J. Microbiol. Biotechnol. (2014), 24(5), 648–660
http://dx.doi.org/10.4014/jmb.1401.01009

jmb

Bacterial Community Migration in the Ripening of *Doenjang*, a Traditional Korean Fermented Soybean Food

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Received: January 6, 2014
Revised: February 15, 2014
Accepted: February 16, 2014

First published online
February 19, 2014

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pISSN 1017-7825, eISSN 1738-8872

Doenjang, a traditional Korean fermented soybean paste, is made by mixing and ripening *meju* with high salt brine (approximately 18%). *Meju* is a naturally fermented soybean block prepared by soaking, steaming, and molding soybean. To understand living bacterial community migration and the roles of bacteria in the manufacturing process of *doenjang*, the diversity of culturable bacteria in *meju* and *doenjang* was examined using media supplemented with NaCl, and some physiological activities of predominant isolates were determined. Bacilli were the major bacteria involved throughout the entire manufacturing process from *meju* to *doenjang*; some of these bacteria might be present as spores during the *doenjang* ripening process. *Bacillus siamensis* was the most populous species of the genus, and *Bacillus licheniformis* exhibited sufficient salt tolerance to maintain its growth during *doenjang* ripening. *Enterococcus faecalis* and *Enterococcus faecium*, the major lactic acid bacteria (LAB) identified in this study, did not continue to grow under high NaCl conditions in *doenjang*. Enterococci and certain species of coagulase-negative staphylococci (CNS) were the predominant acid-producing bacteria in *meju* fermentation, whereas *Tetragenococcus halophilus* and CNS were the major acid-producing bacteria in *doenjang* fermentation. We conclude that bacilli, LAB, and CNS may be the major bacterial groups involved in *meju* fermentation and that these bacterial communities undergo a shift toward salt-tolerant bacilli, CNS, and *T. halophilus* during the *doenjang* fermentation process.

Keywords: *Meju*, *doenjang*, fermented soybean food, bacterial community, *Bacillus*, *Staphylococcus*, *Enterococcus*, *Tetragenococcus halophilus*

Introduction

Ganjang and *doenjang*, the traditional Korean fermented soybean sauce and soybean paste, are the essential flavors and nutritional bases of authentic Korean cuisine. *Ganjang* is used as an essential condiment to enhance saltiness and flavor. *Doenjang* can be eaten as a sauce for vegetables, fish, and meats and as an ingredient in soups for additional protein and for flavor. These soybean products are prepared by mixing *meju* (fermented soybean block) with high salt brine (approximately 18%), followed by ripening in a porcelain pot. The liquid portion is separated and boiled after approximately two months, resulting in *ganjang*. *Doenjang* is the remaining solid portion, which is subsequently mashed and fermented for 30 to 180 days in the porcelain pot [37]. Traditional *meju* is prepared from soybeans by soaking, steaming, and mashing, followed by molding the soybean paste into blocks approximately 8 × 12 × 20 cm in size and allowing it to ripen for one or two months under natural environmental conditions. Generally, *meju* production begins in November. During ripening, naturally occurring microorganisms supply the enzymes that degrade macromolecules in the soybean block, which acquires the characteristic taste and flavor of *meju*. *Meju* is later used to prepare traditional Korean fermented seasonings, including *gochujang* (hot pepper

paste), as well as *doenjang* and *ganjang*. *Meju* functions as a source of nutrients, flavors, enzymes, and microorganisms [27, 42].

Fermented soybean products are a major source of the flavor and nutrition for a variety of foods in Korea. A number of studies have examined several aspects of these food products, including determining the microorganisms responsible for the quality and flavors of fermented foods. In microbial studies, the presence of specific microorganisms has been determined, including fungal species of the genera *Mucor*, *Penicillium*, *Scopulariopsis*, and *Aspergillus*, yeasts in the genera *Rhodotorula*, *Torulopsis* (amended as *Candida*), and *Saccharomyces*, and bacterial species in the genus *Bacillus* and lactic acid bacteria (LAB) [3, 24]. In subsequent studies, more diverse fungi and yeasts were detected, and the involvement of LAB was confirmed in *meju* [15, 25, 26]. Until the application of culture-independent methods for the evaluation of microorganisms in fermented soybean foods, little microbiological diversity was observed [17, 43], and *Bacillus* spp. and *Aspergillus oryzae* were considered to play major roles in the fermentation process because they were frequently isolated and exhibited high amylase and protease activities [5, 13, 28].

Culture-independent methods suggested that a wider variety of microorganisms were present in soybean fermentations compared with the classical microbiological methods. Recent data have shown that LAB and staphylococci, as well as bacilli, are present [4, 18, 19, 29]. However, microbial community migration from *meju* to *doenjang* has not been elucidated because these products have not been sequentially examined. Recently, a pyrosequencing analysis of a bacterial community in the *doenjang* ripening process was reported [20]. *Bacillus* spp. were determined to be the most populous species in *meju* and were apparently transferred to *doenjang*. Species in the genera *Clostridium* and *Enterococcus* were also evidently transferred from *meju* as part of the dominant microbiota of *doenjang*. Bacterial community migration from *meju* to *doenjang* was suggested by bacterial diversity comparisons, but the roles of bacterial transfer in the course of the manufacturing process from *meju* to *doenjang* has not been clearly demonstrated. Therefore, several culture-independent studies on *meju* and *doenjang* have produced a rough view of the microbial community composition for subsequent in-depth analysis. To understand bacterial community migration and the roles of bacteria in the *doenjang* manufacturing process, we evaluated the diversity of bacteria using bacterial growth media supplemented with NaCl, and some physiological activities of predominant isolates were determined.

Materials and Methods

Meju and *Doenjang* Samples and Bacterial Strain Isolation

Beginning in November of 2012, we began to purchase *meju* and *doenjang* samples from two manufacturers in the Gyeonggi Province of Korea. The manufacturers are located at a 50 km distance. *Doenjang* samples were made with the same batches of *meju* samples that were supplied for this research. After the first collections of *meju* and *doenjang* samples, the second samplings were collected after 60 days.

Meju samples were ground and homogenized with an equal amount of sterilized water and filtered through sterilized cheesecloth. *Doenjang* samples were homogenized with an equal amount of sterilized water and filtered through sterilized cheesecloth. The filtrates were used for measuring the NaCl content and pH and for isolating bacteria. The NaCl content was measured by titration with silver nitrate according to the Mohr method [1]; the pH was measured with a pH meter. For microbial counts, the filtrates were spread on agar media after appropriate dilutions with saline. Tryptic soy agar (TSA; Becton Dickinson, USA) and TSA containing 7% or 14% NaCl (w/v) were used for isolation of bacteria, and all media were incubated at 30°C until distinguishable colonies appeared. The cell counts on TSA and TSA containing 7% or 14% NaCl were determined after 1-, 2-, and 4-day incubations, respectively. Over 20 different types of colonies were collected from each plate based on differences in morphology, growth characteristics, and the numbers of colonies on each plate. The collected colonies were purified by successive transfer on the same type of agar medium used for isolation.

Identification of Isolates by 16S rRNA Gene Sequence Analysis

Genomic DNA of isolates was extracted using a DNeasy tissue kit (Qiagen, Germany). Amplification of the 16S rRNA gene was performed with eubacterial universal primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) [23], using a T3000 Thermocycler (Biometra, Germany). The PCR mixtures were preheated for 5 min at 95°C and were amplified using 30 cycles of 1 min at 95°C, 1 min at 58°C, and 1 min at 72°C. The PCR products were purified and sequenced using a custom service provided by GenoTech (Korea). The 16S rRNA gene sequence similarities were searched using the web-hosted BLASTn algorithm with the National Center for Biotechnology Information database and EzTaxon server 2.1 [6]. The phylogenetic positions of the isolates were inferred by 16S rRNA gene sequence analysis.

Confirmation of the Endospore Formation of Bacilli

Strains of *Bacillus licheniformis*, *Bacillus methylotrophicus*, and *Bacillus siamensis* isolated in this study were cultured overnight in TSB at 30°C. The stationary-phase cultures were inoculated to TSB and TSB containing 7% or 14% NaCl. After a 24 h incubation, cell numbers were counted on TSA with and without heat treatment at 73°C for 2 min to determine spore counts.

Determinations of Salt Tolerance, Enzyme Activities, and Acid Production

Salt tolerances of the predominant isolates were determined by assessing growth on TSA with up to 21% NaCl added. Growth on 0.5%, 7%, 14%, and 21% NaCl was determined after 1-, 2-, 4-, and 7-day incubations, respectively.

For the determination of amylase, protease, and lipase, and acid-producing activities of dominant isolates, agar media containing 1% soluble starch (w/v), 2% skim milk (w/v), 1% tributyrin (v/v; Sigma, USA), and 0.7% $CaCO_3$ (w/v) were used, respectively. Soluble starch, skim milk, and $CaCO_3$ were added to TSA, whereas tributyrin was added to tributyrin agar (Sigma) and emulsified by sonication before autoclaving. NaCl was added to confirm its effect on each activity. Colonies cultured on TSA were transferred to each substrate-supplemented agar medium and incubated at 30°C for 48 h. The size of the clear zone around the colony was used as the indicator of enzyme activity. Iodine staining was applied before amylase activity determination.

Results

Sample Conditions and the Growth of Bacteria on Media

Two types of *meju* and *doenjang* samples had average NaCl concentrations of 2.5% and 12.6%, respectively (Table 1). Samples from both manufacturers had nearly identical NaCl concentrations, whereas each sample at a stage had a different pH value. The difference in pH values between *meju* samples was greater than the difference between *doenjang* samples. Differences in the manufacturing conditions and first *meju* sampling time could be the cause of the pH difference in *meju* samples. During the ripening of *meju* for 60 days, the pHs of two samples were increased. Nitrogen compounds, such as amines and amino acids, produced from soy protein degradation were considered to contribute to the increase in pH [30, 36]. The *doenjang* samples had very similar initial pH values, which decreased after the 60-day ripening period. The high salt conditions from the *meju* brining process may contribute to the initial pH of the *doenjang* ripening process; the decrease in pH during the ripening of *doenjang* might originate from the acids produced by fermentation at high salt condition.

Table 1. The pHs and NaCl concentrations of samples.

Sample	pH		Salt concentration (%)	
	A	B	A	B
Meju (day 1)	7.0	5.5	2.3 ± 0.4	2.8 ± 0.1
Meju (day 60)	8.0	6.8	2.3 ± 0.9	2.5 ± 0.4
Doenjang (day 1)	5.9	5.9	13.3 ± 2.2	12.0 ± 1.3
Doenjang (day 60)	5.3	5.7	12.0 ± 1.6	13.1 ± 1.1

The first sampling days are denoted as day 1, and day 60 denotes the second sampling. The results are presented as the average values of three replicates.

The *meju* and *doenjang* samples had average bacterial counts of 9.1×10^8 and 2.9×10^8 CFU/g on TSA, respectively (Table 2). The bacterial cell counts were greatly influenced by the NaCl concentration of the plating medium and were proportionally decreased by the addition of NaCl. The bacterial counts in the *meju* and *doenjang* samples, counted on each NaCl concentration, were generally increased during the 60-day ripening period. The largest and smallest increases in bacterial counts were detected in *meju* sample B at 14% (9-fold increase) and 0.5% (1.1-fold increase) NaCl concentrations, respectively. In *meju* sample A, the bacterial counts on 0.5% and 7% NaCl concentrations were decreased to 80% and 64% of the first samplings, respectively, but that on 14% was increased 3-fold. The increased bacterial counts on the medium containing 14% NaCl suggests that salt-tolerant bacteria are involved in *meju* ripening and then transferred from *meju* to *doenjang*.

Structure of Culturable Bacterial Communities Isolated from *Meju* and *Doenjang*

In total, 554 isolates were identified from the samples of *meju* and *doenjang* from the two different manufacturers (Tables 3 and 4). Bacilli and LAB were successfully isolated on TSA. The addition of NaCl to TSA enabled frequent detection of staphylococci and supported the growths of halotolerant or halophilic bacteria; specifically, *Oceanobacillus*

Table 2. Numbers of bacteria counted on TSA and NaCl-supplemented TSA (CFU/g).

Sample	A			B		
	0.5%[a]	7%	14%	0.5%[a]	7%	14%
Meju (day 1)	1.2×10^9	2.2×10^8	3.3×10^7	6.9×10^8	8.5×10^5	2.2×10^5
Meju (day 60)	9.6×10^8	1.4×10^8	1.0×10^8	7.8×10^8	5.9×10^6	2.0×10^6
Doenjang (day 1)	1.6×10^8	8.0×10^7	4.3×10^7	1.6×10^8	7.2×10^7	4.1×10^6
Doenjang (day 60)	3.8×10^8	3.3×10^8	6.2×10^7	4.5×10^8	1.3×10^8	8.7×10^6

[a]The NaCl concentration in TSA is 0.5% (w/v), and others indicate the final concentrations of NaCl in the media.

Cell counts were repeated three times independently and the mean values of the replicates are presented.

Table 3. Numbers of isolates from *meju* and *doenjang* summarized at the species level (sample A).

Species	*Meju* (day 1)			*Meju* (day 60)			*Doenjang* (day 1)			*Doenjang* (day 60)			Total
	0.5%[a]	7%	14%	0.5%[a]	7%	14%	0.5%[a]	7%	14%	0.5%[a]	7%	14%	
Bacillus aryabhattai							2						2
Bacillus licheniformis								1	2		5	5	13
Bacillus methylotrophicus				14	2			5		7	2		30
Bacillus siamensis	8	3		1	1		21			18	17		69
Oceanobacillus oncorhynchi									1		1	19	21
Enterococcus faecalis	7	16											23
Enterococcus faecium	7												7
Weissella cibaria	1												1
Tetragenococcus halophilus								12	15				27
Staphylococcus equorum					2	4							6
Staphylococcus nepalensis				1	1	1			3				6
Staphylococcus saprophyticus	1	4	21	2	15	19		3	1				66
Staphylococcus succinus		1	1										2
Staphylococcus warneri					2								2
Staphylococcus xylosus			1	3	2				1				7
Total	24	24	23	21	25	24	23	21	23	25	25	24	282

[a]0.5% indicates that NaCl was not supplemented to TSA, and others indicate the final concentrations of NaCl in the media.

oncorhynchi, *Oceanobacillus picturae*, *Oceanobacillus sojae*, and *Tetragenococcus halophilus* were detected. More diverse species were isolated from sample B than from sample A, but the genera detected in both samples were similar except for the genus *Leuconostoc*. Our culture conditions covered most of the major genera identified by culture-independent methods [4, 18–20, 29]. Seven species in the genus *Bacillus*, three species in the genus *Enterococcus*, and eight species in the genus *Staphylococcus* were identified in this study. As the NaCl concentration increased, the isolated bacterial population shifted from *Enterococcus* spp. to *Staphylococcus* spp.; *Bacillus* spp. were not influenced by the NaCl concentrations as much as were *Enterococcus* species.

Detection frequencies of bacilli, LAB, and staphylococci at each stage of the two samples did not coincide, but the bacterial community structures and migrations occurred in similar patterns. *Bacillus* spp. were the predominant genera of bacteria isolated in this study (40% and 50% of the total isolates from samples A and B, respectively). Most of the *Bacillus* spp. were isolated on TSA and TSA containing 7% NaCl; however, the *Bacillus* species identified on the medium containing 14% NaCl was *B. licheniformis* (Tables 3 and 4). Notably, *B. siamensis* was most populous of the isolates, followed by *B. licheniformis* and *B. methylotrophicus*. Previous studies [3–5, 18, 22, 24, 29, 43] indicated that *B. subtilis* was the predominant organism; however, only six strains were isolated from sample B. *Bacillus* spp. were not the predominant bacteria at the beginning of *meju* fermentation, but they became the majority in *doenjang* through the ripening process.

In the case of LAB, species in the genera *Enterococcus*, *Leuconostoc*, and *Weissella* were identified, but *Enterococcus faecalis* and *Enterococcus faecium* were the predominant species. *Enterococcus* spp. proliferated in the early *meju* stage, and later, their proportion was decreased in the *meju* ripening process owing to the increase of bacilli. Two *Enterococcus durans* strains were isolated from *doenjang*, but they did not show growth in medium containing 7% NaCl (data not shown). This observation is in agreement with reports that the maximum concentration of NaCl for *E. durans* growth is 6.5% [10] and supports the hypothesis that the high salt concentration of *doenjang* is inhibitory to this organism. *Weissella cibaria* was isolated from *meju* samples of both manufacturers, and this report is the first to demonstrate its existence in *meju*.

Staphylococcus spp. were primarily isolated from *meju* on NaCl-containing media, although *meju* samples contain only 2.5% NaCl. *Staphylococcus saprophyticus* was the major species, representing 61% of *Staphylococcus* isolates. *Staphylococcus xylosus* and *Staphylococcus succinus* were the next most prevalent staphylococci, but their bacterial

Table 4. Numbers of isolates from *meju* and *doenjang* summarized at the species level (sample B).

Species	*Meju* (day 1)			*Meju* (day 60)			*Doenjang* (day 1)			*Doenjang* (day 60)			Total
	0.5%[a]	7%	14%	0.5%[a]	7%	14%	0.5%[a]	7%	14%	0.5%[a]	7%	14%	
Bacillus aerophilus								1					1
Bacillus licheniformis				1	4	20		1	2		7	9	44
Bacillus methylotrophicus					1			1		11	2		15
Bacillus siamensis		1		13	17		16			13	6		66
Bacillus subtilis		1									5		6
Bacillus tequilensis		1								1	3		5
Oceanobacillus oncorhynchi												2	2
Oceanobacillus picturae												1	1
Oceanobacillus sojae											1		1
Enterobacter cowanii				1									1
Enterococcus durans							2						2
Enterococcus faecalis				1									1
Enterococcus faecium	9			4				1					14
Leuconostoc lactis	3												3
Leuconostoc mesenteroides	5												5
Weissella cibaria	3												3
Tetragenococcus halophilus								17	22			11	50
Staphylococcus saprophyticus		5	12			2		1	1				21
Staphylococcus sciuri		2				1							3
Staphylococcus succinus		2	10										12
Staphylococcus vitulinus		4											4
Staphylococcus warneri		2											2
Staphylococcus xylosus		6	4			1							11
Total	20	24	26	19	22	24	18	22	25	25	24	23	272

[a]0.5% indicates that NaCl was not supplemented to TSA, and others indicate the final concentrations of NaCl in the media.

counts were only 21% and 16% of *S. saprophyticus* counts, respectively. The numbers of *Staphylococcus* spp. were dramatically decreased after brining, and these species were not isolated from either *doenjang* samples after 60 days of ripening.

T. halophilus and *Oceanobacillus* spp. were detected in *doenjang* but not in *meju*. The presence of *T. halophilus* was at its peak during the early *doenjang* stage, and later, the proportion of *T. halophilus* decreased during ripening. Decreased detection of *T. halophilus* was most likely influenced by the increase of bacilli and bacilli relatives. *Oceanobacillus* spp., increased during the ripening of *doenjang*, was also firstly identified in this research. The appearance of the halophilic and halotolerant bacteria after brining suggests that the source of these organisms was the salt used for *doenjang* preparation, which is produced in solar salterns.

The results of this bacterial community analysis indicate that the microbiota in *meju* and *doenjang* differ, but that certain microorganisms in *meju* were transferred to *doenjang*, whereas certain salt-intolerant species in *meju* were not able to survive in *doenjang*. Additionally, sea salt is an important microbial source for *doenjang* fermentation.

Influence of NaCl on the Growth of Predominant Species

To confirm the involvement of the predominant species isolated from the *meju* and *doenjang* ripening process, growth was tested on TSA containing 0.5% to 21% NaCl (Table 5). The growth of *B. methylotrophicus* and *B. siamensis* was inhibited by 14% NaCl in the medium, but *B. licheniformis* exhibited growth in this condition. High frequency isolation of *B. licheniformis* from *doenjang* might be the result of its salt tolerance. *O. oncorhynchi* isolates exhibited halotolerant growth, and the salt tolerance of the species is high enough

Table 5. Growth of the predominant species on TSAs containing 0.5%, 7%, 14%, and 21% NaCl.

Species	Growth			
	0.5%[a]	7%	14%	21%
Bacilli				
B. licheniformis 14BML10	++	++	W	-
B. licheniformis 14BML11	++	++	W	-
B. licheniformis 14BML12	++	++	+	-
B. methylotrophicus 0AML10	++	++	-	-
B. methylotrophicus 0AML11	++	++	-	-
B. methylotrophicus 0AML12	++	++	-	-
B. siamensis 0BDE1	++	++	-	-
B. siamensis 0BDE2	++	++	-	-
B. siamensis 0BDE3	++	++	-	-
O. oncorhynchi 14ADL12	+	+	+	+
O. oncorhynchi 14ADL13	+	+	+	+
O. oncorhynchi 14ADL14	+	+	+	+
LAB				
E. faecalis 7AME16	++	+	-	-
E. faecalis 7AME17	++	+	-	-
E. faecalis 7AME18	++	+	-	-
E. faecium 0BME1	++	+	-	-
E. faecium 0BME2	++	+	-	-
E. faecium 0BME3	++	+	-	-
T. halophilus 7BDE22	+	++	+	+
T. halophilus 7BDE23	+	++	+	+
T. halophilus 7BDE24	+	++	+	+
CNS				
S. saprophyticus 14BME5	++	++	++	+
S. saprophyticus 14BME6	++	++	++	+
S. saprophyticus 14BME7	++	++	++	+
S. succinus 14BME1	++	++	++	+
S. succinus 14BME2	++	++	++	+
S. succinus 14BME3	++	++	++	+
S. xylosus 7BME21	+	+	+	-
S. xylosus 7BME22	+	+	+	-
S. xylosus 7BME22	+	+	+	-

[a]The NaCl concentration in TSA is 0.5% (w/v), and others indicate the final concentrations of NaCl in the media.
Abbreviations: +, positive growth; -, negative growth; W, weak growth.

to involve in *doenjang* ripening. *E. faecalis* and *E. faecium* showed growths in the medium containing 7% NaCl but was inhibited by 14% NaCl. Both species cannot grow in *doenjang* but could be actively involved in *meju* ripening. *T. halophilus* exhibited sufficient salt tolerance to contribute in *doenjang* fermentation. The growths of *S. saprophyticus* and *S. succinus* determined by the colony size on media were highest among the tested strains, but that of *S. xylosus* did not grow as well as either of these two species. The active growth of *S. saprophyticus* and *S. succinus* on TSA containing 14% NaCl suggests that both have a role in the entire *doenjang* manufacturing process.

Endospore Formation of Bacilli

B. licheniformis was the only isolated *Bacillus* species that grew on the medium containing 14% NaCl (Table 5), suggesting the existence of *Bacillus* isolates that do not continue to grow during the *doenjang* ripening process. To investigate the effect of high salt (14%) on the *Bacillus* population, endospore counts were performed (Fig. 1). Bacterial cells grown on TSA without heat treatment represent the total viable cells, whereas colonies forming after heat treatment at 73°C for 2 min are the endospore-forming cells. Less than 0.02% of *B. methylotrophicus*, *B. siamensis*, and *B. licheniformis* cells cultured in TSB formed endospores. In the medium containing 7% NaCl, the growths of *B. methylotrophicus* and *B. siamensis* were decreased to 1.4% and 70% of TSB culture levels, respectively, but spore formation did not occur. Dramatic growth inhibition of the three species occurred in the medium containing 14% NaCl; the salt-tolerant *B. licheniformis* strain 14BML12 was the exception. The spore formation ratios of *B. methylotrophicus* and *B. siamensis* were over 51% of viable cells in the medium containing 14% NaCl, whereas those of *B. licheniformis* strains 14BML10 and 14BML12 were 4.1% and less than 0.002%, respectively. These results indicate that most *Bacillus* isolates form endospores at 14% NaCl concentration, except *B. licheniformis*. Considering the growth inhibition in medium with 7% NaCl, the salt tolerance of these species is as expected for *B. licheniformis*, *B. siamensis*, and *B. methylotrophicus*.

Enzyme Activities and Acid Production of the Predominant Species

To investigate the roles of predominant isolates in *meju* and *doenjang* ripening, the isolates' amylase, protease, lipase, and acid-producing activities were measured by the degradation of substrates supplemented in the medium (Table 6). Generally, the activities of strains within a species were notably similar, but certain strain-specific activities were detected.

Most of strains in the genus *Bacillus* showed amylase activity at 0.5% NaCl concentration, protease activity at up to 7% NaCl concentration, and tributyrin hydrolysis at up to

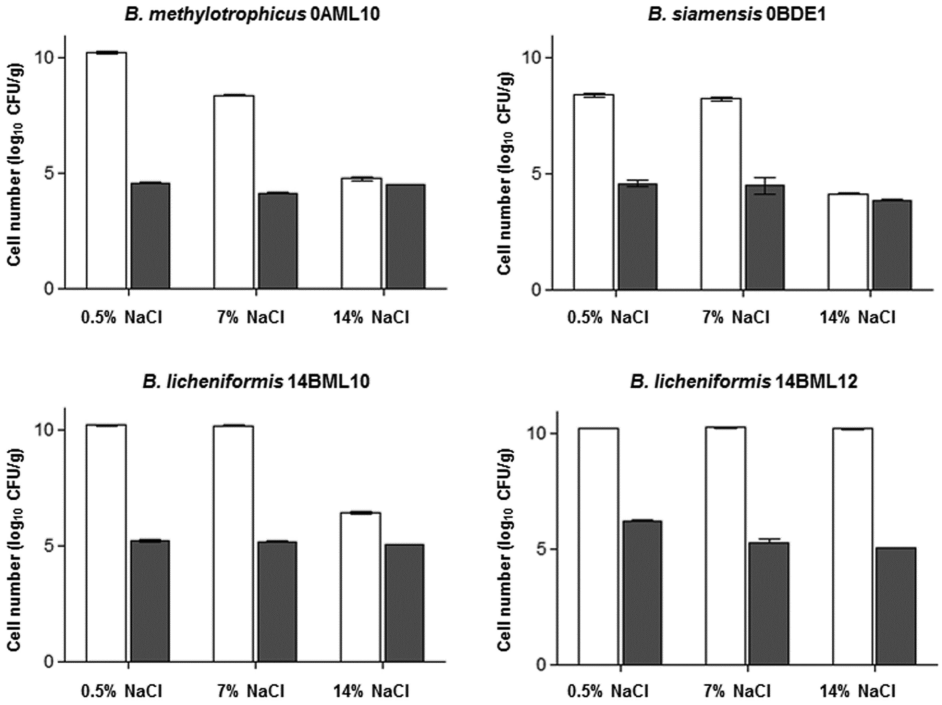

Fig. 1. Effect of NaCl on bacilli endospore formation.
Cells were grown in TSB and TSB containing 7% or 14% NaCl. After 24 h of incubation, cell numbers were counted on TSA before (□) and after (■) heat treatment at 73°C for 2 min.

3% NaCl concentration. *B. licheniformis* strains could grow on media containing tributyrin and 6% NaCl but could not hydrolyze tributyrin. *B. licheniformis* strain 14BML12 exhibited protease activity in the presence of 14% NaCl, but the other *B. licheniformis* strains did not. When soluble starch was added to TSA, *B. methylotrophicus* and *B. siamensis* showed growth in the presence of 14% NaCl, although amylase activities were not expressed. Addition of skim milk enhanced the salt tolerance of *B. siamensis*, whereas the addition of tributyrin inhibited the growth of *B. methylotrophicus* and *B. siamensis* in the presence of 6% NaCl. $CaCO_3$ did not influence the growths of *Bacillus* strains at the various NaCl concentrations. Additional organic substances could influence the salt tolerance of *Bacillus* strains. All *Bacillus* species contributed to the degradation of macromolecules in soybeans but did not produce acids, regardless of salt concentrations. Even *O. oncorhynchi*, a species with high growth in 14% NaCl condition, did not demonstrate any of the activities tested in this study; this species may not be involved in the flavor formation of *doenjang*.

E. faecalis and *E. faecium* showed protease, lipase, and acid-producing activities in low salt conditions. Interestingly, *E. faecalis* showed the highest protease activity among all of the tested species, and this activity was not inhibited in the presence of 7% NaCl. High involvement of *Enterococcus* spp. in the *meju* ripening process was indicated by their protease, lipase, and acid-producing activities. *T. halophilus* inoculated after brining presented lipase activity and acid production in all the salt conditions applied in this study. Strain 7BDE23 expressed proteinase activity but was not active in 14% NaCl condition. *T. halophilus* may contribute to the acid and flavor productions in *doenjang* by fermentation.

Strains of three *Staphylococcus* species showed protease activity under 7% NaCl condition and that of *S. succinus* was the highest, but amylase activity was observed in *S. succinus* only with low salt conditions (Table 6). We could detect the lipase activity from *S. saprophyticus* and *S. succinus* on tributyrin agar in the presence of 6% NaCl, but not from *S. xylosus*. The addition of more than 6% NaCl inhibited proper detection of lipase activity on the tributyrin agar; therefore, the maximum salt concentration for the lipase activity of both species could not be determined. However, the growth inhibition of bacilli and enterococci on tributyrin agar with 6% NaCl indirectly suggests that lipase activity from *S. saprophyticus* and *S. succinus* is possible in high salt conditions. Acid production of *S. saprophyticus*

Table 6. Amylase, protease, lipase, and acid-producing activities of the predominant species on the media containing substrates and NaCl.

Species	Amylase			Protease			Lipase			Acid production		
	0.5%[a]	7%	14%	0.5%[a]	7%	14%	0.5%[a]	3%	6%	0.5%[a]	7%	14%
Bacilli												
B. licheniformis 14BML10	+	-	-	+	-	-	+	+	-	-	-	-
B. licheniformis 14BML11	+	-	-	+	-	-	+	+	-	-	-	-
B. licheniformis 14BML12	+	-	-	++	+	+	+	+	-	-	-	-
B. methylotrophicus 0AML10	+	-	-	+	+	N	+	+	N	-	-	N
B. methylotrophicus 0AML11	+	-	-	+	+	N	+	+	N	-	-	N
B. methylotrophicus 0AML12	+	+	-	+	+	N	-	-	N	-	-	N
B. siamensis 0BDE1	-	-	-	+	+	-	+	+	N	-	-	N
B. siamensis 0BDE2	+	+	-	+++	+	-	++	++	N	-	-	N
B. siamensis 0BDE3	-	-	-	+	+	W	+	+	N	-	-	N
O. oncorhynchi 14ADL12	-	-	-	-	-	-	-	-	-	-	-	-
O. oncorhynchi 14ADL13	-	-	-	-	-	-	-	-	-	-	-	-
O. oncorhynchi 14ADL14	-	-	-	-	-	-	-	-	-	-	-	-
LAB												
E. faecalis 7AME16	-	-	N	+++	+	N	+	W	N	+	+	N
E. faecalis 7AME17	-	-	N	+++	+	N	+	W	N	+	+	N
E. faecalis 7AME18	-	-	N	+++	+	N	+	W	N	+	+	N
E. faecium 0BME1	-	-	N	+	-	N	+	-	N	+	-	N
E. faecium 0BME2	-	-	N	+	-	N	+	-	N	+	-	N
E. faecium 0BME3	-	-	N	+	-	N	+	-	N	+	-	N
T. halophilus 7BDE22	-	-	-	-	-	-	+	+	+	+	+	+
T. halophilus 7BDE23	-	-	-	+	+	-	+	+	+	+	+	+
T. halophilus 7BDE24	-	-	-	-	-	-	+	+	+	+	+	+
CNS												
S. saprophyticus 14BME5	-	-	-	-	W	-	+	+	+	+	+	+
S. saprophyticus 14BME6	-	-	-	-	W	-	+	+	+	+	+	+
S. saprophyticus 14BME7	-	-	-	-	W	-	+	+	+	+	+	+
S. succinus 14BME1	+	-	-	++	+	-	+	+	+	+	+	+
S. succinus 14BME2	+	-	-	++	+	-	+	+	+	+	+	+
S. succinus 14BME3	+	-	-	++	+	-	+	+	+	+	+	+
S. xylosus 7BME21	-	-	-	+	W	-	-	-	-	-	-	-
S. xylosus 7BME22	-	-	-	+	W	-	-	-	-	-	-	-
S. xylosus 7BME23	-	-	-	+	W	-	-	-	-	-	-	-

[a]The NaCl concentration in TSA is 0.5% (w/v), and others indicate the final concentrations of NaCl in the media.

Abbreviations: +, positive activity; -, negative activity; W, weak activity; N, non-growth.

and *S. succinus* was not affected by NaCl concentrations up to 14%, but acid production by *S. xylosus* was not detected. The activities of *S. saprophyticus* and *S. succinus* in 14% NaCl condition suggests that these species are actively involved in the fermentation and flavor formation of *meju* and *doenjang*.

We found that the major acid-producing bacteria in the *meju* ripening process are enterococci and staphylococci; those in the *doenjang* ripening process are staphylococci and *T. halophilus*. Our results are unique in identifying staphylococci as acid- and flavor-producing bacteria in Korean fermented soybean products.

Discussion

Culture-independent approaches to microbial community analysis have been introduced as powerful tools that provide more complete information on the microbial diversity in specimens than traditional plating methods. However, culture-dependent methods might be better for investigating the microbial succession of viable cells in food fermentations. The composition of microbial populations in *meju* and *doenjang* has been investigated by culture-dependent and -independent methods, but the succession of microbial communities from *meju* to *doenjang* and their roles in each stage have not been clearly defined. This lack of definition may be due to a lack of understanding of the food matrix and the physiological characteristics of microorganisms involved in the process. In this study, we focused on the differences in salt concentration between *meju* and *doenjang*, which reflected the differences in populations of culturable bacteria.

Bacillus was the major bacterial genus isolated during this research project, as in previous studies [3–5, 18–20, 22, 24, 29, 43]. Furthermore, in this study, *B. siamensis*, *B. licheniformis*, and *B. methylotrophicus* were the predominant species in *meju* and *doenjang* (Tables 3 and 4). *B. siamensis*, the most populous species detected, was newly registered in 2010 [41]. Shortly thereafter, this species was detected in a pyrosequencing study that identified *B. siamensis* as the predominant OTU (operational taxonomic unit) of *meju* [20]. *B. methylotrophicus*, one of the populous bacilli in this study, was not detected in previous culture-dependent studies. The *B. methylotrophicus* type strain was isolated from the rhizoplane of rice collected from Chungwon, Korea and registered in 2010 [31]. After harvesting rice, the bags, ropes, and mats weaved with rice straw are typically used for the traditional *meju* manufacturing process. The morphology and physiological characteristics of *B. methylotrophicus* are notably similar to *B. subtilis*, and the 16S rRNA genes have 99.7% identity. Therefore, the studies performed before 2010 may have identified *B. methylotrophicus* as *B. subtilis*. Based on these considerations, it is likely that *B. methylotrophicus* was transferred from the soil to *meju* and thus became one of the dominant species in *meju* fermentation. The increase of isolation frequency of *B. methylotrophicus* during the ripening of *doenjang* suggests the growth of the species in *doenjang*. This species has the option of spore formation because the growth of the species was decreased in the presence of 7% NaCl and its spore formation occurred at 14% NaCl condition (Fig. 1). The possible reason for the growth of *B. methylotrophicus* in *doenjang* may be attributed to the complexity of the *doenjang* matrix. As shown in the results of Table 6, organic substances in *doenjang* may enhance the salt tolerance of the species.

B. licheniformis and *B. subtilis* have been isolated from culture-dependent studies for fermented soybean foods in Korea [3, 5, 22, 24, 43]. *B. licheniformis* was the second most populous *Bacillus* species in this study, but only six strains of *B. subtilis* were isolated. However, these strains were not detected as predominant species in two pyrosequencing analyses [19, 20]. In a pyrosequencing analysis [19], *Bacillus sonorensis* was the most populous and most commonly identified OTU (83.8%) of nine *meju* samples, and *B. licheniformis* consisted of 0.24% of OTUs. Phenotypic characteristics, including metabolic tests with API strips, did not provide the diagnostic ability to distinguish *B. sonorensis* from *B. licheniformis* [35], and 99.2% of their 16S rRNA gene sequence is identical. Molecular taxonomic methods were effective in distinguishing these two species, and salt tolerance is a phenotypic trait distinct to *B. licheniformis* compared with *B. sonorensis*. *B. licheniformis* was the only species among the *Bacillus* isolates of this study to demonstrate growth on TSA containing 14% NaCl. Among the *B. licheniformis* isolates of this study, 66.7% of strains showed growth in 14% NaCl condition. Salt tolerance and spore formation tests confirmed the possibility of their dominance in *doenjang* (Table 5, Fig. 1). *B. sonorensis* was not identified in the *meju* and *doenjang* samples of this study and does not have enough salt tolerance to maintain its growth in *doenjang* even it would proliferate in *meju* [35]. According to the results from the other pyrosequencing study [20], *B. siamensis* OTUs were at maximum in *meju* (32.5% of total sequence) and decreased to 0.1% in *doenjang*. Conversely, *Bacillus amyloliquefaciens* OTUs were constantly increased, and it became the predominant species in *doenjang* (67.3% of total sequence). *B. amyloliquefaciens* was not detected in this study, and its existence has not been reported in other *meju* and *doenjang* studies. The type strain of *B. amyloliquefaciens* has over 98.3% 16S rRNA gene sequence identities with *B. siamensis*, *B. methylotrophicus*, *B. tequilensis*, and *B. subtilis* type strains, and they cannot be distinguished by the available phenotypic and physiological tests [41]. The differential characteristics of *B. siamensis* compared with related *Bacillus* species were its growth in acidic conditions and in the presence of NaCl up to 14% [41]. However, the growths of *B. siamensis* isolates on TSA were not decreased by the increase of NaCl to 7%, but 14% NaCl caused sporulation (Table 5, Fig. 1). The salt tolerance of *B. siamesis* may be a strain-specific trait, and the salt concentrations of *doenjang* samples of this research (12.5%

NaCl) allowed the species to grow. Considering the salt tolerance of *B. siamensis*, the species is suited for sustaining growth during *doenjang* ripening, compared with related species.

The advent of molecular taxonomy has enabled the precise separation of the species with close physiological and genetic relatedness. The recent registrations of the novel species *Bacillus aryabhattai* [40], *Bacillus aerophilus* [39], *B. methylotrophicus* [31], *B. siamensis* [41], *B. sonorensis* [35], and *B. tequilensis* [11] have influenced the results of culture-independent microbial community analyses of *meju* and *doenjang*, including in the present study. The frequent detections of *B. subtilis* and *B. licheniformis* in the early culture-dependent studies are most likely the result of the above-mentioned newly registered species detected in the recent studies [4, 18–20, 29]. However, when the culture-independent community analysis using the partial 16S rRNA gene sequence is applied, there is a possibility of misidentification within these species because of their high 16S rRNA gene sequence similarity.

Several relatives of *B. subtilis* and *B. licheniformis* originated from the environment can proliferate during the *meju* ripening period. After *meju* brining, *B. licheniformis* and *B. siamensis* may continue proliferating during the *doenjang* ripening period, while the salt concentration of *doenjang* may inhibit the growth of other bacilli. *Bacillus* species tolerant to the salt concentration of *doenjang* may continue growing, but the intolerant species may form spores. The differences in raw materials and manufacturing environment, as well as NaCl concentration, in *doenjang* may determine the proportion of each species in the bacterial community, because the traditional *doenjang* manufacturing process has been dependent on the artisan's empirical methods. Regardless of species, the involvement of bacilli in *meju* and *doenjang* production has been clearly shown by several studies; however, sporulation in high salt conditions has not previously been considered to occur during the *doenjang* ripening process. The research presented in this report showing bacilli spore formation during the *doenjang* ripening process will aid future research on *doenjang* microbial ecology.

Since the detection of LAB in *meju* was proposed [15], few studies have demonstrated their involvement in fermented soybean foods until culture-independent methods were employed. Cho and Seo [4] reported that *Leuconostoc mesenteroides*, *Lactobacillus sakei*, and *T. halophilus* were present in higher numbers than bacilli in *doenjang* and *ganjang*, by the analysis of cloned 16S rRNA gene sequences. PCR-DGGE analysis of *doenjang* also showed predominant involvement of *Lc. mesenteroides*, *T. halophilus*, and *E. faecium* [18]. In the PCR-DGGE study of *meju*, *E. durans* was detected in all *meju* samples [29]. Two pyrosequencing bacterial community studies of *meju* indicated that *E. durans* was the predominant species [19, 20]. Although several studies suggested a dominance of *E. durans* in *meju* fermentation, this report is the first describing the isolation of *E. durans* from *doenjang*. However, two *E. durans* isolates did not have sufficient salt tolerance to maintain growth in *doenjang*. In the course of bacterial selection, TSA might support the growth of cells in the culturable but inactive conditions. In our *meju* samples, the predominant *Enterococcus* species were *E. faecalis* and *E. faecium* and were mainly isolated from *meju*. Three *Enterococcus* species identified in this research have over 99.0% 16S rRNA gene sequence identity, and the high similarity can lead to a cause of misidentification in the PCR-based community analysis. *Enterococcus* spp. together with other LAB may contribute major roles in protein degradation, flavor formation, and acid production during the *meju* ripening period. The origins of *Enterococcus* species vary from environmental to animal and human sources. As enterococci are an essential part of the microflora of humans and animals, their distribution is very similar in these sources [10]. In accordance with their widespread occurrence in the intestinal tract of animals, enterococci are present in many foods, especially those of animal origin such as cheese and sausage [21]. *E. faecalis* and *E. faecium* are the most common enterococci in the human gastrointestinal tract. High frequency isolation of enterococci in the early stage of *meju* causes us to hypothesize that they are inoculated from humans in the course of mashing and molding the steamed soybean.

A halophilic LAB, *T. halophilus*, has been detected by the application of culture-independent methods with *doenjang* samples, but not in *meju* [4, 18–20]. *T. halophilus* was also isolated from *miso*-paste and known to produce acids and preferable flavors in food fermentations as well as masking offensive flavors [34]. Lipase and acid-producing activities of the isolates at the high osmotic conditions confirmed the involvement of *T. halophilus* in the flavor formation of *doenjang* during fermentation.

A *Staphylococcus* species was identified in *meju* in an early microbial study of *ganjang*, but the species was considered to be a casual contaminant [3]. Yoo *et al*. [43] isolated numbers of staphylococci from *ganjang* and identified them as *S. vitulinus* (formerly *S. vitulus*). Since the application of culture-independent microbial community analyses for fermented soybean foods, staphylococci have

been detected in several studies [4, 18–20, 29] but have only been briefly mentioned for having a role in the fermentation [4]. This oversight may have occurred because staphylococci have not been identified as a dominant species, and related species are pathogenic. We found that staphylococci constituted a non-negligible portion of the microflora of *meju* and *doenjang* (25.6% of the total number of isolates). The increase of cell counts on the TSA containing 14% NaCl during *meju* ripening was an indirect proof of their growth (Table 2). We hypothesize that the low frequency of detection of staphylococci in previous microbial community studies resulted from biases due to the media used for isolation and PCR amplification. The results of PCR-based bacterial community analyses are well-known to be subjected to bias, due to the selective extraction of nucleic acids, selective amplification of 16S rRNA gene, and the presence of dead cells. All the species of staphylococci found in this research were coagulase-negative staphylococci (CNS). CNS are widespread in nature and often present in food samples, especially in fermented products (*e.g.*, cheese and dry sausage) [2, 9, 16, 32]. These fermented foods use common raw materials from domestic animals. Thus, these species can originate from the animals, as these bacteria are part of the flora of the skin and mucous membranes of animals [12, 33]. These organisms are considered as normal flora involved in the development of organoleptic characteristics for fermented foods. Some CNS are even used as starter cultures in the production of dry fermented sausage and cheese owing to their aromatic and pigmentary/color abilities [7, 14, 38]. The common CNS isolates from *meju* and *doenjang* of both manufacturers were *S. saprophyticus* (87 strains), *S. xylosus* (18), *S. succinus* (14), and *S. warneri* (4). Among these species, *S. saprophyticus* was frequently isolated from cheese and meat products and sporadically isolated from clinical samples [8]. This reinforces our assumption that the house-microflora surviving in the environment and the equipment of processing units can be the source of CNS in *meju* and that humans are another potent source as well.

This is the first study to show active involvements of enterococci and CNS in the beginning of *meju* fermentation, CNS and *T. halophilus* in *doenjang* fermentation, and the sporulation of bacilli in the high salt environment of *doenjang*. Our conclusion is that the soybean-steaming process sterilizes their inhabiting bacteria and that the following mashing and molding by manufacturers may inoculate enterococci and CNS species from humans to *meju*, thus shaping the bacterial community migration during the ripening of *doenjang*. The house-microflora in the environment and processing units are also sources of LAB, CNS, and bacilli. During the ripening of *meju*, the number of bacilli increases rapidly. By adding *meju* to high salt brine during the *doenjang* manufacturing process, halotolerant or halophilic bacteria from sea salt are inoculated. Over 18% NaCl concentration in the brine renders *T. halophilus* predominant, the CNS survive, a portion of bacilli sporulate, and the LAB counts are reduced. In the process of *doenjang* ripening, *T. halophilus* may play significant roles in acid and flavor production; the bacilli can survive under the osmotic conditions of *doenjang*, and their halotolerant relatives maintain their viability. The decrease of CNS numbers during *doenjang* ripening is in question because CNS can sustain their viability and acid-producing ability in high salt conditions. The population of CNS may be influenced by the final salt concentration of *doenjang* after brining, because the growth of bacilli may influence the proportion of CNS. These results can contribute to the development of starters for massive *doenjang* manufacturing process standardization.

Acknowledgments

This research was supported by the R&D Convergence Center Support Program, Ministry of Agriculture, Food and Rural Affairs, Republic of Korea and by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2012R1A1A2039955).

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