and K10 samples, respectively.

4. Discussion

4.1. Application of barcoded pyrosequencing to bacterial community dynamics in kimchi fermentation

Scientific understanding of microbial community composition is particularly poor for food ecosystems, even though the diversity and composition of microbial and viral communities in foods are thought to have a direct influence on the gut microbial consortia in humans (Zhang et al., 2006; Hehemann et al., 2010). Only few of fermented foods have been used for recent microbial ecological studies of metagenomes by using high-throughput sequencing (Humblot and Guyot, 2009; Roh et al., 2010). Metagenomic approaches give a fascinating opportunity to comprehensively analyze uncultured microbial communities of environmental samples, including foods. In this study, we have presented a detailed survey of the phylogenetic and comparative diversities and community structure of members of the fermentative bacteria during kimchi fermentation, thus demonstrating the power of this method. To our knowledge, this represents the first wide-scale culture-independent sequence analysis of bacteria involved in vegetable food fermentation. The resulting pictures of metagenomic bacterial communities will provide a deeper understanding of kimchi ecosystems from the early to late stages of the fermentation.

Although the average length of sequence reads (400 bases) obtained were significantly shorter than the full-length sequences of a highly variable region of the 16S rRNA in bacteria and archaea, several studies have previously demonstrated that short sequence lengths (100–400 bases for the pyrosequencing instruments) are sufficient to capture the same pattern as nearly full-length sequences (Humblot and Guyot, 2009; Liu et al., 2007; Roh et al., 2010). Moreover, the barcoded 16S rRNA amplicons from multiple samples can be analyzed in parallel and provide an overall view of kimchi community profiling, thereby allowing both faster and cheaper analysis than was previously possible.

4.2. Bacterial diversity comparison of ten representative kinds of kimchi

Rarefaction curves generated from our data indicate that the phylotype richness in the sequenced samples is decreased during fermentation (Tables S1–S5), reflecting the bacterial community at the early stage of fermentation was converged onto the dominance of lactic acid bacteria such as genera *Leuconostoc*, *Lactobacillus* and *Weisella* during kimchi fermentation. Diversity of the bacterial community in kimchi, as reflected by the Shannon index, decreased from 4.11 to 1.74 during the fermentation progress. This is a higher Shannon index than was observed in fermented seafood (1.22–1.97, depending on the variety) (Roh et al., 2010). Hence, the pH profile during fermentation appears to be related to the Shannon diversity index. The scores were highest in the early stage of fermentation and lowest in the late stage in samples K1 to K6, while there were no significant differences for samples K7 to K10 during fermentation. It could also be because the genera *Leuconostoc* and *Weisella*, belonging to the order Lactobacillales, were predominant in samples K7 to K10, and the composition of the bacterial community in them was relatively simple compared to K1 to K6 at the initial stage (Fig. 2). As can be seen from the relationships between pH and bacterial community composition in samples K1 to K6, most of the bacterial diversities at 6 or 9 days fermentation were relatively

simple, even though the pH levels were consistent with initial levels. This indicates that the initial bacterial communities of the samples K7 to K10 were seemed to be in progressive status of fermentation with the corresponding period of 6–9 days in samples K1 to K6. One hypothesis is that changes in bacterial community structure are a result of the long period of time at room temperature (22 ± 2 °C) during the preparation of ingredients and production stages, which induces pre-fermentation.

Kimchi pH not only reflected the bacterial diversity, it also predicted the overall community composition during the fermentation process (Table 2, Tables S1–S5, Figs. 1 and 2). The observed patterns of kimchi pH are likely to be predictor of bacterial community composition. It has also been observed that the soil bacterial diversities, as estimated by species richness and diversity, were strongly correlated with pH values <8.5 in soil (Fierer and Jackson, 2006). Since kimchi with pH values 8.5< are rare, the bacterial richness and diversity of kimchi are likely to be dependent on the levels of pH due to the excessive growth of LAB regardless of the major ingredient and the starter inoculation.

4.3. Comparison of bacterial community compositions in ten representive kinds of kimchi

Given that the ingredients of kimchi are raw vegetables that have been grown in soil and fermented seafood products, it is not surprising that the various environmental microorganisms are present even during the early stages of the fermentation, despite adequate washing of the raw ingredients. The dominant microbial groups shifted sequentially from less acid tolerant at the early stages of fermentation (up to 2–3 days) to more acid tolerant LAB groups adapted to the acidic environmental condition of kimchi (Fierer and Jackson, 2006; Plengvidhya et al., 2007) observed that the bacterial communities within ecosystems corresponded to the measured pH; therefore, the relatively low pH of the sample may predict a low microbial diversity. The quantity of total bacterial populations are consistently maintained during fermentation (Park et al., 2009), even though the pH and bacterial diversity decreased (Bae et al., 2005; Chang et al., 2008). Moreover, we showed that the highest similarity of the genus abundance between kimchi samples occurred after the middle stage of fermentation (12–15 days), which indicates a highly stable microbial community in the kimchi ecosystem. The predominant LAB throughout the fermentation was similar to that reported earlier: the genera *Weisella*, *Leuconostoc*, *Lactobacillus* and *Pediococcus* (Bae et al., 2005; Kim and Chun, 2005; Nam et al., 2009).

In kimchi samples fermented with starter culture *L. mesenteroides* DRC 0211 (K1, K3 and K5; Fig. 2), the genus *Leuconostoc* was highly abundant at the early stage of fermentation, thereby, bringing relatively simple bacterial complexity. Through the application of the starter cultures, we expected a reduction in the unexpected microbial population present in the raw vegetables.

As expected, the bacterial community of baek-kimchi (K3 and K4 in Fig. 2) made of few ingredients was less diverse than those of baechu-kimchi (K1 and K2) and chonggak-kimchi (K5 and K6) at the early stage of fermentation. This is in accordance with the major ingredients, red pepper powder and fermented seafood product, which act as major microbial sources in the kimchi ecosystem. The salinity is also a crucial factor in determining the rate of fermentation. The relatively low salinity of baek-kimchi (approximately 1–1.5%) compared with baechu-kimchi and chonggak-kimchi (2.5–3%) may influence its rapid progress. Among the same varieties of kimchi made of different components, baechu-kimchi (samples K2, K7 and K8) and chonggak-kimchi (samples K6 and K10), there were significant differences in the community profiles at the early stage (Fig. 2). This may also be due to differences in the washing methods for the raw vegetables, the proportions of the ingredients and the temperature of the workplace. The shape of Chinese cabbage, whole (samples K7 and 8) or cut (sample K9), does not seem to be crucial factor in bacterial abundance. Overall, after the transition time between the heterofermentation and homofermentation stages (7–10 days fermentation), all of the bacterial communities were affiliated with the genera *Leuconostoc*, *Lactobacillus* and *Weisella*, and were stable throughout the following periods.

The variations in bacterial community structures that occur on the variety of kimchi, including the ordering of abundant and rare phylotypes, have implications for understanding how bacterial community compositions in kimchi ecosystem are affected by environmental factors, such as major ingredients, manufacturing process and storage temperature. We used both starter-inoculated and non-inoculated kimchi samples to examine the influence of starter culture on pH profile and bacterial community composition. In many of previous studies focused on the microbial community ecology of kimchi, starter-inoculated kimchi was employed as a representative kimchi for the investigation of bacterial community, and thus the comparative study of bacterial community between starter-inoculated and natural fermented kimchi is required. Consequently, we have shown that the disturbance of bacterial composition in early fermentation time may be caused by the environmental factors such as temperature of working place, major vegetable ingredient and seasoning, although the most abundant microbial genera display and persist of their relative abundance after that. The integration of bacterial ecology into the field of research concerning the kimchi ecosystem is likely to provide a more comprehensive understanding of the factors controlling the biodiversity of kimchi.

In this study, high-throughput barcoded pyrosequencing has been used to examine questions about the bacterial community profiles of kimchi ecosystems. Our results evaluated with high-throughput sequencing technique appear to be partly consistent with the previously reported observation based on the classical culture-dependent analysis that several species of *Welsella*, *Leuconostoc* and *Lactobacillus* belonging to the order Lactobacillales are predominant in kimchi fermentation. However, bacterial populations in the early stage of fermentation are more diverse in several kimchi samples than we expected. In addition, this modern pyrosequencing tecnhology provide differences in relatively population quantities between phylogenetically nearest microorganisms. This is the most extensive sequencing to identify even rare phylotypes in a wide variety of kimchi during fermentation periods reported to date. The results from this study elucidate, for the first time, the temporal scales of bacterial communities in commercial kimchi fermentations. Metagenomic analysis of bacterial community in kimchi improves the understanding of bacterial ecology in the kimchi limited in the classical knowledge of the kimchi ecosystem.

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fm.2011.10.011.

References

Ashelford, K.E., Chuzhanova, N.A., Fry, J.C., Jones, A.J., Weightman, A.J., 2005. At least 1 in 20 16S rRNA sequence records currently held in public repositories is

estimated to contain substantial anomalies. Applied and Environmental Microbiology 71, 7724–7736.
Bae, J.W., Rhee, S.K., Park, J.R., Chung, W.H., Nam, Y.D., Lee, I., Kim, H., Park, Y.H., 2005. Development and evaluation of genome-probing microarrays for monitoring lactic acid bacteria. Applied and Environmental Microbiology 71, 8825–8835.
Chang, H.W., Kim, K.H., Nam, Y.D., Roh, S.W., Kim, M.S., Jeon, C.O., Oh, H.M., Bae, J.W., 2008. Analysis of yeast and archaeal population dynamics in kimchi using denaturing gradient gel electrophoresis. International Journal of Food Microbiology 126, 159–166.
Cho, K.M., Math, R.K., Asraful Islam, S.M., Lim, W.J., Hong, S.Y., Kim, J.M., Yun, M.G., Cho, J.J., Yun, H.D., 2009. Novel multiplex PCR for the detection of lactic acid bacteria during kimchi fermentation. Molecular and Cellular Probes 23, 90–94.
Chun, J., Lee, J.H., Jung, Y., Kim, M., Kim, S., Kim, B.K., Lim, Y.W., 2007. EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. International Journal of Systematic and Evolutionary Microbiology 57, 2259–2261.
Chun, J., Kim, K.Y., Lee, J.H., Choi, Y., 2010. The analysis of oral microbial communities of wild-type and toll-like receptor 2-deficient mice using a 454 GS FLX Titanium pyrosequencer. BMC Microbiology.
Claesson, M.J., O'Sullivan, O., Wang, Q., Nikkilä, J., Marchesi, J.R., Smidt, H., de Vos, W.M., Ross, R.P., O'Toole, P.W., 2009. Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. PLoS ONE 4.
Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M., Tiedje, J.M., 2009. The ribosomal database project: improved alignments and new tools for rRNA analysis. Nucleic Acids Research 37, D141–D145.
Edwards, R.A., Rodriguez-Brito, B., Wegley, L., Haynes, M., Breitbart, M., Peterson, D.M., Saar, M.O., Alexander, S., Alexander Jr., E.C., Rohwer, F., 2006. Using pyrosequencing to shed light on deep mine microbial ecology. BMC Genomics 7.
Ercolini, D., 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. Journal of Microbiological Methods 56, 297–314.
Fierer, N., Jackson, R.B., 2006. The diversity and biogeography of soil bacterial communities. Proceedings of the National Academy of Sciences of the United States of America 103, 626–631.
Fierer, N., Lauber, C.L., Zhou, N., McDonald, D., Costello, E.K., Knight, R., 2010. Forensic identification using skin bacterial communities. Proceedings of the National Academy of Sciences of the United States of America 107, 6477–6481.
Hamady, M., Walker, J.J., Harris, J.K., Gold, N.J., Knight, R., 2008. Error-correcting barcoded primers for pyrosequencing hundreds of samples in multiplex. Nature Methods 5, 235–237.
Hehemann, J.H., Correc, G., Barbeyron, T., Helbert, W., Czjzek, M., Michel, G., 2010. Transfer of carbohydrate-active enzymes from marine bacteria to Japanese gut microbiota. Nature 464, 908–912.
Humblot, C., Guyot, J.P., 2009. Pyrosequencing of tagged 16S rRNA gene amplicons for rapid deciphering of the microbiomes of fermented foods such as pearl millet slurries. Applied and Environmental Microbiology 75, 4354–4361.
Huys, G., Vanhoutte, T., Joossens, M., Mahious, A.S., De Brandt, E., Vermeire, S., Swings, J., 2008. Coamplification of eukaryotic DNA with 16S rRNA gene-based PCR primers: possible consequences for population fingerprinting of complex microbial communities. Current Microbiology 56, 553–557.
Keijser, B.J.F., Zaura, E., Huse, S.M., Van Der Vossen, J.M.B.M., Schuren, F.H.J., Montijn, R.C., Ten Gate, J.M., Crielaard, W., 2008. Pyrosequencinq analysis of the oral microflora of healthy adults. Journal of Dental Research 87, 1016–1020.
Kim, B.S., Kim, B.K., Lee, J.H., Kim, M., Lim, Y.W., Chun, J., 2008. Rapid phylogenetic dissection of prokaryotic community structure in tidal flat using pyrosequencing. Journal of Microbiology 46, 357–363.
Kim, M., Chun, J., 2005. Bacterial community structure in kimchi, a Korean fermented vegetable food, as revealed by 16S rRNA gene analysis. International Journal of Food Microbiology 103, 91–96.
Kirchman, D.L., Cottrell, M.T., Lovejoy, C., 2010. The structure of bacterial communities in the western Arctic Ocean as revealed by pyrosequencing of 16S rRNA genes. Environmental Microbiology 12, 1132–1143.
Liu, Z., Lozupone, C., Hamady, M., Bushman, F.D., Knight, R., 2007. Short pyrosequencing reads suffice for accurate microbial community analysis. Nucleic Acids Research 35.
Liu, Z., DeSantis, T.Z., Andersen, G.L., Knight, R., 2008. Accurate taxonomy assignments from 16S rRNA sequences produced by highly parallel pyrosequencers. Nucleic Acids Research 36, e120.
Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A., Berka, J., Braverman, M.S., Chen, Y.J., Chen, Z., Dewell, S.B., Du, L., Fierro, J.M., Gomes, X.V., Godwin, B.C., He, W., Helgesen, S., Ho, C.H., Irzyk, G.P., Jando, S.C., Alenquer, M.L.I., Jarvie, T.P., Jirage, K.B., Kim, J.B., Knight, J.R., Lanza, J.R., Leamon, J.H., Lefkowitz, S.M., Lei, M., Li, J., Lohman, K.L., Lu, H., Makhijani, V.B., McDade, K.E., McKenna, M.P., Myers, E.W., Nickerson, E., Nobile, J.R., Plant, R., Puc, B.P., Ronan, M.T., Roth, G.T., Sarkis, G.J., Simons, J.F., Simpson, J.W., Srinivasan, M., Tartaro, K.R., Tomasz, A., Vogt, K.A., Volkmer, G.A., Wang, S.H., Wang, Y., Weiner, M.P., Yu, P., Begley, R.F., Rothberg, J.M., 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature 437, 376–380.
Nam, Y.D., Chang, H.W., Kim, K.H., Roh, S.W., Bae, J.W., 2009. Metatranscriptome analysis of lactic acid bacteria during kimchi fermentation with genome-probing microarrays. International Journal of Food Microbiology 130, 140–146.
Park, E.J., Chang, H.W., Kim, K.H., Nam, Y.D., Roh, S.W., Bae, J.W., 2009. Application of quantitative real-time PCR for enumeration of total bacterial, archaeal, and yeast populations in kimchi. Journal of Microbiology 47, 682–685.
Plengvidhya, V., Breidt Jr., F., Lu, Z., Fleming, H.P., 2007. DNA fingerprinting of lactic acid bacteria in sauerkraut fermentations. Applied and Environmental Microbiology 73, 7697–7702.
Roesch, L.F.W., Fulthorpe, R.R., Riva, A., Casella, G., Hadwin, A.K.M., Kent, A.D., Daroub, S.H., Camargo, F.A.O., Farmerie, W.G., Triplett, E.W., 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. ISME Journal 1, 283–290.
Roh, S.W., Kim, K.H., Nam, Y.D., Chang, H.W., Park, E.J., Bae, J.W., 2010. Investigation of archaeal and bacterial diversity in fermented seafood using barcoded pyrosequencing. ISME Journal 4, 1–16.
Sieuwerts, S., de Bok, F.A., Hugenholtz, J., van Hylckama Vlieg, J.E., 2008. Unraveling microbial interactions in food fermentations: from classical to genomics approaches. Applied and Environmental Microbiology 74, 4997–5007.
Sogin, M.L., Morrison, H.G., Huber, J.A., Welch, D.M., Huse, S.M., Neal, P.R., Arrieta, J.M., Herndl, G.J., 2006. Microbial diversity in the deep sea and the underexplored "rare biosphere". Proceedings of the National Academy of Sciences of the United States of America 103, 12115–12120.
Yeates, C., Gillings, M.R., Davison, A.D., Altavilla, N., Veal, D.A., 1998. Methods for microbial DNA extraction from soil for PCR amplification. Biological Procedures Online 1, 40–47.
Zhang, T., Breitbart, M., Lee, W.H., Run, J.Q., Wei, C.L., Soh, S.W.L., Hibberd, M.L., Liu, E.T., Rohwer, F., Ruan, Y., 2006. RNA viral community in human feces: prevalence of plant pathogenic viruses. PLoS Biology 4, 0108–0118.