

In situ analysis of the bacterial community associated with the Korean salty fermented seafood jeotgal

Hyunjun Kim, Yoomin Ahn, Chulhee Park and Eungbin Kim*

Department of Systems Biology, Yonsei University, Seoul 03722, Republic of Korea

*Corresponding author

Eungbin Kim

Tel. 02-2123-2651

E-mail. eungbin@yonsei.ac.kr

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Abstract: Jeotgal is a salty and fermented traditional Korean fish sauce. Unlike most other previous studies that investigated samples purchased from retail markets, this study focused on samples of jeotgal with traceable history to Yeonggwang, a time-honored fishing village in Korea. Three jeotgal samples, which were made from small yellow croakers, largehead hairtail, and miscellaneous fish, were selected based on information obtained from interviews with local craftsmen and literature reviews. Bacterial community profiles of the three jeotgal samples were investigated to identify indicator (and potentially core) bacteria for jeotgal ripening. The 16S rRNA gene-based metagenomic analysis revealed that the dominant phyla and classes, (Gammaproteobacteria, Betaproteobacteria, Bacilli, and Clostridia) of the three different jeotgal were identical, albeit with different composition ratios. Diversification was evident beginning at the order level. Interestingly, each dominant order was mainly comprised of single members even at the genus level. The dominant genera included *Halomonas*, *Tetragenococcus*, *Halanaerobium*, *Pseudomonas*, *Massilia*, and *Lentibacillus*. This observed genus-level heterogeneity suggests that there are diverse bacterial signatures in jeotgal and that these can be used as indicators for jeotgal ripening and/or as starters to increase its sensory quality and functionality.

Keywords: jeotgal, 16S rRNA gene, metagenome, halophilic bacteria, fermentation

INTRODUCTION

Jeotgal (or jeot) is a salty and fermented traditional seasoning that is essential in a variety of Korean cuisine. There are many jeotgal types, depending on the raw materials and preparation methods. Jeotgal varieties are usually used to enhance the taste of a dish. For example, these seasonings are added as condiments to pickled kimchi and used in some Korean soups and stews instead of salt or soy sauce. Jeotgal is also served as a side dish or a dipping sauce. Jeotgal is prepared from a diverse variety of seafood, such as various fishes, shrimps, oysters, and shellfish, and is produced via fermentation by the combined action of microorganisms and

self-digestion. During fermentation, which lasts for several months to years, jeotgal acquires a unique taste and flavor derived from the various enzymatic reaction products, such as amino acids, fatty acids (palmitic acid, oleic acid, linoleic acid, eicosapentaenoic acid [EPA], and docosahexaenoic acid [DHA]), and small molecule peptides (Shim *et al.* 2017; Jung *et al.* 2018). This composition is rich in essential nutrients, including amino acids (Glu, Arg, and Leu), minerals (sodium, calcium, and potassium), and fatty acids (palmitic acid, oleic acid, linoleic acid, EPA, and DHA; Song *et al.* 2018), which increase the nutritional value of jeotgal.

During the past decade, studies have attempted to cha-

racterize microbial communities in various jeotgal using culture-dependent and culture-independent methods, and these studies have indeed identified many bacterial and archaeal species. As summarized in Table 1, although halophilic bacilli are most prevalent in jeotgal, several novel species have been isolated. It has been reported that generally the dominant bacterial group in jeotgal changes from the phylum Proteobacteria at the beginning stage to the phylum Firmicutes at the maturing stage (Koo *et al.* 2016). When making jeotgal, salts are essential ingredients because they inhibit spoilage by microorganisms during fermentation. The final salt concentration of jeotgal is generally 20–30% (w/w) to prevent putrefaction (Kim *et al.* 2019). In addition to their antifouling function, salts have important effects on the microbial community composition of jeotgal (Shim *et al.* 2017). Until now, most research on jeotgal has been conducted with samples purchased from retail markets. In these samples, it is rarely possible to

specify the source of the salt, which likely exerts remarkable effects on the microbial community of jeotgal. In order to obtain samples of jeotgal with traceable history, we searched for local producers that adhere to traditional craftsmanship processes to make jeotgal using local catch and salt.

Yeonggwang (longitude 126°39'–125°59' E and latitude 35°10'–35°26' N), a famous fishing village in Korea, was determined to be the optimal place to find such a jeotgal producer according to the National Federation of Fisheries Cooperatives. Also, Yeonggwang is the key production site of sun-dried salt in Korea. Interviews with the local craftsmen as well as literature review led us to examine the bacterial community of jeotgal from this village. In this study, we characterized and compared the bacterial communities in three kinds of jeotgal indigenous to Yeonggwang, providing insight into the variations of jeotgal microbiota and the role of these communities in jeotgal fermentation.

Table 1. Selected bacterial species isolated from various jeotgal samples

Jeotgal	Isolates	Reference
Jeone (gizzard shad)	<i>Proteus cibarius</i>	Hyun <i>et al.</i> 2016
Jogae (clam)	<i>Lelliottia jeotgali</i>	Yuk <i>et al.</i> 2018
	<i>Weissella jogaejeotgali</i>	Lee <i>et al.</i> 2015
Myeolchi (anchovy)	<i>Salimicrobium jeotgali</i>	Choi <i>et al.</i> 2014
	<i>Staphylococcus saprophyticus</i>	Lee <i>et al.</i> 2019
	<i>Tetragenococcus halophilus</i>	Kim <i>et al.</i> 2018
Ojingeo (squid)	<i>Bacillus</i> sp. SJ-10	Lee <i>et al.</i> 2015
	<i>Psychrobacter alimentarius</i>	Yoon <i>et al.</i> 2005
	<i>Staphylococcus</i> sp. strain OJ82	Sung <i>et al.</i> 2012
Meonggae (sea squirt)	<i>Bacillus velezensis</i> BS2	Yao <i>et al.</i> 2019
	<i>Lentibacillus alimentarius</i>	Sundararaman <i>et al.</i> 2018
	<i>Paenibacillus tyraminigenes</i>	Mah <i>et al.</i> 2008
	<i>Virgibacillus jeotgali</i>	Sundararaman <i>et al.</i> 2017
Saeu (shrimp)	<i>Bacillus subtilis</i> JS2	Yao <i>et al.</i> 2018
	<i>Brevibacterium jeotgali</i>	Choi <i>et al.</i> 2013
	<i>Carnobacterium jeotgali</i> MS3	Whon <i>et al.</i> 2015
	<i>Halakalicoccus jeotgali</i>	Roh <i>et al.</i> 2007
	<i>Halomonas cibimaris</i>	Jeong <i>et al.</i> 2013
	<i>Haloterrigena jeotgali</i>	Cha <i>et al.</i> 2015
	<i>Kocuria atrinae</i> C3-8	Nam <i>et al.</i> 2012
	<i>Leucobacter salsicium</i>	Yun <i>et al.</i> 2011
	<i>Natronococcus jeotgali</i>	Roh <i>et al.</i> 2007
	<i>Staphylococcus equorum</i> KS1039	Jeong <i>et al.</i> 2016

MATERIALS AND METHODS

1. Jeotgal preparation

The freshly caught fish was covered with local sun-dried salt to a thickness of 2–3 cm immediately on board. On arrival at the fermentation house, the pre-salted fish were dumped into low-density polyethylene bags that were placed in polyethylene barrels (220 L), and salt was added at 20% of the fish weight. Fermentation was performed in tied bags under ambient temperature for 2 years. The pH of jeotgal was measured using a pH electrode (Orion 8157BNURCA; Thermo Scientific, USA), and salinity was calculated using a refractometer (RSM-1000; HM Digital, Korea). The refractometer has a limit at 28% NaCl, so the results were obtained following dilution of the jeotgal.

2. DNA extraction and sequencing

Metagenomic DNA was extracted from the jeotgal using NucleoSpin kit for soil (Macherey-Nagel, Düren, Germany). About 0.5 g jeotgal was used for DNA extraction following homogenization of the fish and liquid in jeotgal in a blender. For bacterial amplification, 341F and 805R primers were used, and i5 forward primer and i7 reverse primer were used for secondary amplification for attaching the Illumina NexTera barcode. After quality control, sequencing of the V3-V4 region from 16S rDNA was performed by Chunlab Inc. (Seoul, Korea) using the Illumina MiSeq platform. Primers sequences were trimmed using the Chunlab program cut-off of 0.8. Bioinformatics analysis was performed using EzBioCloud program (Chunlab Inc.), which uses USEARCH to detect chimeras on reads that have < 97% similarity.

3. Statistical analyses

The Shannon index is an indicator of species evenness (proportional distribution of the number of each species in a sample) and is represented by values greater than 0 (Magurran 2003). Chao1 is a species richness (total number of species in a sample) indicator that is sensitive to rare OTUs (operational taxonomic units), and higher values indicate higher diversity (Chao 1987). The Simpson index is a species evenness (proportional distribution of the number of each species in a sample) indicator that displays the probability that two randomly selected sequences are of the same species (Magurran 2003).

RESULTS AND DISCUSSION

Small yellow croakers (Jogi in Korean, *Pseudosciaena polyactis*) annually migrate to the Yellow Sea to spawn in the spring. The spawning season spans April to June, with a peak in May, when passing Chilsan in the coastal waters of Yeonggwang (Kim *et al.* 2008). For the past 1,000 years, people in Yeonggwang have prepared jogi-jeot, the specific type of jeotgal made from small yellow croakers, every June as described in the Materials and Methods. During fermentation, inflation-deflation cycles were observed in the fermentation bags. The inflation began in July and lasted through September with a peak in the middle of August. The correspondence of the cycle with temperature curves is apparently due to higher microbial activity, including gas production, under warmer conditions. For microbiological analysis, 300 mL liquid samples were scooped directly from 2-month-old (2MF) and 2-year-old (2YF) fermentation bags. The salinity and pH were measured as 22.5‰ and 5.7 for 2MF and 28.8‰ and 6.6 for 2YF, respectively.

Metagenomic DNA was isolated directly from each homogenized sample, and the 16S rRNA gene was amplified and sequenced as described in the Materials and Methods. Application of the conventional sequence similarity cut-off of 97% on 16S amplicons identified a total of 359 OTUs in the 2YF sample. Subsequent taxonomic classification of OTUs showed that the two phyla Proteobacteria (57.9%) and Firmicutes (41.9%) accounted for more than 99% of the OTUs. Five additional phyla with relative abundance of less than 1% of the total abundance were identified: Bacteroidetes, Actinobacteria, Fusobacteria, Rhodothermaeota, and Acidobacteria. The Proteobacteria almost solely consisted of class Gammaproteobacteria (57.8%). Furthermore, single members were detected almost exclusively at the order, family, and even genus levels: Oceanospirillales (55.7%), Halomonadaceae (55.7%), and *Halomonas* (55.5%). The Firmicutes was further split into two classes, Bacilli (37.6%) and Clostridia (4.3%). Each class was also predominantly occupied by single members at the order, family, and genus levels: Lactobacillales (37.6%), Enterococcaceae (37.4%), and *Tetragenococcus* (37.4%), respectively, and Halanaerobiales (4.3%), Halanaerobiaceae (4.3%), and *Halanaerobium* (4.2%), respectively.

In the 2MF sample, a total of 572 OTUs were identified. Further taxonomic examination of the bacterial community revealed that 2MF was populated by four phyla: Proteobacteria (86.3%), Firmicutes (8.1%), Bacteroidetes (2.6%), and Actinobacteria (2.1%). Phyla comprising less

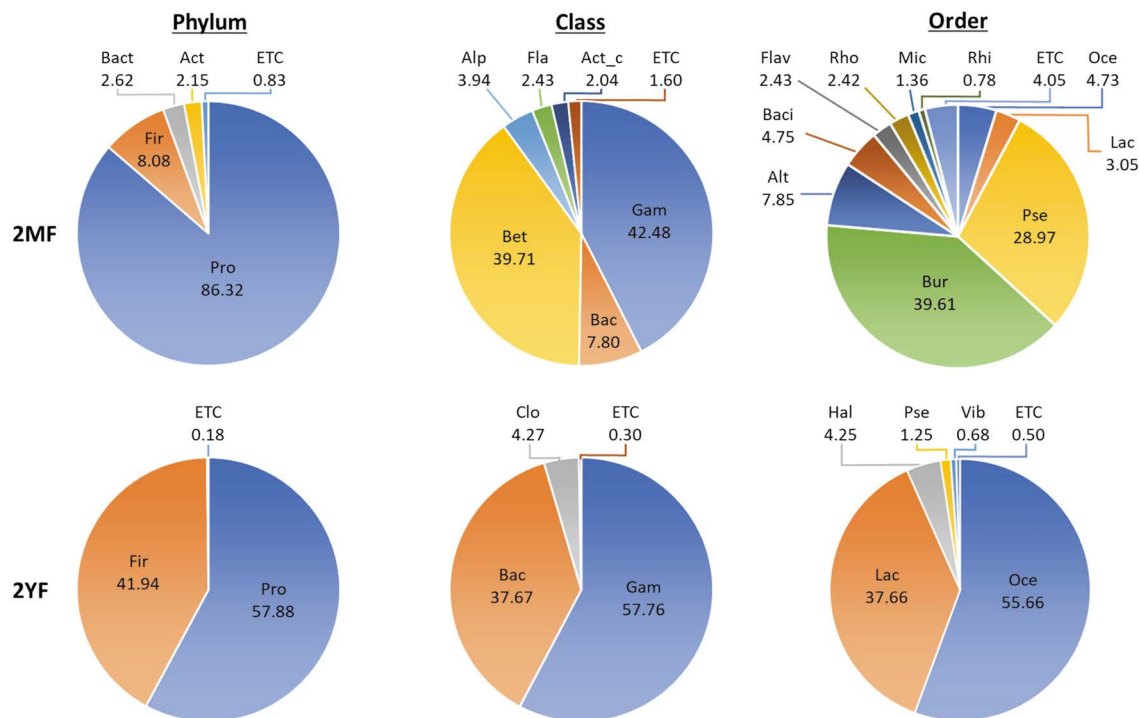


Fig. 1. Bacterial composition at the phylum, class, and order levels in two-month and two-year-fermented jogi-jeot. ETC indicates the sum of the remaining minor taxa. Numbers in parentheses indicate the percentage of each taxon at the corresponding taxonomic rank. Abbreviations: 2MF, 2-month-old fermentation; 2YF, 2-year-old fermentation. Abbreviations: (Phylum) Pro, Proteobacteria; Act, Actinobacteria; Bact, Bacteroidetes; Fir, Firmicutes; (Class) Alp, Alphaproteobacteria; Bet, Betaproteobacteria; Gam, Gammaproteobacteria; Fla, Flavobacteria; Act_c, Actinobacteria_c; Bac, Bacilli; Clo, Clostridia; (Order) Pse, Pseudomonadales; Bur, Burkholderiales; Alt, Alteromonadales; Baci, Bacillales; Flav, Flavobacteriales; Rho, Rhodobacterales; Mic, Micrococcales; Rhi, Rhizobiales; Oce, Oceanospirillales; Lac, Lactobacillales; Hal, Halanaerobiales; Vib, Vibrionales; Clos, Clostridiales.

than 1% of the total bacterial community were identified as Planctomycetes, Parcubacteria_OD1, Rhodothermaeota, Acidobacteria, Saccharibacteria_TM7, and Cyanobacteria. The higher bacterial diversity of 2MF is visualized in Fig. 1. In addition, the Shannon index, which estimates the diversity of the species present in a sample, of 2MF was 3.153, and this value was more than two times greater than that of 2YF (1.386), confirming that the bacterial diversity is much higher in 2MF than 2YF.

Members of Firmicutes were increased 5-fold, while Proteobacteria members were decreased by almost half over the 2 y. Also, the population sizes of Bacteroidetes and Actinobacteria were reduced to 0.14% and 0.02%, respectively, in the 2YF samples. It is generally assumed that during fermentation, the bacterial community shifts from Proteobacteria to Firmicutes, which is thus considered to be a common indicator for jeotgal ripening (Rho *et al.* 2010). Since our results reinforced this assumption, we then searched for such an indicator bacteria at the genus level. Com-

parison of members of Proteobacteria and Firmicutes at the genus level demonstrated that the dominant bacterial members were totally different (Table 2). The three genera, *Halomonas*, *Tetragenococcus*, and *Halanaerobium*, represented a total of 97.1% of the Proteobacteria in the 2YF samples. Notably, these three genera were present at negligible percentages in the 2MF samples: 0.27% for *Halomonas* and undetectable for *Tetragenococcus* and *Halanaerobium*.

Bacteria in jogi-jeot can come from the fish itself, from the added salt, from the seawater, and even from the handlers. The initial diverse community of bacteria was then subjected to increased osmotic pressure and decreased water activity, and this condition would exert a filtering effect on the species that can colonize and persist in the jogi-jeot. In order to gain a more comprehensive understanding of this filtering effect during jeotgal fermentation, two other indigenous jeotgal, 2-year-fermented jap-jeot and galchi-jeot were chosen for taxonomic analysis. Unlike most other jeotgal, jap-jeot, is made from the miscellaneous fish that

Table 2. Comparison of dominant members of Proteobacteria and Firmicutes in 2MF and 2YF jogi-jeot

	Phylum	Class	Order	Genus
2MF	Proteobacteria (86.3)	Gammaproteobacteria (42.5)	Pseudomonadales (29.0)	<i>Psychrobacter</i> (27.9)
			Alteromonadales (7.9)	<i>Pseudoalteromonas</i> (6.0)
			Oceanospirillales (4.7)	<i>Chromohalobacter</i> (4.3)
	Firmicutes (8.1)	Bacilli (7.8)	Burkholderiales (39.6)	<i>Ralstonia</i> (37.9)
			Bacillales (4.7)	<i>Alkalibacillus</i> (4.0)
			Lactobacillales (3.1)	<i>Lactobacillus</i> (1.1) <i>Streptococcus</i> (0.9) <i>Carnobacterium</i> (0.8)
2YF	Proteobacteria (57.9)	Gammaproteobacteria (57.8)	Oceanospirillales (55.7)	<i>Halomonas</i> (55.5)
	Firmicutes (41.9)	Bacilli (37.7)	Lactobacillales (37.7)	<i>Tetragenococcus</i> (37.4)
		Clostridia (4.3)	Halanaerobiales (4.3)	<i>Halanaerobium</i> (4.2)

Numbers in parentheses indicate percentage of each taxon at the corresponding taxonomic rank. Abbreviations: 2MF, 2-month-old fermentation; 2YF 2-year-old fermentation

have almost no commercial value due to a variety of reasons, such as small size and minor damage. *Jap* means “mixed” or “miscellaneous” in Korean, thus the name itself suggests the hybrid ingredients. Largehead hairtail, which are also called cutlass fish (Galchi in Korean, *Trichiurus lepturus*) migrate annually in the spring and spawn in the summer in the Yellow Sea. These fish are caught mainly between July and November.

Using the same method as described for the jogi-jeot, a total of 393 and 490 OTUs were identified in jap-jeot and galchi-jeot, respectively. Taxonomic analysis of the bacterial communities showed that the prevalence of Actinobacteria (5.3%) in jap-jeot was more than 250-fold greater than that in jogi-jeot, although a dominance of Proteobacteria (47.9%) and Firmicutes (45.8%) was observed in these jeotgal as well. It is also noteworthy that in addition to all the infrequent phyla detected in jogi-jeot (Bacteroidetes, Acidobacteria, Fusobacteria, and Rhodothermaeota), jap-jeot harbored *Deinococcus-Thermus*. Meanwhile, galchi-jeot was overwhelmingly dominated by phylum Firmicutes (93.1%) with only a small percentage of Proteobacteria (4.9%; Fig. 2). As summarized in Table 3, the dominant phyla and classes of these two jeotgal were found to be almost the same as those of jogi-jeot, albeit with different composition ratios. However, diversification between the samples was present at order level, and each dominant order

was predominantly occupied by single members even at the genus level. The dominant genera were *Halomonas*, *Tetragenococcus*, *Halanaerobium*, *Pseudomonas*, *Massilia*, and *Lentibacillus*. This genus-level heterogeneity suggests that there are diverse bacterial signatures in jeotgal.

From a microbiological point of view, the 2-year fermentation corresponds to a 2-year enrichment culture, which would select for some bacteria and against others. Considering that the preparation methods were basically the same for all three jeotgal examined in this study, the notable difference among the dominant bacterial species seemingly resulted from fish ingredients rather than the culture conditions. In recent years, jeotgal has been considered a rich repository of novel and useful microorganisms, mainly because this fermentation product is produced from various seafood types under region-specific fermentation conditions. In fact, in order to exploit the beneficial attributes of jeotgal, efforts have been made to isolate and characterize autochthonous bacteria from jeotgal.

The use of proper starter cultures to produce jeotgal is attracting increasing interest, as such cultures could not only increase product consistency and microbiological quality but also shorten the fermentation time. Furthermore, these cultures can improve the sensory characteristics of jeotgal. The Korean traditional fermented fish product, jeotgal, is one of the best preservation methods of raw seafood to pro-

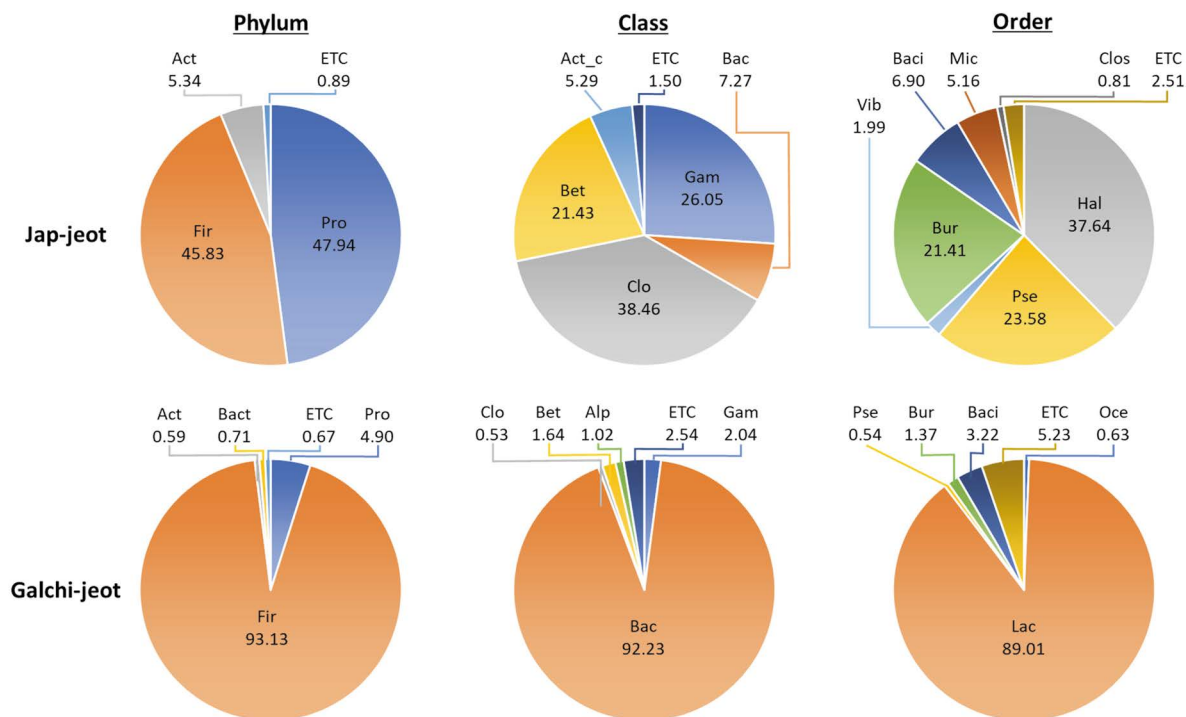


Fig. 2. Bacterial composition at the phylum, class, and order levels in jap-jeot and galchi-jeot. ETC indicates the sum of the remaining minor taxa. Numbers in parentheses indicate the percentage of each taxon at the corresponding taxonomic rank. Abbreviations: (Phylum) Pro, Proteobacteria; Act, Actinobacteria; Bact, Bacteroidetes; Fir, Firmicutes; (Class) Alp, Alphaproteobacteria; Bet, Betaproteobacteria; Gam, Gammaproteobacteria; Fla, Flavobacteria; Act_c, Actinobacteria_c; Bac, Bacilli; Clo, Clostridia; (Order) Pse, Pseudomonadales; Bur, Burkholderiales; Alt, Alteromonadales; Baci, Bacillales; Flav, Flavobacteriales; Rho, Rhodobacteriales; Mic, Micrococcales; Rhi, Rhizobiales; Oce, Oceanospirillales; Lac, Lactobacillales; Hal, Halanaerobiales; Vib, Vibrionales; Clos, Clostridiales.

Table 3. Comparison of Proteobacteria and Firmicutes bacteria in jap-jeot and galchi-jeot

Phylum		Class - Order - Family - Genus			
Jap-jeot pH 5.4 Salinity 36%	Proteobacteria (47.9)	Gammaproteobacteria - Pseudomonadales - Pseudomonadaceae - <i>Pseudomonas</i>			
		(26.1)	(23.6)	(23.4)	(23.4)
	Firmicutes (45.8)	Betaproteobacteria - Burkholderiales - Oxalobacteraceae - <i>Massilia</i>			
		(21.4)	(21.4)	(21.3)	(21.3)
Galchi-jeot pH 5.4 Salinity 36%	Firmicutes (93.1)	Clostridia - Halanaerobiales - Halanaerobiaceae - <i>Halanaerobium</i>			
		(38.5)	(37.7)	(37.7)	(37.6)
		Bacilli - Bacillales - Bacillaceae - <i>Lentibacillus</i>			
		(7.3)	(6.9)	(6.9)	(6.8)
		Bacilli - Lactobacillales - Enterococcaceae - <i>Tetragenococcus</i>			
		(92.2)	(89.0)	(88.1)	(88.0)

Number in parentheses indicate percentage of each taxon at the corresponding taxonomic rank.

tect it from deterioration; however, it is also true that jeotgal is not palatable to everyone, mainly due to the strong smell. Therefore, it is important to develop a way to dampen the

smell of jeotgal. Currently, halophilic members of LAB (lactic acid bacteria), such as *Tetragenococcus* spp., which are dominant bacteria in many jeotgal, are considered promi-

sing candidates for starter cultures for jeotgal (Kim *et al.* 2019). In this context, the diversity among dominant genera observed in the present study expands the list of indicator (and potentially core) bacteria that can be used as starters to increase the sensory quality and functionality of jeotgal products.

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