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Pyrosequencing-Based Analysis of the Bacterial Community in Korean Traditional Seafood, *Ojingeo Jeotgal*

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Jeotgal fermentation is dependent upon a diverse microbial community, although a detailed understanding of its microbial composition is limited to a relatively small number of *jeotgal*. Pyrosequencing-based bacterial community analysis was performed in fermented squid, *ojingeo jeotgal*. *Leuconostoc* was identified as the predominant bacterial genus, with *Bacillus* and *Staphylococcus* also accounting for a large proportion of the bacterial community. Phylogenetic analysis with 16S rRNA genes of *Leuconostoc* type species indicated that *L. citreum*- and *L. holzapfelii*-like strains could be the major *Leuconostoc* strains in *jeotgal*. High concentrations of NaCl were thought to be an important factor determining the makeup of the bacterial community in the fermented squid; however, a genomic survey with osmotic stress-related genes suggests the existence of more complex factors selecting the dominant bacterial species in fermented squid.

Keywords: *Staphylococcus*, *Leuconostoc*, *ojingeo jeotgal*, pyrosequencing-based bacterial community analysis

Fermented food has held an important place in human diet, and it is widely produced and consumed in a variety of countries [18]. Because the fermentation process is entirely driven by the metabolic actions of microorganisms, there have been numerous studies performed to understand the relationship between microorganisms and food fermentation *via* the isolation of novel species [12, 21], genome sequencing of food isolates [10], and analysis of metabolites and the microbial community [9, 15, 17]. General features of food fermentation can be summarized from those studies as follows: (i) Biochemical characteristics of fermented food such as pH and metabolite profiles are significantly different from the beginning of the fermentation. (ii) The succession of the bacterial community results a simpler composition and a predominant species appears. The predominant species in the fermented food is considered more competitive than other minor group of microorganisms, regarding the efficient utilization of nutritional sources, resistance to diverse stresses, and competition with other microbial communities [20].

Jeotgal is a Korean traditional fermented food. There are many types of *jeotgal* depending on the main ingredient, such as squid, shrimp, oyster, fish, and fish eggs. They are seasoned with various kinds of vegetables, red pepper, ginger, sesame, and garlic. The most important feature is the high concentration of salt (10–20%) [8]. Culture-dependent bacterial community analysis of *jeotgal* determined that the genera *Staphylococcus*, *Bacillus*, *Halomonas*, and *Kocuria* were major constituents of the *jeotgal* microbial community. Viable bacterial cell counts of these communities were approximately 10^3–10^5 colony-forming units per gram [8]. The growth of *Staphylococcus* and *Virgibacillus* isolated from *jeotgal* was confirmed in high-NaCl condition [8]. Culture-independent analysis of the bacterial community of our unpublished data and other previous studies indicated that lactic acid bacteria were also frequently dominant. Metabolite analysis of *jeotgal* and *jeotgal*-containing fermented foods indicated that sugars and amino acids, including fructose, glucose, alanine, glycine, and valine, were of major metabolites and their concentrations were changed during the fermentation processes [11].

The aims of this study were to investigate the bacterial

community of Korean traditional fermented squid, *ojingeo jeotgal* by culture-independent methods. Any microbiological analysis has not been investigated in fermented squid. To achieve our research purpose, we analyzed the bacterial community of fermented squid *via* pyrosequencing of the 16S rRNA gene.

The general recipe for fermented squid is as follows; the skin of the squid was peeled off and 10% (w/w) coarse salt was spread on the squid to ripen for 1 day. After ripening of the squid, it was cut into pieces and seasoned with 3% (w/w) fish sauce, 3% (w/w) red pepper powder, 3% (w/w) sesame, 3% (w/w) scallion, 4% (w/w) crushed garlic, 10% (w/w) chopped pear, 12% (w/w) chopped white radish, and 14% (w/w) starch syrup. The mixed ingredients were fermented at room temperature for 5 days and stored at 4°C. Because there could be subtle differences in the recipe by regions, we purchased *jeotgal* samples from five different manufacturers in Seoul, Korea and mixed them into a sample.

To analyze the culture-independent microbial community of fermented squid, the total genomic DNA was extracted twice from a pellet obtained from 50 ml of fermented squid filtrate using a NucleoSpin Soil kit (Macherey-Nagel, Germany) according to manufacturer instructions, and the concentrations of the extracted genomic DNA were measured with a Nanodrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, USA). The V1 to V3 hypervariable regions of the bacterial 16S rRNA gene from the genomic DNA were amplified using primers V1-27F (5′-CCTATCCCCTGTGTG CCTTGGCAGTC-TCAG-AC-GAGTTTGATCMTGGCTCAG-3′) and V3-541R (5′-CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TCAGATG-AC-WTTACCGCGGCTGCTGG-3′ and 5′-CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-ATAGCT CTCG-AC-WTTACCGCGGCTGCTGG-3′) [2, 16]. All of the polymerase chain reactions (PCR) were conducted in a MyCycler Thermal Cycler (Bio-Rad, USA) in 20 μl volumes containing 30 ng of genomic DNA, 50 pmol of each primer, and Han-Taq (Genenmed, Republic of Korea) polymerase. The following PCR protocol was used: 94°C for 90 sec (1 cycle); 94°C for 45 sec, 55°C for 45 sec, and 72°C for 45 sec (30 cycles); and 72°C for 5 min (1 cycle). Pyrosequencing of the PCR Product was performed by Macrogen (Korea) using a 454 GS FLX Titanium system (Roche, Germany). To assess bacterial species richness estimators, diversity indices and rarefaction curves, we applied the pyrosequencing pipeline (http://pyro.cme.msu.edu) of the Ribosomal RNA Database Project (PCR) [3].

A total of 566 reads resulted from a single run. The Shannon and Chao1 indices were 2.45 and 42, respectively, which were close to the previously determined bacterial diversity from other fermented seafoods [1, 16, 18, 19]. Although the number of reads from the pyrosequencing data was small, a rarefaction curve approached to a plateau, indicating that pyrosequencing results reflected the composition of the whole bacterial community (Fig. 1). The phylogenetic classification of bacterial phyla and genera from the fermented seafood is summarized in Fig. 2. Six phyla – Proteobacteria (37.1%), Firmicutes (32.9%), Actinobacteria (14.6%), Cyanobacteria (13.3%), Bacteroidetes (1.8%), and Deinococcus-Thermus (0.4%) – were identified. The predominant genus was *Leuconostoc* (37.6%), followed by *Bacillus* (11.0%), *Staphylococcus* (9.9%), and *Psychrobacter* (8.0%). *Leuconostoc* was frequently identified as a predominant genus of another Korean traditional fermented food, *kimchi* (fermented Chinese cabbage), and used as a fermentation starter culture in a commercial product [11]. It is of note that the bacterial community of *kimchi* was usually taken up by only a small number of genera such as *Leuconostoc*, whereas *jeotgal* often contained a number of minor groups of bacteria [8]. Therefore, squid fermentation was considered as a result of a more complicated interaction between bacterial communities.

Since the PCR did not assign the sequence reads to the species level, we performed phylogenetic analysis of *Leuconostoc*-assigned reads with the previously described *Leuconostoc* type species, as it is the predominant genus and frequently identified in other fermented foods. *Leuconostoc*-associated reads were extracted from the total sequencing data and aligned with 16S rRNA gene sequences of *Leuconostoc* type species. Unaligned sequences were trimmed and 450 bp was used for a neighbor-joining phylogenetic

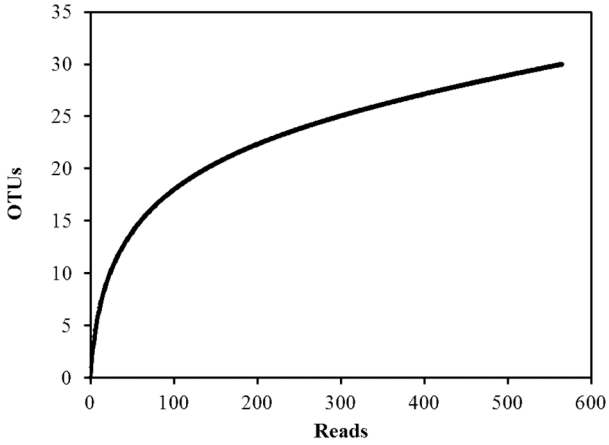

Fig. 1. Rarefaction curve of the bacterial community from fermented squid.

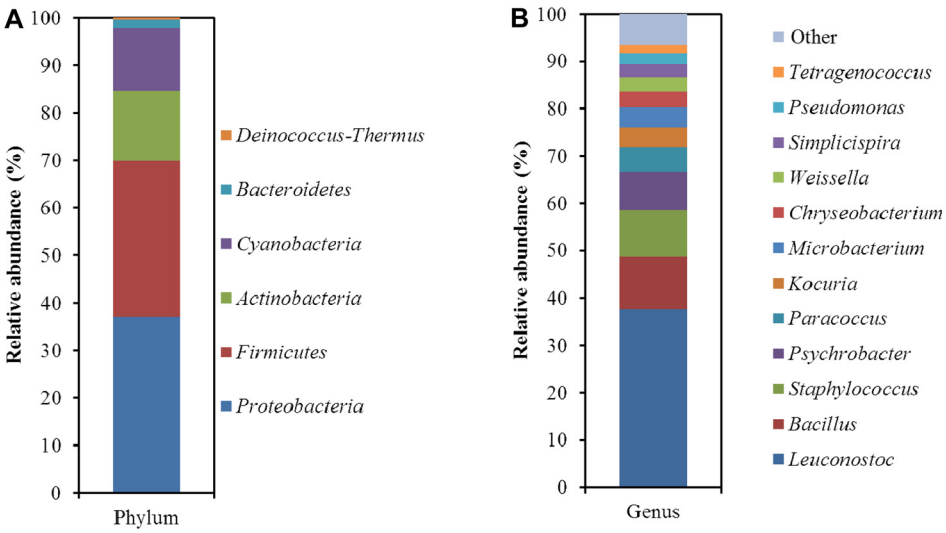

Fig. 2. Bacterial community composition of fermented squid.
(**A**) Phylum and (**B**) genus. Genera with <1% were included in the Other category. Unidentified bacterial sequences were not included in relative abundance.

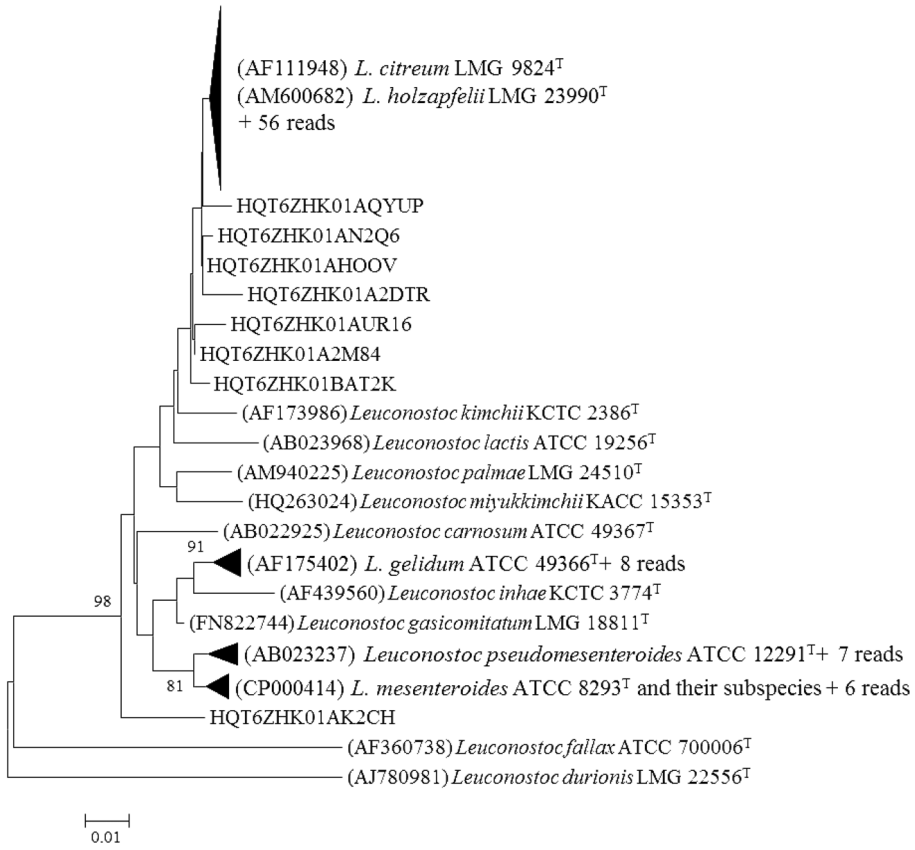

Fig. 3. Phylogenetic analysis of *Leuconostoc*-associated sequencing reads with 16S rRNA gene sequences of *Leuconostoc* type species.
The tree was constructed using the neighbor-joining method. Bootstrap values over 70 are shown. Bar, 0.01 changes per nucleotide position. GenBank accession numbers are in parenthesis.

tree. Sequence alignment and construction of a phylogenetic tree were performed using MEGA 5. The phylogenetic tree shown in Fig. 3 demonstrates that the *Leuconostoc* identified in fermented squid is primarily associated with *L. citreum* and *L. holzapfelii*. A small portion of sequencing reads were related to *L. gelidum*, *L. pseudomesenteroides*, and *L. mesenteroides*. *L. mesenteroides* is used as a starter culture in *kimchi* fermentation for commercial products, and its effect on *kimchi* production was previously evaluated [11]. Although *Leuconostoc* was identified as the predominant species in fermented squid and other fermented food, taxonomic affiliation at the species level could be different by kinds of fermented foods.

High concentrations of NaCl can be an important characteristic to determine the bacterial community in fermented seafood. Therefore, we reasoned that halotolerant bacterial species are likely to be the dominant species in *jeotgal*. To verify our hypothesis, we investigated the

Table 1. Distribution of potassium uptake system, glycine betaine transport and biosysnthesis, and proline biosynthesis genes in the three most abundant genera and three rarest genera of fermented squid.

		Abundant genera																						Rare genera											
		Leuconostoc						*Bacillus*						*Staphylococcus*						*Enterococcus*					*Halanaerobium*		*Enterobacter*									
		Leuconostoc sp. C2	*L. citreum* KM20	*L. gasicomitatum* LMG 18811	*L. gelidum* JB7	*L. kimchii* IMSNU11154	*L. mesenteroides* subsp. *mesenteroides* ATCC 8293	*B. cellulosilyticus* DSM 2522	*B. cereus* ATCC 10987	*B. halodurans* C-125	*B. licheniformis* ATCC 14580	*B. subtilis* subsp. *subtilis* str. 168	*B. amyloliquefaciens* DSM 7	*Staphylococcus* sp. OJ82	*S. aureus* subsp. *aureus* ED133	*S. epidermidis* ATCC 12228	*S. haemolyticus* JCSC1435	*S. saprophyticus* subsp. *saprophyticus* ATCC 15305	*S. pseudintermedius* ED99	*E. casseliflavus* ATCC 12755	*E. faecalis* PC1.1	*E. faecium* DO	*E. hirae* ATCC 9790	*E. italicus* DSM 15952	*H. hydrogeniformans*	*H. praevalens* DSM 2228	*E. asburiae* LF7a	*E. aerogenes* KCTC 2190	*E. cancerogenus* ATCC 35316	*E. hormaechei* ATCC 49162	*E. cloacae* subsp. *cloacae* ATCC 13047
K^+ uptake	KdpA	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+	-	+	+	-	-	-	-	-	-	+	+	+	+	+
	KdpB	-	-	-	-	-	-	+	+	-	-	-	-	-	+	-	+	-	+	+	-	-	-	-	-	-	+	+	+	+	+
	KdpC	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	-	+	+	-	-	-	-	-	-	+	+	+	+	+
	TrkG	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
	TrkH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
	KtrA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
	KtrB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
Glycine betaine transport and synthesis	BetT	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	+	+	+
	ProV	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	ProW	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	ProX	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	+	+
	BetA	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+
	BetB	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+
	GbsA	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-
	GbsB	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-
	OpuA	-	-	-	-	-	-	-	-	-	+	+	+	+	-	+	-	-	-	+	+	+	-	+	-	-	-	-	-	-	-
	OpuB	-	-	-	-	-	-	-	-	-	+	+	+	+	-	+	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-
	OpuC	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-
	OpuD	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-
Proline biosynthesis	ProH	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	ProJ	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+

The presence and the absence of the homologs are marked with + and -, respectively.

presence of potassium uptake, glycine betaine transport and biosynthesis, and proline biosynthesis. Uptake of potassium ion is known as the first response to acute osmotic stress [5]. Glycine betaine and proline can act as compatible solutes. We selected 18 genomes and 12 genomes from the three most abundant genera and three rarest genera, respectively. A BLASTP search used reference sequences from *E. coli*, *Bacillus,* and *Staphylococcus* species, because previously published studies have confirmed their relatedness to osmotic stress [7]. Our results suggested the prevalence of osmotic stress-related genes in all tested genomes, regardless of their abundance in the fermented squid (Table 1). However, *Leuconostoc* species did not possess homologous osmotic stress-related genes, such as potassium transport system genes (KdpABC and TrkGH), glycine betaine transport and biosynthesis genes (BetT, ProX, and BetAB) that we investigated. This may indicate that *Leuconostoc* possesses an alternate osmotic-stress defense system with low sequence identity. Alternatively, the *Leuconostoc* physiology may be well adapted to the high-NaCl fermentation environment in the absence of osmotic stress-related genes. Therefore, osmotic stress did not have strong selective pressure on the bacterial community in the fermented squid.

The scientific understanding of microbial communities in fermented seafood is insufficient, even though the microbial composition of fermented foods has a substantial positive impact on the gut microbial community, nutrition, and overall health in humans [6]. Our pyrosequencing-based community analysis demonstrated that the dominant genera of bacteria in fermented seafood are *Leuconostoc*, *Bacillus*, and *Staphylococcus*. *Leuconostoc*, *Chryseobacterium*, *Pseudomonas*, *Ochrobactrum*, and *Enterobacter* were frequently identified in both *kimchi* and *jeotgal*, whereas *Staphylococcus*, *Kocuria*, *Tetragenococcus*, *Brachybacterium*, *Deinococcus*, and *Enterococcus* are usually identified in *jeotgal* [8, 14]. Interestingly, our unpublished culture-dependent community analysis data determined that many isolates were identified as *Staphylococcus*, although *Staphylococcus* was the third predominant genus. A previous culture-dependent community analysis of *jeotgal* samples reported that *Staphylococcus* isolated from *jeotgal* did not possess proteolysis activity; however, *Staphylococcus* were described as "too numerous to ignore" [8]. Many other studies have also shown that culturable isolates from fermented seafood were predominantly *Staphylococcus* species [4, 8, 12, 13]. Therefore, *Staphylococcus* appeared to be relatively easily isolated in laboratory culture conditions compared with the other predominant species.

To our best knowledge, our study is the first report on the bacterial community in traditional Korean fermented seafood (*ojingeo jeotgal*). The bacterial community was dominated by *Leuconostoc*, whereas other bacterial groups were maintained in small proportions. Detailed phylogenetic analysis of *Leuconostoc*-assigned sequencing reads indicated that *L. citreum*- and *L. holzafelii*-like strains could be the dominant strains, unlike the bacterial community of *kimchi*. A genome survey conducted with osmotic stress-related genes showed that osmotic stress may not be the primary factor shaping the bacterial community. More detailed genomic and transcriptomic analyses with the fermented seafood will be performed to understand the fermentation microbiome.

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