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Bacterial community structure in kimchi, a Korean fermented vegetable food, as revealed by 16S rRNA gene analysis

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Abstract

Kimchi is a traditional Korean food fermented from a variety of vegetables. We elucidated the microbial community structure of five commercially produced kimchis made from Chinese cabbage by examining culture-independent 16S rRNA gene clone libraries. Most of the clones (347 out of 348) belonged to lactic acid bacteria and included several species of the genera *Lactobacillus*, *Leuconostoc* and *Weissella*. *Weissella koreensis* was found in all the samples and predominated in three of them (42.6–82%). *Leuconostoc gelidum*, *Leuconostoc gasicomitatum* and *Lactobacillus sakei* were common in the remaining kimchi clone libraries (>34%). The composition of bacterial phylotypes in kimchi varied between samples. Our approach revealed different community structures from those reported in previous culture-dependent studies based on phenotypic identification methods. The culture-independent method used here proved to be efficient and accurate and showed that the bacterial communities in kimchi differ from those in other fermented vegetable foods.
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Keywords: Kimchi; 16S rRNA; Microflora; Lactic acid bacteria; ARDRA

1. Introduction

Kimchi is a well known traditional Korean food; the product of lactic acid fermentation of more than 100 types of vegetables. A typical adult Korean consumes an average of 50–200 g of kimchi per day and its market in Korea exceeded 130 million USD in 2002. Taxonomically diverse groups of lactic acid bacteria (LAB) have been identified during the fermentation of kimchi. A few studies have been carried out on the microbial composition of kimchi, using conventional methods of isolation and phenotypic identification (Lim et al., 1989; Park et al., 1990; Shin et al., 1996). Some important species thought to be responsible for kimchi fermentation are *Leuconostoc* (*Lc.*) *mesenteroides*, *Lc. pseudomesenteroides* and *Lc. lactis*, as well as lactobacilli including *Lactobacillus* (*Lb.*) *brevis* and *Lb. plantarum*. *Lc. mesenteroides* was reported to predominate in the early stages of fermentation and to be responsible for the initial

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anaerobic state of kimchi; as the pH gradually falls to 4.0, *Lb. plantarum* becomes predominant (Mheen and Kwon, 1984).

These earlier studies of the microbial community in kimchi have serious limitations. First, the organisms present were isolated by conventional culture methods using limited types of media and culture conditions, and it is now well known that bacteria in nature cannot all be cultured under general laboratory conditions. Second, the species were identified by phenotypic methods, especially by biochemical analysis such as sugar fermentation patterns and by cell morphology. This inevitably leads to misidentification and misinterpretation in microbial community studies. Recently, LAB isolates from kimchi were re-examined using a battery of polyphasic methods including 16S rRNA sequencing and DNA–DNA hybridization. This led to the discovery of several novel species: *Lc. kimchii* (Kim et al., 2000), *Lb. kimchii* (Yoon et al., 2000), *Weissella kimchii* (Choi et al., 2002), *Weissella koreensis* (Lee et al., 2002) and *Lc. inhae* (Kim et al., 2003).

In the present work, we examined the structure of the bacterial communities of mature Chinese cabbage kimchi produced by five major Korean commercial manufacturers. We used direct DNA extraction, 16S rRNA gene amplification, amplified ribosomal DNA restriction analysis (ARDRA) and sequencing. To our knowledge, this is the first study to examine kimchi microbiota using culture-independent molecular methods.

2. Materials and methods

2.1. Sampling and DNA extraction

Kimchi samples were purchased from 5 major kimchi manufacturers, designated A to E. Sampling was carried out when the pH of the kimchi reached approximately 4.2. Each sample (ca. 5 g) was diluted with 20 ml phosphate buffered saline, mixed by vortexing and sonicated mildly for 40 min. Bacteria were collected by successive filtration through Whatman paper no. 4 (Whatman, Maidstone, Kent, UK) and a 0.22 μm pore size filter (GE Osmonics, Westborough, MA). The organisms collected were removed from the 0.22 μm pore size filters and total genomic DNA was extracted with a Soil DNA extraction kit (Mo Bio, Solana Beach, CA).

2.2. Amplification and cloning of prokaryotic 16S rDNA

The prokaryotic 16S rRNA gene was amplified by PCR with a thermal cycler (model 9600, Applied Biosystems, Foster City, CA). The PCR reaction contained 25 μl of final solution consisting of: 2.5 μl 10×PCR buffer for *Taq* polymerase (ExTaq, TaKaRa, Tokyo, Japan), 200 μM of each deoxynucleoside triphosphate (dNTP; TaKaRa), 0.4 μM of each primer and 1 U of *Taq* polymerase (ExTaq, TaKaRa). The oligonucleotide primers used for the bacterial 16S rRNA gene were: 5′-AGAGTTTGATC[A,C]TGGCTCAG-3′ and 5′-GG[C,T]TACCTTGTTACGACTT-3′ (Chun and Goodfellow, 1995) and for the archaeal 16S rRNA gene, 5′-TTCCGGTTGATCC[C,T]GCCGGA-3′ and 5′-[C,T]CCGGCGTTGA[A,C]TCCAATT-3′ (DeLong, 1992). The thermocycle program was as follows: 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min; and a final extension step at 72 °C for 7 min. After cycling, the PCR products were detected by electrophoresis on a 1% agarose gel, staining with ethidium bromide and visualizing under UV light.

The bacterial 16S rRNA gene amplicons were purified with a PCR Clean-up Kit (Mo Bio), ligated into the pGEM-T easy vector (Promega, Madison, WI), following the manufacturer's instructions, and transformed into *E. coli* DH10B. The 16S rRNA gene inserts in the *E. coli* transformants were amplified by a colony PCR method (Chun et al., 1999) with primers prGTf (5′-TACGACTCACTATAGGGCGA-3′) and prGTr (5′-CTCAAGCTATGCATCCAACGC-3′) targeting the flanking regions of the multi-cloning site of pGEM-T easy vector. The same PCR protocol, as for the amplification of 16S rRNA genes, was used.

2.3. Amplified ribosomal DNA restriction analysis (ARDRA) and nucleotide sequencing

One microliter of each colony PCR product was digested with *Msp*I (NEB, Beverly, MA) at 37 °C for 2 h and the resulting fragments were analyzed by 2% agarose gel electrophoresis together with a 100 bp

ladder (Amersham, Piscataway, NJ) as DNA marker. The ARDRA band patterns from different clones were compared with the Dice coefficient using GelCompar v.3.0. software (AppliedMath, Kortrijk, Belgium). The PCR products were cleaned up with shrimp alkaline phosphatase and *Exo*I, following the PRISM SnaPshot Multiplex Kit protocol (Applied Biosystems, Foster City, CA). Sequencing was carried out with an automated DNA sequencer (Applied Biosystems GeneScan 3100) and the primer sets used previously (Chun and Goodfellow, 1995).

2.4. Sequence analysis

All sequences were checked for chimeric artifacts by the CHECK_CHIMERA program of the Ribosomal Database Project (RDP) (Maidak et al., 1997), and manually aligned with closely related sequences obtained from the Ribosomal Database Project and GenBank databases. Sequence similarity and phylogenetic trees were calculated with the PHYDIT program (available at http://plaza.snu.ac.kr/~jchun/phydit/). An evolutionary tree was generated by the neighbor-joining method (Saitou and Nei, 1987) and evolutionary distance matrices were generated according to Jukes and Cantor (1969). Sequences were deposited in GenBank under accession numbers AY323815, AY323816 and AY421793–AY421961.

3. Results and discussion

Only bacterial clones were considered in this study as no archaeal 16S rRNA gene was detected in the kimchi samples. We initially sequenced about 20 clones per sample to identify predominant phylotypes. The 16S rRNA gene clones were then screened by ARDRA, and only clones giving restriction patterns different from the predominant phylotypes were sequenced. High-throughput techniques such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (t-RFLP) have proved useful in revealing microbial diversity in foods (Meroth et al., 2003). However, direct examination of PCR-amplified clones in a given sample is essential in the first stage of molecular ecology study because minor components of microbiota cannot be accurately identified by the high-throughput methods mentioned. A total of 191 16S rRNA gene sequences and 157 ARDRA patterns were obtained from the five kimchi samples. 16S rRNA gene sequences that were >98% similar to the type strains were regarded as belonging to the corresponding species or phylotypes. In the case of ARDRA, identical restriction patterns to the type strains led to assignment to the corresponding species.

We chose kimchi made from Chinese cabbage for this study since it is the most popular type of kimchi in Korea. The overall bacterial community structures of the five commercial kimchi samples are summarized in Table 1. Of 348 clones examined, only one (KMD-0-40) was identified as non-LAB and assigned a unique phyletic line equivalent to a novel genus in the family Caulobacteraceae (data not shown). This alpha-proto-bacterial clone showed a close relationship to an uncultured clone from insect (*Zophobas mori*) larval intestine, with 99.3% similarity. Forty-six percent of the LAB clones consisted of species of the genus *Weissella*, followed by *Leuconostoc* (39%) and *Lactobacillus* (15%). *W. koreensis* was the only species found in all kimchi samples, and it was generally the most abundant. We did not find in our clone libraries *Pediococcus* spp. and *Enterococcus faecalis*, which had been reported in kimchi by culture-dependent studies (Mheen and Kwon, 1984; Lim et al., 1989; Lee et al., 1992; Shin et al., 1996). This discrepancy is probably due to inaccurate identification in those studies, which depended on morphology and rather few biochemical tests. Those particular bacteria may also be more easily cultured than other LAB.

W. koreensis, a recently described novel species isolated from kimchi (Lee et al., 2002), was the predominant species in kimchi samples A, B and E (Table 1). We did not find *Weissella kimchi*, another *Weissella* species from kimchi (Choi et al., 2002). Over 88% of the clones derived from samples A and E consisted of just two LAB species, whereas sample B was relatively diverse. One clone (KMJ-0-43; GenBank accession number AY323815) from sample A could not be assigned to any known LAB taxa; it had the greatest 16S rRNA similarity (97.0%) to the type strain of *Lactobacillus sakei*; this clone points to the presence of a hitherto unknown *Lactobacillus* species in kimchi. *Lb. sakei* and *Leuconostoc gelidum* were the most abundant groups in sample C, followed by *W. koreensis* (Table 1).

Table 1
Bacterial community structure of five commercial kimchi samples

Species	Kimchi sample				
	A (n[a]=94)	B (n=54)	C (n=52)	D (n=103)	E (n=45)
Weissella koreensis	82.0[b] (98.2–100)[c]	42.6 (99.4–100)	23.1 (99.6–100)	3.9	77.8 (98.4–100)
Leuconostoc gasicomitatum	13.7 (98.6–100)	9.3 (99.6–99.9)		42.7 (99.5–99.9)	2.2 (99.8)
Leuconostoc gelidum		11.1 (99.3–99.6)	34.6 (98.1–99.8)	53.4 (99.3–99.8)	
Leuconostoc mesenteroides		7.4 (99.4–99.9)			
Leuconostoc carnosum		1.9 (99.6%)			
Leuconostoc inhae	3.1 (99.37–100)	9.3 (98.0–99.6)			4.4 (98.4–98.6)
Leuconostoc kimchii		1.9 (99.6)			
Lactobacillus sakei		13 (99.3–99.8)	38.5 (98.9–99.8)		11.1 (99.7–99.8)
Lactobacillus curvatus			1.9 (99.6)		
Lactobacillus brevis		1.9 (97.9)	1.9 (97.6)		
Lactobacillus algidus					4.4 (99.4)
Others	Clone KMJ-0-43	Clone KMD-0-40			

[a] n=the number of clones examined.
[b] % compositions.
[c] Range of 16S rRNA gene sequence similarity values between kimchi clones and the type strain.

Considerable numbers of *Lb. sakei* were also found in samples B and E. *Lc. gelidum* was the predominant bacterial component of sample D, with *Leuconostoc gasicomitatum* close behind. The former was the second most dominant species in sample C and the third most dominant in sample B. *Lc. gasicomitatum* was also the second most frequent species in sample A. Samples A and E had similar bacterial compositions, whereas the remaining samples had unique bacterial communities.

It is clear from the results presented in this study that the major players in mature kimchi are *W. koreensis*, *Lb. sakei* and *Leuconostoc* spp. in the samples tested. Our data differ from the previous studies based on culture-dependent methods. Mheen and Kwon (1984) examined the microbial community of laboratory-made kimchi using tryptone–glucose–yeast extract agar and an identification scheme based on acid production and cell morphology, and found that *Lc. mesenteroides* and *Lb. plantarum* were the predominant species, though *Lb. brevis*, *Pediococcus* sp. and *E. faecalis* were also found. Using MRS and a medium made from kimchi extract for isolation, and conventional phenotypic tests for identification, Lim et al. (1989) found that *Lb. plantarum*, *Lb. fructivorans* and *Lc. mesenteroides* were the most abundant species. Similarly, Shin et al. (1996) examined commercially produced kimchi using plate counting agar and API identification systems, and reported that *Lb. brevis*, *Lb. plantarum* and *Lb. acidophilus* were the most frequent isolates, followed by *Lc. mesenteroides* and *Pediococcus pentosaceus*. In contrast we did not find many clones of *Lb. plantarum* and *Lc. mesenteroides* in our clone libraries.

Possible reasons for this disagreement between our study and the others are (i) that the kimchi samples examined by those authors had different ingredients from our samples; (ii) that the previous studies were based on culturing whereas ours is culture-independent and (iii) that the identification methods used by those workers were phenotype-based and relatively inaccurate, whereas our study is based on more reliable 16S rRNA sequence analysis. Because exactly identical kimchi samples cannot be reproduced, it is not clear whether *W. koreensis*, the predominant species in our study, was indeed absent from the samples examined in the previous studies, or whether it was misidentified, perhaps as leuconostocs. In 1993 the genus *Weissella* was proposed to harbor a phylogenetically coherent group previously known as the "*Lc. paramesenteroides*" subgroup (Collins et al., 1993) and resembles to the present member of the genus *Leuconostoc* on the basis of morphological and physiological characteristics. Moreover, *W. koreensis* was only formally recognized in 2002 (Lee et al., 2002). Therefore, it is possible that studies prior to these taxonomic proposals identified weissellae as leuconostocs.

Lactic acid bacteria are major players in many fermented vegetable foods. *Lc. mesenteroides*, *Lb. brevis*, *P. pentosaceus* and *Lb. plantarum* are known to contribute to the complex sauerkraut fermentation process (Pederson and Albury, 1969). *Lb. plantarum* and *Lb. manihotivorans* are the predominant species in cassava sour starch fermentation (Omar et al., 2000). Similarly, lactobacilli are responsible for the fermentation of Mexican pozol, an acid beverage produced by the natural fermentation of nixtamal (heat- and alkali-treated maize) dough (Ampe et al., 1999; Escalante et al., 2001). Streptococci, found in Mexican pozol, were not detected in kimchi. It is interesting that the bacterial community in kimchi is somewhat different from those of other fermented vegetable foods, since a *Weissella* species, namely *W. koreensis*, was the major component of four of the kimchi samples examined. The fact that many of the clones in our libraries were assigned to a recently described species isolated from kimchi strengthens the argument that kimchi has a unique bacterial community structure.

In this study, we have shown that culture-independent methods can be successfully applied to a mixed food environment such as kimchi. It is clear from our results that even though *W. koreensis* is the predominant species in three samples, the bacterial community structure differs between mature kimchi samples, probably depending on the initial ingredients that serve as natural LAB inocula. The data presented in this study should provide a useful framework for further studies of the population dynamics of kimchi fermentation, the use of starter cultures, control of the fermentation process and long-term preservation.

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