#### RESEARCH NOTE



The Korean Society of Food Science and Technolog

# Analysis of the Bacterial Composition During *Kochujang*, a Korean Traditional Fermented Hot Pepper-soybean Paste, Fermentation

Sun-Jung Park<sup>1</sup>, Jin-Hee Chang<sup>1</sup>, Seong-Kwan Cha<sup>1</sup>, and Gi-Seong Moon\*

Division of Food and Biotechnology, Chungju National University, Jeungpyeong, Chungbuk 368-701, Korea <sup>1</sup>Traditional Food Research Group, Korea Food Research Institute, Seongnam, Gyeonggi 463-746, Korea

Abstract In this study we analyzed the dynamic changes in microbiota composition during *kochujang* fermentation at  $30^{\circ}$ C. During fermentation, the viable cell counts slowly increased and reached  $3.2 \times 10^{7}$  for aerobic bacteria,  $8.3 \times 10^{3}$  for yeast, and  $1.4 \times 10^{3}$  CFU/mL for fungi after 60 days. Bacilli were found to be the most dominant microorganisms throughout the fermentation process. Using the culture dependent method *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus amyloquefaciens* were found to be the main species during the early stages of fermentation; however, *Bacillus pumilus* and *Bacillus stearothermophilus* became the most dominant species during the late stage of fermentation. In contrast, when the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) method was used *Bacillus ehimensis* was found to be the dominant species during the early stage of fermentation and *Bacillus megaterium*, *B. pumilus*, *B. subtilis*, and *B. licheniformis* were dominant in the ate stages. These results indicate various other *Bacillus* species rather than just *B. subtilis* and *B. licheniformis* might be involved in the fermentation of *kochujang*.

Keywords: kochujang, fermentation, Bacillus species, denaturing gradient gel electrophoresis

#### Introduction

Kochujang is a traditional fermented food and a very famous hot sauce in Korea. Various methods for manufacturing kochujang have been developed in Korea and these different methods generally involve using the following ingredients; red pepper powder, soybean, glutinous rice, salt, and water (1). In a previous paper, 10 different bacterial genera, Bacillus, Corynebacterium, Enterococcus, Flavimonas, Flavobacterium, Gemella, Pasteurella, Pseudomonas, Staphylococcus, and Streptococcus were found in traditional kochujang where Bacillus subtilis was the dominant species (2). In addition to bacterial strains, yeast and fungi were also found in kochujang during fermentation. Jin et al. (3) analyzed the microbial composition of a traditional kochujang and found that the genus Bacillus constituted 97% of the bacterial compositions, where Bacillus licheniformis was found to be the most dominant species. In addition, Zygosaccharomyces pseudorouxii and Aspergillus oryzae were determined to be the most dominant yeast and fungi species, respectively.

Determining the microbial composition of fermented foods is very important for understanding the fermentation profile and providing valuable information for the commercial production of fermented foods. In the past, the methods used to analyze microbial composition have been dependent on culture based techniques; however, these methods are time-consuming, laborious, and uneconomical. In this respect, the use of a culture-independent molecular

technique, such as polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) in combination with 16S rRNA gene sequencing, would be a powerful and superior tool to analyze the microbial composition from complex matrices such as fermented foods (4-7). In this study we used this molecular technique as well as a culture-dependent method using API kit to analyze the dynamic changes in microbiota composition during *kochujang* fermentation although these methods still have limitations to discriminate one *Bacillus* sp. from another in species level (8).

# **Materials and Methods**

**Kochujang** fermentation Freshly prepared *kochujang* from a factory (Sunchang MoonOkRae Food) in the Sunchang province, which is famous for its Korean traditional sauces such as *kochujang*, *doenjang* (a fermented soybean paste), and *cheonggukjang* (9), was used for the experiments. After preparation, it was immediately stored at 30°C, which temperature is for the fermentation of commercial *kochujang*.

**Culture-dependent method** To determine the microbial composition of the fermented *kochujang* samples using the culture-dependent method, the samples (1 g) were diluted by 10 fold with a peptone buffer (0.85% NaCl, 0.01% peptone, 0.003% KH<sub>2</sub>PO<sub>4</sub>, and 0.006% Na<sub>2</sub>HPO<sub>4</sub>) and then spread on a plate count agar (PCA; Difco, Sparks, MD, USA) plate to determine the aerobic bacterial composition, an MRS agar (Difco) plate to assess the lactic acid bacteria and a 3M petrifilm (St. Paul, MN, USA) to determine the yeast and fungi. Viable cell counts were measured by counting the colonies on the plates after incubation for 24

Received December 22, 2008; Revised March 12, 2009;

Accepted March 17, 2009

<sup>\*</sup>Corresponding author: Tel: +82-43-820-5251; Fax: +82-43-820-5272 E-mail: gsmoon@cjnu.ac.kr

1036 S. -J. Park et al.

hr at 30°C. All colonies on the PCA plate, which included 45-57 colonies, were selected and subjected to Gramstaining, catalase and oxidase activities assays and morphological examination for preliminary identification. Finally, the API 50 CHB kit (BioMérieux, Marcy l'Etoile, France) was used to identify the different isolates.

**PCR-DGGE** To determine the microbial composition using the PCR-DGGE technique, whole genomic DNAs were isolated with the Blood & Cell Culture DNA Maxi kit (Qiagen, Hilden, Germany) according to manufacturer's instruction. 16S rRNA gene sequences from the isolated DNAs were partially amplified with primers gc338f [5'-gaggcagcag-3'; a gc clamp (10) is underlined] and 518r (5'attaccgcggctgctgg-3'), which span the V3 region of the 16S rRNA gene (5). The PCR products were then separated by DGGE using the DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA) as described previously (7). The DNA in the stained gel were excised and eluted with 30 µL of sterile water. The eluents of the PCR amplicons were re-amplified with the 338f (5'actccgacgggaggcagcag-3')-518r primers and sequenced by Macrogen (Seoul, Korea). Sequence homology searches were performed using the BLAST program (blastn) from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/).

## **Results and Discussion**

Viable counts of all the organisms tested were gradually increased throughout kochujang fermentation at 30°C for 60 days (Fig. 1). The cell count of aerobic bacteria increased by 1.8 log scale at day 60 when compared with day 0 and the yeast and fungi cell counts increased by 1.5 and 1.4 log scale, respectively. However, lactic acid bacteria were not detected throughout the fermentation process. As expected, organisms belonging to the genus of Bacillus were the most dominant during kochujang fermentation by both the culture-dependent and cultureindependent methods (Table 1 and Fig. 2). In the culturedependent method, B. subtilis, B. licheniformis, and B. amyloquefaciens were dominant at day 0 and B. amyloquefaciens was the dominant species at day 28, which changed into B. pumilus and B. stearothermophilus at day 60 (Table 1). In contrast, B. ehimensis (arrow 11 in Fig. 2) was shown to be most dominant at the early stage (day 0 and 7) of the fermentation from the cultureindependent PCR-DGGE method. In addition, B. megaterium (arrow 5), which was not dominant in the culture-dependent analysis, was found to be dominant throughout the fermentation process. B. pumilus (arrows 2, 4), B. licheniformis (arrow 6, 16, and 17), and B. subtilis (arrow 8, 10, and 18) were also dominant at the mid and late stages of the fermentation. The arrows 3, 9 corresponded to *Bacillus* sp. and an arrow 15 to *Paenibacillus* motobuensis. The other arrows (1, 7, 12, 13, 14, and 19) were not matched with 16S rRNA gene sequences from the databases. Similar to our previous study (7), the results of the PCR-DGGE method were different from those of the culture-dependent method. In relation to this discrepancy, Comi et al. (4) suggested that the bacterial DNA from

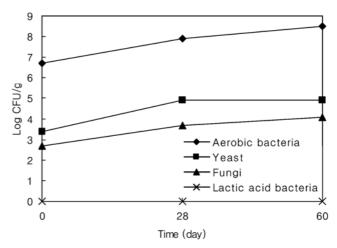


Fig. 1. Growth curves of microorganisms during *kochujang* fermentation.

Table 1. Identification of isolates from *kochujang* during fermentation at 30°C

Fermentation time (day)	Identities <sup>1)</sup>	No. of colonies
0	Bacillus amyloquefaciens	11
	Bacillus circulans	3
	Bacillus lentus	3
	Bacillus licheniformis	16
	Bacillus megaterium	2
	Bacillus pumilus	2
	Bacillus stearothermophilus	1
	Bacillus subtilis	19
28	Bacillus amyloquefaciens	30
	Bacillus circulans	2
	Bacillus lentus	1
	Bacillus licheniformis	3
	Bacillus pumilus	1
	Bacillus stearothermophilus	1
	Bacillus subtilis	9
60	Bacillus amyloquefaciens	4
	Bacillus brevis	3
	Bacillus firmus	7
	Bacillus laterosporus	3
	Bacillus lentus	1
	Bacillus licheniformis	2
	Bacillus pumilus	12
	Bacillus stearothermophilus	10
	Bacillus subtilis	3

<sup>&</sup>lt;sup>1)</sup>The isolates were identified by a culture-dependent method using API 50 CHB kit.

cellular autolysis in the PCR-DGGE method and media selectivity in the culture-dependent method may be problematic for analyzing microbial compositions from food samples. In addition, they emphasized the importance of using a combination of both methods for a more accurate analysis. Currently, neither method can correctly analyze the microbiota from complex samples such as

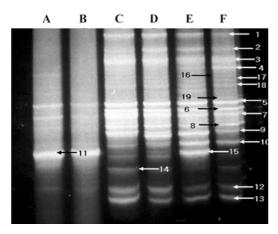


Fig. 2. DGGE analysis of partial 16S rRNA gene fragments from the microbiota of *kochujang* samples at 30°C. Day 0 (A), 7 (B), 14 (C), 21 (D), 28 (E), and 60 (F). The DNA bands correspond to partial 16S rRNA gene products.

fermented foods; however, it would be highly beneficial to develop powerful tools based on molecular techniques that can resolve the current problems associated with analyzing complex samples.

In general, it has been established that *B. licheniformis* and *B. subtilis* are the prevailing bacteria that exist during the fermentation of commercial and traditional *kochujang* (2,3). However, based on our results various other *Bacillus* species, such as *B. amyloquefaciens*, *B. megaterium*, *B. pumilus*, *B. stearothemophilus* as well as *B. licheniformis* and *B. subtilis*, could also be the main species in *kochujang* fermentation. However, we cannot conclusively state that these results represent the microbiota composition during *kochujang* fermentation since many different varieties of *kochujang* are manufactured by various methods in Korea.

Kochujang is a representative Korean hot sauce and a very important ingredient in many foods in Korea (11). As the result, there have been many Korean food researchers that have studied the physicochemical, microbiological, and sensory properties of kochujang (2,3,9,12,13). Particularly, understanding the microbial composition during fermentation is quite important because kochujang is a fermented food that is controlled by the microorganisms that exist during fermentation. By better understanding the microbial composition we can attain useful information about the correlation between the fermentation process and microbial composition. Despite this, the analysis of microbial composition during fermentation has been rarely studied. In this study, we systematically analyzed the major microorganisms of a traditional kochujang during

fermentation using a culture-dependent and a culture-independent method. To the best of our knowledge, this is the first report that used the PCR-DGGE method to analyze the microbiota of *kochujang* during fermentation.

# Acknowledgments

The research was supported by a grant from the Academic Research Program of Chungju National University in 2006. This work was also partially supported by a grant (20080401034067) from BioGreen 21 Program, Rural Development Administration, Republic of Korea.

## References

- Shin DH, Ahn EY, Kim YS, Oh JY. Changes in the microflora and enzyme activities of *kochujang* prepared with different *koji* during fermentation. Korean J. Food Sci. Technol. 33: 94-99 (2001)
- Lee JM, Jang JH, Oh NS, Han MS. Bacterial distribution of kochujang. Korean J. Food Sci. Technol. 28: 260-266 (1996)
- Jin HS, Kim JB, Lee KJ. Major microbial composition and its correlation to the taste of Sunchang traditional *kochujang*. Korean J. Food Nutr. 20: 363-368 (2007)
- Comi G, Citterio B, Manzano M, Cantoni C. Evaluation and characterization of *Micrococcaceae* strains in Italian dry fermented sausages. Fleischwirtschaft 72: 1679-1683 (1992)
- ben Omar N, Ampe F. Microbial community dynamics during production of the Mexican fermented maize dough pozol. Appl. Environ. Microb. 66: 3664-3673 (2000)
- Endo A, Okada S. Monitoring the lactic acid bacterial diversity during shochu fermentation by PCR-denaturing gradient gel electrophoresis. J. Biosci. Bioeng. 99: 216-221 (2005)
- Park SJ, Jang JH, Cha SK, Moon GS. Microbiological analysis of dongchimi, Korean watery radish kimchi, at the early and mid-phase fermentation. Food Sci. Biotechnol. 17: 892-894 (2008)
- Porwal S, Lal S, Cheema S, Kalia VC. Phylogeny in aid of the present and novel microbial lineages: Diversity in *Bacillus*. PLoS ONE 4: e4438 (2009)
- Kim JW, Kim YS, Jeong PH, Kim HE, Shin DH. Physicochemical characteristics of traditional fermented soybean products manufactured in folk village of Sunchang region. J. Food Hyg. Saf. 21: 223-230 (2006)
- Muyzer G, de Waal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microb. 59: 695-700 (1993)
- 11. Hong SP, Kim EM, Jo GH. Preparation of *kochujang* sauce and its characteristics. Korean J. Food Culture 19: 239-249 (2004)
- Bang HY, Park MH, Kim GH. Quality characteristics of kochujang prepared with Paecilomyces japonica from silkworm. Korean J. Food Sci. Technol. 36: 44-49 (2004)
- Oh JY, Kim YS, Shin DH. Changes in microorganisms and enzyme activities of low-salted *kochujang* added with horseradish powder during fermentation. Korean J. Food Sci. Technol. 37: 463-467 (2005)