Korean Journal of Microbiology (2022) Vol. 58, No. 3, pp. 169-178
DOI https://doi.org/10.7845/kjm.2022.2057

pISSN 0440-2413
eISSN 2383-9902

Changes in bacterial communities during the aging of traditional *Gochujang*, a Korean fermented red pepper paste

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한국 전통 고추장의 숙성기간에 따른 미생물 군집의 변화

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(Received August 23, 2022; Revised September 19, 2022; Accepted September 27, 2022)

The changes in bacterial communities during aging were investigated in Sunchang traditional *Gochujang* in order to clarify the fermentation process by the naturally occurring microorganisms. The community composition at the beginning of aging in both the preparations was similar to that on the 90th day of aging, in which *Bacillus* species were dominant. However, on the 180th day of aging, a drastic change among the *Bacillus* species was observed in the *Gochujang* of one company, while a change in dominant species, from *Bacillus* to *Lactobacillus*, was observed in the other. The community composition on the 520th day of aging was similar to that on the 180th day, suggesting that the time needed to mature traditional *Gochujang* could be linked to changes in the composition of bacterial communities.

Keywords: bacterial community, fermentation, *Gochujang*, pyrosequencing, Sunchang

Gochujang (a Korean fermented red pepper paste) is one of Korea's most representative fermented foods made with glutinous rice, red pepper, *Meju* (dried brick of naturally fermented soybeans), and salt and has a unique blend of sweet, salty, savory, and sour flavors. Because of this characteristic, it is widely used as a key ingredient in Korean food along with *Deongjang* (soybean paste) and *Ganjang* (soy sauce). The taste of *Gochujang* is internationally recognized and represents Korean food culture. *Gochujang*, like western cheese, was originally produced in every Korean household but is now mostly produced in factories with mass production systems using *Koji*, so the traditional production methods are used only in small local companies. Since the traditional *Gochujang* is fermented by a variety of microorganisms from the air and rice straw, its flavor differs from that of the modified *Gochujang* fermented by *Aspergillus oryzae* as the sole starter (Kim *et al.*, 1994).

Studies have been conducted to elucidate the types of microorganisms that contribute to the fermentation and flavor of the *Gochujang* manufactured in Sunchang area. Jin *et al.* (2007) reported that *Bacillus licheniformis* was the most abundant species in 7 out of the 29 samples of traditional *Gochujang*, while *B. subtilis* was a dominant species in 11 samples. Jang *et al.* (2011) reported that *B. licheniformis*, *B. subtilis*, and *B. velezensis* were the major microorganisms in the 7 samples of traditional Sunchang *Gochujang*. These studies examined the randomly selected colonies grown on the agar plates and provided a general pattern of the major bacterial species of the Sunchang *Gochujang*. Since many bacteria are unculturable in a general medium, a limited number of microbial communities of the *Gochujang* were evaluated. On the other hand, Cho *et al.*

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(2017) analyzed the metagenome of the Sunchang *Gochujang* using pyrosequencing, a next-generation sequencing technique. Among 5 *Gochujang* samples aged 2 to 5 years, *Bacillus* was a dominant genus in 3 samples, while *Aneurinibacillus* and *Thermoactinomyces* were dominant in the other two samples. Commonly, the *Bacillus* species was abundant in all the Sunchang samples. These results are similar to the report by Nam *et al.* (2012), where *Bacillus* was the most abundant genus in 8 samples of the traditional *Gochujang* collected from each province in Korea.

The reported findings so far have been the analysis of bacterial communities in the mature *Gochujang*. Since the changes in the bacterial community during aging have not been studied, the fermentation characteristics of the Sunchang *Gochujang* are not well understood. Therefore, it is necessary to investigate not only the changes in the bacterial community during the aging process but also the bacterial communities of the basic *Gochujang* ingredients, such as salt, red pepper powder, and *Meju*. The purpose of this study is to identify the bacteria involved during the aging progress of Sunchang traditional *Gochujang* by the metagenomic analysis.

Materials and Methods

Gochujang manufacturing

Gochujang manufacturing was commissioned by two companies (A and B) in Sunchang Folk Village. The master of each company made *Gochujang* using raw materials produced in Sunchang, according to the traditionally handed down recipe, on March 5, 2012. The manufacturing method of company A, which uses *Meju* powder for saccharification of cooked rice, was as follows: 10 kg of rice was hard-boiled; thereafter 15 L of cool water, and 3 kg of *Meju* powder were mixed well with the hard-boiled rice and saccharified at room temperature for 3 h. Then 8 kg of red pepper powder and 5 kg of bay salt were added to the saccharified porridge and mixed well. The mixture was put into a jar, pressed well and then fermented outdoors. The manufacturing method of company B, which uses barley malt for saccharification of cooked rice, was as follows: glutinous rice (10 kg) was soaked for 24 h in water and made into flour. Barley malt (5 kg) was soaked for 3 h in 15 L of water, filtered and added to the rice flour. The mixture was boiled to make glutinous rice porridge. To the cooled porridge, 3.8 kg of *Meju* powder, 10 kg of red pepper powder, and 5.5 kg of bay salt were added and mixed. The mixture was put into a similar jar, pressed well, and fermented outdoors.

The preparation of *Meju* for *Gochujang* was as follows: At the end of August, 6 kg of soybeans and 4 kg of rice flour were soaked in water for 3 h and 6 h, respectively; mixed well and then cooked for 90 min. The mixture was ground and molded into the shape of donuts. They were tied with straw ropes, hung under the eaves for 4 weeks, broken into pieces, and then dried for 3–4 days before grinding. Red pepper powder for *Gochujang* was prepared using red pepper harvested in the previous year from the Sunchang Farms. Bay salt was purchased from salt farms in the Sunchang county of Jeonbuk province. For the pyrosequencing analysis, samples of bay salt, red pepper powder, *Meju* powder, and *Gochujang* were collected from each company.

Sample preparation

During the aging process, *Gochujang* samples were collected periodically. About 30 g of each sample was collected at a depth of 10–15 cm from the surface of the ripening *Gochujang* at three spots and transferred to sterile tubes for storage at -80°C until analysis. Genomic DNA (gDNA) was extracted with a Fast DNA SPIN Kit for soil (MP Bio Laboratories), which can disrupt both of spores and vegetative cells, following the manufacturer's instructions. The DNA quality and concentration were determined by 1% agarose gel and with a NanoDrop spectrophotometer (ND-1000; Thermo Scientific), respectively.

Pyrosequencing

For pyrosequencing, a fusion forward primer 27F was constructed for the V1, V2, and V3 polymorphic regions 27–518 of *Escherichia coli* 16S rRNA using a 5'-CCTATCC CCTGTGTGCCTTGGCAGTC-(adapter)-TCAG-(key)-AC-linker-GAGTTTGATCMTGGCTCAG-3' and a reverse primer 518R was composed of 5'-CCATCTCATCCCTGCGTGTCT CCGAC-(adapter)-TCAG-(key)-Barcode-AC-linker-WTTA CCGCGGCTGCTGG-3'. For DNA amplification, a reaction mixture containing 1 μl template DNA, 1 μl each primer (50 pmol), 1 μl dNTP mix (100 mM each), 2 μl 10x polymerase

chain reaction (PCR) buffer, 1 μl Taq polymerase (Roche), and 14 μl H_2O was subjected to PCR amplification in a thermal cycler (Bio-Rad). The PCR cycling was performed at 94°C for 5 min; 10 cycles of 94°C for 30 sec, 60°C for 45 sec, and 72°C for 90 sec; 20 cycles of 94°C for 30 sec, 55°C for 45 sec, and 72°C for 90 sec. The PCR products were separated by electrophoresis on a 2% agarose gel and then purified using a QIAquick PCR purification kit (Qiagen). Equal concentrations of the purified products were pooled and purified using an AMPure bead kit (Agencourt Bioscience). The quality of the PCR products were assessed on a Bioanalyzer 2100 (Agilent) using a DNA 7500 chip. The amplicons were subjected to pyrosequencing using a GS Junior Titanium system sequencer (Roche). The methods and reactions used for pyrosequencing were according to the manufacturer's manual by Chunlab Inc.

Sequence processing and bacterial community analysis

The raw sequence data files were processed in the following order: demultiplexing, trimming of primer sequence, quality filtering, sequencing error correction, taxonomic assignment, and detection of chimeras. Each sample was identified by a unique barcode during the demultiplexing step. After sorting of each sequence, the bar code sequence, primer, and linker were subtracted using GL FLX software (Roche) and sequences 300 bp or less were excluded from the analysis. Non-16S rRNA sequences were removed via the BLASTN search and the statistical Hidden Markov Model (HMMER 3.0). To correct sequence errors, taxonomic identification was carried out by selecting representative sequences within clusters of the trimmed sequences. Individual reads were assigned according to the highest pair-wise alignment among the top five BLASTN hits using the EzTaxon® extended database (Yoon *et al*., 2017). Chimera sequences were removed using UCHIME (Edgar, 2010). The read number of each sample was normalized by random subsampling. The number of operational taxonomic units (OTUs), which is the number of species present in the sample, was calculated using the CD-HIT program (Fu *et al*., 2012) based on the 3% sequence inconsistency. For statistical analysis, the rarefaction curve (Heck *et al.*, 1975), abundance-based coverage estimator (ACE) index (Chao *et al*, 1992), Chao1 richness index (Chao, 1984), Shannon (Shanoon, 1948) and Simpson diversity indices (Simpson, 1949), and Good's coverage index (Good, 1953) were obtained using the EzTaxon® program (Chunlab Inc.). The phylogenetic distance between communities was estimated using Fast UniFrac (Hamady *et al*., 2010).

Chemical analysis

After aging for 520 days, the pH of the *Gochujang* from each company was measured with a pH meter (Mettler Toledo) by diluting 5 g of each sample 10-fold with distilled water. For the analysis of organic acids, the diluted *Gochujang* was filtered using a 0.45 μm membrane. The filtrate was passed through a Sep-Pak C18 cartridge and analyzed by high-performance liquid chromatography (HPLC). The HPLC conditions were as follows: 0.01 N H_2SO_4 was used as the mobile phase and passed through an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad) heated to 60°C at a 0.6 ml/min flow rate.

Accession numbers

The sequence data from this study has been deposited in the GenBank Sequence Read Archive with the accession number SRP151448.

Results and Discussion

The results of the community richness and the relevant analysis of the ingredients of *Gochujang* and the aging period, which were obtained after pyrosequencing, are summarized in Table 1. Among the ingredients for company A's *Gochujang*, bay salt had the highest estimated community richness value (Ace & Chao1) and *Meju* had the lowest. The community diversity index (Shannon index & Inverse Simpson index), which is a mixed index of species richness and species evenness, was relatively higher in bay salt than in red pepper powder and *Meju*. In company B's *Gochujang*, the estimated community richness value was the highest in bay salt and lowest in pepper powder, while the community diversity index was the highest in *Meju* and the lowest in pepper powder. Although companies A and B were located close to each other in the Sunchang Folk Village and followed the same method for preparing *Meju*, it was interesting that the species diversity of company B's *Meju* differed from that of company A's *Meju*. The rarefaction curve

Table 1. Richness and diversity estimates of bacterial communities based on a cut-off of 97% sequence identity of 16S rRNA sequences

Sample[a]	Valid reads	OTUs	Ace[b]	Chao1[b]	Shannon[b]	Inverse Simpson[b]	Goods lib. coverage
Red pepper powder A	13010	258	509 (452, 584)	414 (351, 520)	2.05 (2.01, 2.09)	2.64 (2.57, 2.70)	0.99
Meju (fermented soybean lump) A	16664	72	90 (79, 119)	95 (79, 145)	2.30 (2.28, 2.32)	6.08 (5.97, 6.19)	0.99
Bay salt A	4709	802	1747 (1625, 1888)	1317 (1193, 1479)	5.55 (5.51, 5.60)	108.38 (101.60, 116.14)	0.92
Aging 5 A	26047	292	538 (479, 616)	459 (391, 576)	3.29 (3.27, 3.31)	12.65 (12.38, 12.93)	0.99
Aging 90 A	16436	76	166 (133, 218)	139 (99, 249)	2.05 (2.03, 2.06)	5.31 (5.22, 5.41)	0.99
Aging 180 A	23256	121	170 (149, 205)	148 (131, 190)	2.28 (2.26, 2.30)	4.98 (4.89, 5.07)	0.99
Aging 520 A	11962	98	105 (100, 119)	106 (100, 129)	2.87 (2.84, 2.89)	9.57 (9.31, 9.86)	0.99
Red pepper powder B	30352	352	461 (425, 514)	443 (407, 505)	3.03 (3.00, 3.05)	7.85 (7.70, 8.01)	0.99
Meju (fermented soybean lump) B	9490	734	1239 (1159, 1335)	1099 (998, 1239)	5.22 (5.19, 5.25)	81.48 (78.03, 85.24)	0.97
Bay salt B	1775	564	3808 (3436, 4229)	1874 (1521, 2357)	5.08 (4.99, 5.16)	49.38 (44.34, 55.72)	0.77
Aging 5 B	11642	483	885 (811, 976)	749 (663, 877)	3.85 (3.81, 3.89)	10.33 (9.88, 10.83)	0.98
Aging 90 B	8592	417	850 (771, 945)	688 (594, 830)	4.20 (4.16, 4.23)	26.02 (24.98, 27.15)	0.98
Aging 180 B	23740	120	146 (132, 180)	158 (134, 223)	2.79 (2.77, 2.81)	7.80 (7.62, 7.99)	0.99
Aging 520 B	8244	266	319 (298, 355)	338 (304, 404)	4.04 (4.00, 4.07)	24.41 (23.26, 25.67)	0.99

[a]A or B after the sample name refers to company A or B.
[b]Values in parentheses are 95% confidence interval.

showing the average species richness of the *Meju* from the two companies also exhibited a similar tendency (data not shown).

For company A, the Good's library coverage was 0.92 in salt and 0.99 in red pepper powder and *Meju*, whereas for company B, the Good's library coverage was 0.97 in *Meju* and 0.99 in red pepper powder. Thus, the sequence information from these samples was sufficient to cover the diversity of the actual sample. However, the Good's library coverage was 0.77 in salt in the case of company B, indicating that more sequence information is needed to analyze the community diversity. The Good's library coverage of the samples collected from the two according to the aging period ranged from 0.98 to 0.99, suggesting that sufficient sequence information was obtained from the community diversity analysis.

Bacterial communities at the phylum level

The relative abundance of the bacterial community in the *Gochujang* ingredients of company A was investigated at the phylum level. In terms of the common community abundance, phyla *Firmicutes* and *Proteobacteria* were dominant in red pepper powder despite differences in the relative abundance proportion, while the *Firmicutes* was dominant in *Meju*. Bay salt showed a greater variety of phyla than red pepper powder and *Meju*, but there was a relatively high abundance of *Proteobacteria and Bacteroidetes*.

On the other hand, the phylum *Firmicutes* was dominant once aging began, and 180 days after aging it showed absolute dominance (99%) in the *Gochujang* from both the companies. In company A's *Gochujang*, from the beginning of aging to 520 days of aging, the relative abundance of *Firmicutes* was close to 100%, whereas in company B's *Gochujang*, it was dominant after 180 days of aging. These results confirm that *Firmicutes* played a key role in the aging of Sunchang traditional *Gochujang*. This result was consistent with that of a study by Nam *et al.* (2012), which reported that after collecting fermented *Gochujang* from each province in Korea and analyzing the relative abundance at the phylum level, *Firmicutes* had an average dominance of 93.1%.

Bacterial communities at the genus and species level

Table 2 summarizes the top ten taxon species observed after the analysis of *Gochujang* from both the companies based on their aging periods. On the 5th day and 90th day of aging, *B. amyloliquefaciens* was the most dominant species in the *Gochujang* of company A, and the relative abundances were 77.63% and 78.96% of the total taxa, respectively; followed by *B. atrophaeus* (8.09% and 8.47%, respectively) and *B. methylotrophicus* (5.74% and 8.11%, respectively). The total

Table 2. Relative abundance of the top ten taxon species observed after the analysis of *Gochujang* based on their aging periods

Taxon	Company A[a]			
	Aging 5	Aging 90	Aging 180	Aging 520
Bacillus amyloliquefaciens	77.63 (1)	78.96 (1)	2.61 (4)	5.8 (3)
Bacillus atrophaeus	8.09 (2)	8.47 (2)	5.99 (2)	5.54 (4)
Bacillus methylotrophicus	5.74 (3)	8.11 (3)	4.51 (3)	6.6 (2)
Bacillus siamensis	1.15 (4)	0.21	85.35 (1)	79.76 (1)
Enterococcus durans	1.1 (5)	0.1	0	0
Bacillus_uc	1.05 (6)	0.59 (5)	0.09 (8)	0.84 (5)
Bacillus vallismortis	0.84 (7)	0.86 (4)	0.37 (6)	0.47 (6)
Bacillus mojavensis	0.8 (8)	0.53 (6)	0.54 (5)	0.35 (7)
Bacillus subtilis	0.73 (9)	0.26 (9)	0.28 (7)	0.26 (8)
Staphylococcus_uc	0.29 (10)	0.24 (10)	0	0
4P001191_s	0.28	0.31 (8)	0	0
Weissella confusa	0.15	0.32 (7)	0	0
Bacillaceae_uc_s	0.13	0.09	0.03	0.08 (10)
Bacillus aerius	0.11	0.07	0.04 (9)	0.08
Bacillus tequilensis	0.06	0.09	0.03 (10)	0.08 (9)

Taxon	Company B[a]			
	Aging 5	Aging 90	Aging 180	Aging 520
Bacillus mojavensis	17.35 (1)	21.08 (1)	0.05 (4)	10.68 (2)
Bacillus subtilis	13.09 (2)	8.3 (4)	0.01 (10)	5.34 (4)
Bacillus aerius	10.46 (3)	11.73 (3)	0.03 (6)	7.1 (3)
Bacillus thermoamylovorans	9.03 (4)	11.95 (2)	0.03 (9)	3.41 (5)
Enterobacter cowanii	5.23 (5)	5.58 (5)	0.03 (6)	0.07
Bacillus licheniformis	5.17 (6)	5.14 (6)	0.03 (5)	2.48 (6)
Bacillus tequilensis	4.54 (7)	4.01 (7)	0.01	2.11 (7)
4P000779_s	4.42 (8)	3.19 (8)	0	0
Bacillus_uc	2.84 (9)	1.95 (9)	0	1.06 (9)
4P001083_s	2.27 (10)	1.57	0	0
Bacillus smithii	0.5	1.66 (10)	0	0.87
Lactobacillus acidipiscis	0	0	97.66 (1)	60.84 (1)
Lactobacillus pobuzihii	0	0	1.95 (2)	1.04 (10)
Lactobacillus_uc	0	0.01	0.12 (3)	1.57 (8)
Lactobacillaceae_uc_s	0	0.01	0.03 (6)	0.3

[a]The numbers in parentheses refer to top 10 ranks of species.

relative abundances of these three *Bacillus* species were 91.46% and 95.54% on the 5th and 90th day of aging, respectively, showing that company A's *Gochujang* was fermented mostly by the *Bacillus* species in early aging stage. During the same aging period, various *Bacillus* species coexisted in company B's *Gochujang*, unlike company A's *Gochujang*. The species present on the 5th day of aging were *B. mojavensis* (17.35%), *B. subtilis* (13.09%), *B. aerius* (10.46%), *B. thermoamylovorans* (9.03%), *Enterobacter cowanii* (5.23%), and *B. licheniformis* (5.17%). On the 90th day, *B. mojavensis* (21.08%) was the dominant species, followed by *B. thermoamylovorans* (11.95%), *B. aerius* (11.73), and *B. subtilis* (8.3%). In the genus level, the relative abundance was higher, in the order of *Bacillus* (61.15%), *Bacillus*_g5 (15.80%), and *Enterobacter* (7.31%) on the 5th day of aging, and in the order of *Bacillus* (56.98%), *Bacillus*_g5 (17.46%), and *Enterobacter* (7.88%) on the 90th day of

fermentation. Thus, *Bacillus* was the dominant genus at the beginning of aging in company B's *Gochujang*, but other genera were involved in aging. Considering the diversity of the species present on the 5th and 90th day of aging, the community structure of company A was simpler than that of company B's *Gochujang*. Also, the bacterial communities in the *Gochujang* from the two companies on the 5th day of aging were similar to those on the 90th day of aging, indicating that the communities were stabilizing.

Comparison of the dominant bacteria in company A's *Gochujang* on the 90th day and 180th day of aging showed that the abundance of *B. amyloliquefaciens*, which was relatively high (78.96%) on the 90th day, abruptly decreased to 2.61% on the 180th day of aging. In contrast, the abundance of *B. siamensis*, which was only 1.15% on the 90th day of aging, increased to 85.35% on the 180th day of aging. On the other hand, in company B's *Gochujang*, the *Bacillus* species, which belonged to the top rank on the 90th day of aging, disappeared on the 180th day of aging and *Lactobacillus acidipiscis*, which had not been detected at the early stage of aging, showed an abundance of 97.66% and became the absolute dominant bacteria. Thus, a sudden change in the genus level was observed. In addition, the species showing the second and third highest abundance on the 180th day of aging belonged to the genus *Lactobacillus* and the total abundance of these species was 99.73%.

The relationship between temperature and the bacterial community of *Gochugjang*

This result indicates that the microbial community equilibrium, which had been stable until the 90th day of aging, was rapidly disturbed for some reason during the period from the 90th to the 180th day of aging in the *Gochujang* from both the companies. One of the important clues to explain this is that this period overlapped with a significant change in fermentation temperature. According to the data (The Sunchang Traditional *Gochujang* Association, 2005) submitted by the Sunchang Traditional *Gochujang* Association for the registration of the Geographical Indication System, the Sunchang traditional *Gochujang* is fermented for 8 to 18 months after making *Gochujang* from late December to early March of the following year. That is, for Sunchang traditional *Gochujang* to have an appropriate flavor, an aging period of at least 8 months is required. Such characteristics of the aging recipe may be related to the restructuring of the bacterial community observed in the *Gochujang* from the two companies between 3 months (90 days) and 6 months (180 days) of aging.

The aging period according to the Sunchang traditional *Gochujang* recipe was from March 2012 (Spring), when the aging began, to September 2013 (Fall), when the aging was completed. Between the 90th and 180th day of aging, which was from early June to early September, a major change was observed in the composition of the bacterial community. From June to August 2012, the average maximum temperatures in Sunchang were 28.8℃, 30.9℃, and 31.6℃, respectively; the average maximum temperature was 35.5℃ from July 24 to August 8 (The Korea Meteorological Administration, 2012; Fig. 1). The actual temperature of the *Gochujang* that had been fermented in the outdoor pot was expected to be much higher than the highest temperature announced by the Korea Meteorological Administration and this increase in temperature is likely to have caused changes in the composition of these species. In general, the maximum growth temperature of the *Bacillus* strains is known to range from 31−76℃ (Warth, 1978). The *Bacillus* species, which were dominant until the 90th day of aging in the *Gochujang* from both the companies, can withstand microaerophilic conditions such as those inside the pot and form endospores, thereby resisting the temperature changes.

In company A's *Gochujang*, *B. amyloliquefaciens* and *B. siamensis*, which were dominant on the 90th and 180th day of

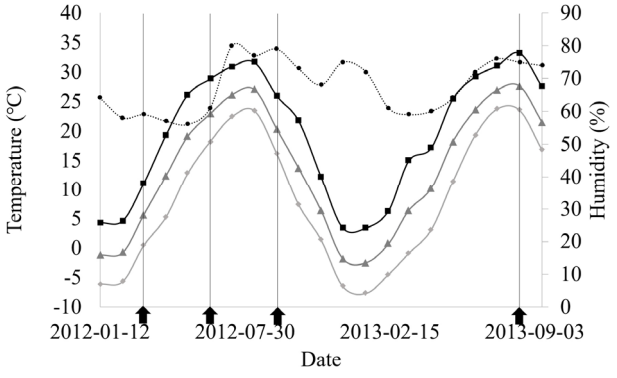

Fig. 1. Changes of temperature and humidity at Sunchang during the aging period (2012.03−2013.09) of *Gochujang*. Each arrow means the sample collect time. (◆, average of temperature; ■, average of high temperature; ▲, average of low temperature; ●, average of relative humidity)

aging, respectively, are both endospore-forming bacteria. In particular, *B. siamensis* can endure a salt concentration of 0–14%, and grow at a temperature range of 4°–55°C and a pH range of 4.5–9 (Sumpavapol *et al*., 2010). *B. siamensis* adapted better to the environmental changes in the *Gochujang* resulting from increased temperature than *B. amyloliquefaciens*. According to an analysis of the bacterial community in Sunchang traditional *Gochujang* fermented for two years, *B. amyloliquefaciens* (88.84% of the total bacterial community) was dominant (Cho *et al*., 2017). Therefore, the change in bacterial dominance in our study is incomprehensible. In company B's *Gochujang*, *Lactobacillus* species were the dominant bacteria during this period. *Lactobacillus acidipiscis*, which had an absolute dominance of 97.66% on the 180th day of aging, accounted for 60.84% of the bacterial community even on the 520th day of aging. It can grow in a microaerophilic environment and endure 10% NaCl (Tanasupawat *et al*., 2000); thus, they are appropriate for growth in the fermentation environment of *Gochujang*. However, the survival and dominance of the non-spore-forming bacteria, which were not detected in the metagenomics analysis of the *Gochujang* ingredients, in the summer temperatures is questionable. They became the absolute dominant species by winning the survival competition against the *Bacillus* strains. In the analysis of traditional fermented *Gochujang* from the Jeonbuk province, however, *Weissella salipiscis* showed dominance with 69% (Nam *et al*., 2012) and in traditional fermented *Gochujang* from the Jeonnam province, *L. rennini* showed the second highest dominance (15%) (Cho *et al*., 2017). These results demonstrate that lactic acid bacteria can also be the dominant species in the traditional *Gochujang* aging, exceeding the populations of other bacteria such as *Bacillus* species.

Røssland *et al.* (2005) reported that in a growth inhibition study of *B. cereus* by *Lactobacillus*, only a *Lactobacillus* strain, which caused the abrupt reduction of the pH to 5.0 at the early stage of culturing when both the bacteria were mixed and cultured, prevented the sporulation of *B. cereus* and completely killed *B. cereus*; only lactic acid inhibited the sporulation of *B. cereus*. The L-lactic acid produced by anaerobic metabolism cannot be used as an energy source by the *Bacillus*, which consumes a lot of energy for sporulation to cope with high temperatures and acidification. On the 520th day of aging, the pH values of the *Gochujang* from companies A and B were 4.64 ± 0.04 and 4.68 ± 0.05, respectively. The major organic acid (malic acid, lactic acid, citric acid) contents (g) per kg of *Gochujang* were as follows: 12.48 ± 0.01, 1.54 ± 0.02, and 4.87 ± 0.03, respectively for company A and 7.39 ± 0.01, 7.19 ± 0.04, and 4.47 ± 0.01, respectively for company B. Thus, the lactic acid content of company B's *Gochujang* was higher, but the L-malic acid content was lower than that of company A's *Gochujang*. L-malic acid, which is mainly produced during fermentation of fungi such as *Aspergillus oryzae* and *Saccharomyces cerevisiae* (Zelle *et al*., 2008), is a stronger acid than L-lactic acid (pKa = 3.86) due to the dissociation of the two carboxyl acids under this pH (pKa_1 = 3.4, pKa_2 = 5.2). In addition to producing L-lactic acid, *L. acidipiscis*, the dominant bacteria in company B's *Gochujang* has a malolactic enzyme (Kazou *et al*., 2017), which converts L-malic acid into L-lactic acid. As a result, company B's *Gochujang* showed a relatively high concentration of L-lactic acid. However, *Lactobacillus* species was not detected in company B's *Gochujang* on the 90th day of aging; thus, it would be unaffected the sporulation of *Bacillus* species because of its low level of lactic acid in *Gochujang* at that time. To achieve explosive growth of *Lactobacillus*, it is highly likely that the dominant *Bacillus* species, with excellent environmental adaptability, face some specific extinction situations, including lysis due to infection with the *Bacillus* prophage.

Possible scenario for a drastic change of bacterial communities

Erez *et al.* (2017) reported that when a phage infects *Bacillus*, it secretes a communication peptide. When the concentration of this peptide is elevated due to mass infection, progeny phages are lysogenized to the *Bacillus* host chromosome. Therefore, it is likely that these infected phages are already present as prophages in the *Bacillus* chromosome in the fermentation stage of *Meju* or the early stage of *Gochujang* fermentation, when the *Bacillus* population rapidly increases. One scenario is that in the dominant *Bacillus* strain, the lysogenic state due to infection with the phages on the 90th day of *Gochujang* aging was converted to the lytic state due to high temperatures in summer; thus, the bacteria became extinct. This makes the lactic acid bacteria, which were earlier present in a small amount, dominant. Analysis of 189 *Bacillus* genomes registered in the GenBank showed that many genomes included

inserted phage machinery necessary for bacteriophage assembly, such as transposases, integrases, recombinases, and endolysin. The conversion to the lytic cycle by prophage excision has been reported to be induced by nutrient stress, oxidative stress, UV radiations, quinolone antibiotics, heat shock, and quorum sensing (Fortier and Sekulovic, 2013). It is possible that the dominant *Bacillus* strain containing prophages was converted into the lytic state and died in the stressful environment, when the temperature increased in the summer.

A possibility is that *B. subtilis* could contain the *Bacillus subtilis* defective prophage (PBSX) (Wood *et al*., 1990). According to recent reports, when such *Bacillus* strains were stressed, the sequential death process was clarified in detail at the cellular level (Toyofuku *et al*., 2017). Under stress conditions, *B. subtilis* showed increased endolysin expression in the PBSX prophage gene, resulting in damage to the peptidoglycan in the cell membrane and cell death. The endolysin released with the cytoplasmic materials was observed to attack the adjacent *B. subtilis* cells, resulting in the death of the bacteria. Considering that defective phages with functions similar to those of PBSX were also found in *B. amyloliquefaciens*, *B. licheniformis*, and *B. pumilis* genomes (Huang and Marmur, 1970; Steensma *et al*., 1978), which are the major fermentation bacteria of *Gochujang*, the sudden extinction of the *Bacillus* species during the summer may be due to the activation of defective prophages after physical or chemical stress induced by high temperature.

The bacterial community composition after 180 days of aging, when new dominant bacteria appeared in both the companies' products, remained similar until 520 days of aging. The minimum duration of 8 months required for the aging of the Sunchang traditional *Gochujang* belongs to this period. In company B's *Gochujang*, *Bacillus* strains reappeared on the 520th day, after facing almost complete extinction on the 180th day of aging. This suggests that the new dominant bacteria are the strains that may have already had phage immunity, or due to the loss of function in the defective phages, the bacteria regained their dominance.

Relationship between bacterial communities

An unweighted pair-group method with an arithmetic mean (UPGMA) dendrogram was used to compare the relationship between the bacterial communities of *Gochujang* ingredients and aging *Gochujang*, which was obtained by Fast UniFrac analysis (Fig. 2). All bacterial communities during aging were divided into two groups, early aging group (5 and 90 days of aging) and late aging group (180 and 520 days of aging). The community composition of company A's *Gochujang* on the 90th day of aging was almost the same as that of the *Meju*, indicating that the bacteria originating from *Meju* became dominant; the composition on the 90th day was close to that on the 5th day of aging. The bacterial composition of company B's *Gochujang* on the 5th day of aging was closest to that on the 90th day of aging, followed by that in *Meju*. Although the bacterial compositions on the 180th and 520th day of aging were distinguished from those of *Meju* and products of the early aging stages, they were much closer to *Meju*'s community composition compared to those of red pepper powder and salt. These results show that the primary bacterial source for the aging of *Gochujang* is *Meju*, inferring that the unique flavor of Sunchang *Gochujang* may originate from microorganisms originated from *Meju*.

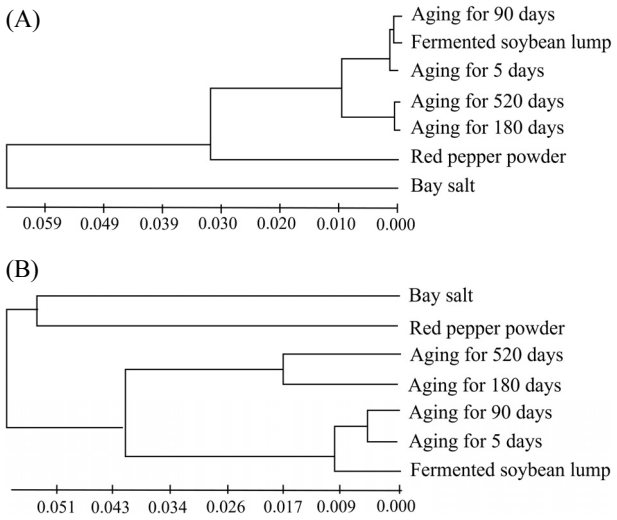

Fig. 2. Clustering analysis for samples collected from company A and B using the UniFrac service after pyrosequencing: (A) company A's *Gochujang*, (B) company B's *Gochujang*. The scale indicates the distance between clusters in UniFrac units.

Involvement of *Bacillus* in the aging progress

A recent study involving bacterial community and metabolite analysis showed that the dominant *Bacillus* species may not play a major role in the fermentation process of *Doenjang*, a Korean fermented soybean paste (Jung *et al*., 2016). Our results

showed that the genus *Bacillus* accounts for over 96% of the community composition in the case of company A's *Gochujang*, through the whole process of aging, suggesting that *Bacillus* is inevitably involved in the aging process of *Gochujang*. The genus *Bacillus* has unique properties, such as an antagonistic ability against other fermentation bacteria (Mannanov and Sattarova, 2001; Chen *et al*., 2009), rapid growth (Mageshwaran *et al*., 2014), spore forming (Kappes and Bremer, 1998; Ikeuchi *et al*., 2003), and strong expression of hydrolytic enzymes (Nijland and Kuipers, 2008; Degering *et al*., 2010), which enable it to become dominant in soy bean fermentation. Therefore, the genus *Bacillus* has been commonly reported as the most dominant bacteria in fermenting *Gochujang* (Jin *et al*., 2007; Jang *et al*., 2011; Nam *et al*., 2012; Cho *et al*., 2017). Further studies need to investigate how the dominant *Bacillus* species participates in the aging process by tracking the function of proteins expressed through transcriptome analysis. To maintain the tradition of Sunchang *Gochujang*, it will also be necessary to examine the microbial communities of *Gochujang* samples in Sunchang area, to analyze the data about the genetic properties of dominant strains, and to compare their metabolites.

적 요

자연적으로 발생된 미생물에 의한 발효 과정을 명확히 하기 위해 순창지역 전통 고추장에서 숙성 중 세균 군집의 변화를 조사하였다. 두 고추장 모두 숙성 초기 군집 구성은 *Bacillus* 종이 우점한 숙성 90일째의 군집 구성과 유사하였다. 그러나 숙성 180일째에 한 회사의 고추장에서는 *Bacillus* 종의 급격한 변화가 관찰되었고, 다른 회사에서는 우점종이 *Bacillus*에서 *Lactobacillus*로 변화됨이 관찰되었다. 숙성 520일째의 군집 구성은 180일째와 유사하여 전통 고추장 숙성에 필요한 시간은 세균 군집의 구성 변화와 연계됨을 보였다.

Acknowledgments

This research was funded by "Functional identification of Korean traditional soybean products(safety monitoring) project" under the Ministry of Agriculture, Food and Rural Affairs and partly Korea Agro-Fisheries and Food trade corporation in 2022 and Chonbuk National University in 2015.

Conflict of Interest

The authors have no conflict of interest to report.

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