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Microbial succession and metabolite changes during fermentation of saeu-jeot: Traditional Korean salted seafood

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ABSTRACT

Saeu-jeot is made by the fermentation of highly salted [approximately 25% (w/v)] shrimp in Korea. Saeu-jeot samples were prepared in triplicate and their cell number, bacterial community, and metabolites were monitored periodically for 183 days. Quantitative PCR showed that bacterial populations were much more abundant than archaeal populations during the entire saeu-jeot fermentation period, which suggested that bacterial populations, not archaeal populations, might be primarily responsible for saeu-jeot fermentation. Pyrosequencing analysis revealed that *Proteobacteria* were dramatically replaced with halophilic *Firmicutes* as the fermentation progressed and members of *Pseudoalteromonas*, *Staphylococcus*, *Salimicrobium*, and *Alkalibacillus* were sequentially dominant and, eventually, *Halanaerobium* predominated after 66 days of fermentation. Halophilic archaeal genera, *Halorubrum*, *Halolamina*, *Halobacterium*, *Haloarcula*, and *Haloplanus* belonging to *Euryarchaeota*, were dominant, but their communities were relatively constant over the entire fermentation period. Metabolite analysis using a ^{1}H NMR spectroscopy showed that the amount of metabolites including amino acids, glycerol, and nitrogen compounds rapidly increased during the early fermentation stage, but their levels were relatively constant or they decreased after approximately 49 days of fermentation. A statistical analysis based on bacterial communities and metabolites demonstrated that members of *Halanaerobium* might be responsible for the production of acetate, butyrate, and methylamines after 66 days of fermentation, which could be considered as a potential indicator to decide the appropriate seafood fermentation time. This study will provide insights into the microbial succession and metabolites of fermented seafood and allow for a greater understanding of the relationships between the microbial community and metabolites in seafood fermentation.

1. Introduction

Salted and fermented seafood, called '*jeotgal*' in Korean, is widely produced and consumed in Korea. It is often used as an additive to improve the taste or flavour of other foods such as kimchi or it is served alone as fermented seafood (Jung et al., 2011). In Korea, *jeotgal* is usually made by the fermentation of highly salted [20–30% (w/w)] marine animals such as shrimp, anchovy, oyster, clam, fish roe, and squid. Based on the major raw materials and the regional fermentation methods, *jeotgal* is classified into more than 160 different kinds, of which approximately 30 are sold commercially in Korea (Guan et al., 2011).

Spontaneous fermentation without the use of starter cultures or sterilization during the seafood fermentation leads to the growth of various microorganisms, which are primarily derived from raw materials, sea animals' associated environments (e.g., seawater or sea-tidal flat), and solar-salts used for fermented seafood preparation. It has been common knowledge that diverse and complex microbial communities including bacteria as well as archaea may be responsible for seafood fermentation due to its high salt conditions (Roh et al., 2010). Many studies that have primarily focused on culture-dependent approaches have been performed in order to obtain an understanding of microbial communities and their function in fermenting seafood (Lee and Choe, 1974; Cha et al., 1983; Paludan-Müller et al., 2002; Kim et al., 2009; Guan et al., 2011). Until now, more than 20 novel bacterial and archaeal species have been identified from fermented seafood using culture-dependent approaches (http://bacterio.net) (Euzeby, 1997). However, little is known about the microbial communities and the dynamics of fermented seafood, because culture-dependent approaches have many limitations (Roh et al., 2010).

Culture-independent approaches based on 16S rRNA gene sequences such as denaturing gradient gel electrophoresis (DGGE)

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and clone library analysis have been developed in order to more accurately explore microbial communities and their application has resulted in additional information that partially circumvents the limitations associated with the traditional culture-based methods (Ercolini, 2004; Dar et al., 2005; Kim et al., 2010, 2011a). However, clone-library and DGGE approaches also have some limitations for expanding microbial information about complex microbial systems such as fermented seafood, because they involve time-consuming steps and produce limited information. A high-throughput pyrosequencing technique has emerged as a powerful technique to unveil complex microbial communities dwelling in natural habitats (Huber et al., 2007; Costello et al., 2009) and has been applied to exploit in detail the microbial communities of non-seafood fermentation (Humblot and Guyot, 2009; Kim et al., 2011b; Sakamoto et al., 2011; Nam et al., 2012; Park et al., 2012). However, until now the pyrosequencing technique was rarely applied to fermented seafood (Roh et al., 2010). Although a little information is available for microbial diversity in fermented seafood, a comprehensive study on the microbial successions during the fermentation processes has not been completed yet.

Organic acids (e.g., lactate, acetate, and butyrate) and many nitrogen compounds (e.g., amino acids, amines, betaine, taurine, and choline) are known to be important components that contribute to the unique tastes, flavours, and food safety of fermented seafood (Chung and Lee, 1976; Mok et al., 2000). Diverse microorganisms, originated from raw seafood, are primarily involved in salted seafood fermentation for developing the taste and flavour (Cha and Lee, 1985). The metabolite compositions reflect a more direct, collective phenotypic view of related microbial populations and endogenous enzymes in fermented seafood. Therefore, studies of not only the metabolite changes, but also the microbial dynamics, might be indispensable in order to evaluate the seafood fermentation processes. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy is one of the most comprehensive, easy, and nondestructive multinuclear techniques for the simultaneous monitoring of multiple compounds present in a given sample (Figueiredo et al., 2006). In addition, it also allows for the understanding of relationships between the microbial communities and the metabolites in fermented food (Ercolini et al., 2011; Jung et al., 2012).

Saeu-jeot, made by fermentation of tiny, salted shrimps (*Acetes japonicus*), is the most representative and best-selling fermented seafood in Korea. However, little is known about the microbial dynamics and the metabolites present during saeu-jeot fermentation. In particular, to the best of our knowledge, no study has been carried out to investigate microbial succession and metabolites during the seafood fermentation period. In this study, we applied a barcoded 454-pyrosequencing strategy and a ^{1}H NMR technique to investigate the saeu-jeot microbial succession and metabolites and the relationships between them during the entire saeu-jeot fermentation period.

2. Materials and methods

2.1. Preparation of saeu-jeot and sampling

Saeu-jeot with a salt concentration of approximately 25% (w/v) was prepared in triplicate using fresh tiny shrimp (*A. japonicus*) with 3–6 cm in length caught from Yellow Sea of South Korea in July 2011 according to a traditional manufacturing method. The fresh shrimp was dispensed into three plastic containers in 10-kg portions and 3 kg of solar salt (Shinan, Korea) were added to each container and mixed well. Finally, approximately 4 l of a 25% (w/v) solar salt solution were poured into each container in order to soak the shrimp and then stored at 15 °C. The same amounts of saeu-jeot soups were periodically sampled from the three containers and their pH values were measured immediately. The saeu-jeot soups were filtered through four layers of sterile coarse gauze (Daehan, Korea) to remove large particles and the filtrates were centrifuged (8000 rpm for 20 min at 4 °C) to harvest microorganisms. The pellets of the triplicates from the same sampling days were combined together and stored at −80 °C prior to microbial community analyses. However, the supernatants from the three containers were stored separately at −80 °C for respective metabolite analyses. The NaCl concentrations of the saeu-jeot were measured by titration with silver nitrate according to the Mohr method (AOAC, 2000). The saeu-jeot samples were identified as Sx, where "x" designates the sampling days during the saeu-jeot fermentation.

2.2. Quantitative PCR to estimate the number of bacteria and archaea

In order to estimate the 16S rRNA gene copy numbers of the total bacteria and archaea during saeu-jeot fermentation using quantitative PCR (qPCR), the total genomic DNA of the pellets derived from 3.0 ml of saeu-jeot filtrate (1.0 ml from each container) was extracted using a FastDNA Spin kit (MPbio, Solon, OH) according to the manufacturer's instructions. Two qPCR primer sets, bac340F (5′-CCTACGGGAGGCAGCAG-3′)/bac758R (5′-CTACCAGGGTATCTAATCC-3′) and arc109F (5′-ACKGCTCAGTAACACGT-3′)–arc344R (5′-TCGCGCCTGCTGCTCCCCGT-3′), were used to measure the bacterial and archaeal 16S rRNA gene copies, respectively (Juck et al., 2000; Ji et al., 2011). The qPCR was performed in triplicate as described previously (Jung et al., 2011). Two standard curves for the calculations of the bacterial and archaeal 16S rRNA gene copies were generated on the basis of the number of pCR2.1 vectors (Invitrogen) carrying bacterial (*Staphylococcus*) and archaeal (*Halarchaeum*) 16S rRNA genes derived from a saeu-jeot sample. The bacterial and archaeal 16S rRNA gene copies of each sample were calculated as described previously (Ritalahti et al., 2006).

2.3. Barcoded pyrosequencing for the community analyses of bacteria and archaea

The genomic DNA was extracted from the combined pellets of the triplicate samples using the FastDNA Spin kit according to the manufacturer's instructions. Bacterial and archaeal 16S rRNA genes containing hypervariable regions were amplified using the universal primer sets, Bac27F (5′-adaptor B-AC-GAG TTT GAT CMT GGC TCA G-3′)/Bac541R (5′-adaptor A-*X*-AC-WTT ACC GCG GCT GCT GG-3′) (Jung et al., 2012) and Arc344F (5′-adaptor B-AG-AYG GGG YGC ASC AGG SG-3′)/Arc927R (5′-adaptor A-*X*-GA-CCC GCC AAT TCC TTT AAG TTT C-3′) (Jurgens et al., 1997; Casamayor et al., 2002), respectively, where *X* denotes unique 7–11 barcode sequences inserted between the 454 Life Sciences adaptor A sequence and the common linkers, AC and GA (Supplementary Table 1). All of the PCR amplifications were carried out as described previously (Jung et al., 2012). The PCR products were purified using a PCR purification kit (Solgent, Korea) and their concentrations were carefully assessed with an ELISA reader equipped with a Take3 multivolume plate (SynergyMx, BioTek, Winooski, VT). Composite samples were prepared by pooling equal amounts of PCR amplicons from each sample and their sequencings were performed using a 454 GS-FLX titanium system (Roche, Branford, CT) at Macrogen (Korea).

2.4. Sequence processing and data analysis

The sequencing reads generated from pyrosequencing were processed using the RDP pyrosequencing pipeline (http://pyro.cme.msu.edu/) (Cole et al., 2009). The sequencing reads were assigned to specific samples based on their unique barcodes and then the

barcodes were removed. Sequencing reads shorter than 300 bp and with 'N' (undetermined nucleotide) and those with average quality values below 20 were removed from the data set. Noisy sequences and putative chimeric sequences were removed from the remaining data set using the PyroNoise algorithm (Quince et al., 2009) and the chimera.slayer command as implemented in MOTHUR (Schloss et al., 2009), respectively.

The sequencing reads with high qualities were aligned using the fast, secondary-structure aware INFERNAL aligner (Nawrocki and Eddy, 2007) and the resulting aligned sequences were clustered into operational taxonomic units (OTUs) defined by 97% similarity using the complete-linkage clustering tool of the RDP pyrosequencing pipeline. The Shannon–Weaver index (Shannon and Weaver, 1963), Chao1 richness indices (Chao and Bunge, 2002), and evenness were calculated using the RDP pyrosequencing pipeline. Rarefaction curves (Colwell and Coddington, 1994) were also generated with a 3% dissimilarity cut-off value using the RDP pyrosequencing pipeline. The high quality sequences were taxonomically classified using the RDP Classifier 2.5 trained on 16S rRNA training set 9 (Wang et al., 2007) at an 80% confidence threshold.

2.5. Metabolite analysis using the ^{1}H NMR technique

The profiles of the total metabolites, including monosaccharides, organic acids, amines, nitrogen compounds, and amino acids, during the saeu-jeot fermentation were analyzed using ^{1}H NMR spectroscopy according to a modification of the methods described previously (Lee et al., 2009; Jung et al., 2011). The saeu-jeot supernatants obtained above were 10-fold diluted with sterile water and were adjusted to a pH value of 6.0. One milliliter of the diluted supernatants was lyophilized and dissolved in 99.9% deuterium oxide (600 μl, D_2O) with 5 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, 97%). The resulting solutions were transferred into 600-MHz NMR tubes and their ^{1}H NMR spectrum data were obtained from a Varian Inova 600-MHz NMR spectrometer (Varian, USA). Identification and quantification of individual metabolites from the ^{1}H NMR spectra were performed using the Chenomx NMR suite program (ver. 6.1, Chenomx, Canada).

2.6. Multivariate statistical analysis

In order to investigate the correlations among the saeu-jeot samples, bacterial communities, and metabolites, a multivariate statistical redundancy analysis (RDA) was performed using the vegan package (Oksanen et al., 2011) of the R programming environment (http://cran.r-project.org/). At first, the correlations between the relative abundances of the bacterial compositions and saeu-jeot samples were statistically evaluated by the ordination biplot of the principal component analysis (PCA). Following the biplot analysis, the individual metabolite concentrations in each saeu-jeot sample were introduced as a set of nominal, passive, explanatory variables onto the ordination biplot, which was plotted as a correlation triplot.

2.7. Nucleotide sequence accession numbers

The pyrosequencing data of the 16S rRNA genes are publicly available in the NCBI Short Read Archive under accession no. SRA058343.

3. Results

3.1. General features of saeu-jeot fermentation

The initial pH of the saeu-jeot samples was approximately 7.1 and the pH values were relatively constant during the early fermentation stage (Fig. 1). However, after approximately 49 days of fermentation, the pH values steadily decreased to approximately 6.6 as the fermentation progressed. This was supposedly the results of the production of organic acids by fermentation. The NaCl concentrations of the saeu-jeot supernatants were maintained to be 25 ± 0.4% (w/v) during the entire fermentation period.

A qPCR approach based on 16S rRNA gene copies was used to enumerate the number of total bacteria and archaea during the saeu-jeot fermentation. The exact enumeration of viable cells using qPCR is almost impossible, because the number of chromosomal 16S rRNA coding gene operons varies with species types (Farrelly et al., 1995) and dead cells are included in samples. However, qPCR may currently be the most efficient way to estimate the microbial cell number of saeu-jeot containing many unculturable microorganisms (Roh et al., 2010). In this study, the total cell number of bacteria and archaea in saeu-jeot samples were estimated using standard curves (bacteria, $R^2 = 0.971$; archaea, $R^2 = 0.973$) generated from the cloned bacterial (*Staphylococcus*) and archaeal (*Halarchaeum*) 16S rRNA genes. The 16S rRNA gene copies of bacteria increased from an initial value of $\sim 1.3 \times 10^8$ copies/ml (day 0) to the highest value of approximately 4.1×10^9 copies/ml (at 49 days) during the early fermentation stage (Fig. 1). After that, the bacterial populations decreased steadily to approximately 1.0×10^7 copies/ml (at 183 days) as the saeu-jeot fermentation progressed. In contrast, the 16S rRNA gene copies of archaea decreased steadily from an initial value of approximately 2.7×10^6 copies/ml (day 0) to the lowest value of approximately 2.6×10^4 copies/ml (at 183 days) over the saeu-jeot fermentation period. In addition, the 16S rRNA gene copies of archaea were unexpectedly much lower than those of bacteria, although the saeu-jeot samples had very high salt concentrations (approximately 25%), which suggested that archaeal populations might play much less important roles in saeu-jeot fermentation than bacterial populations.

3.2. Changes in microbial diversities during the saeu-jeot fermentation

Bacterial and archaeal 16S rRNA genes were PCR-amplified for the analysis of microbial succession during the saeu-jeot fermentation. Enough bacterial 16S rRNA gene amplicons for the barcoded pyrosequencing were successfully produced by one time PCR for all of the saeu-jeot samples. The archaeal 16S rRNA genes were also

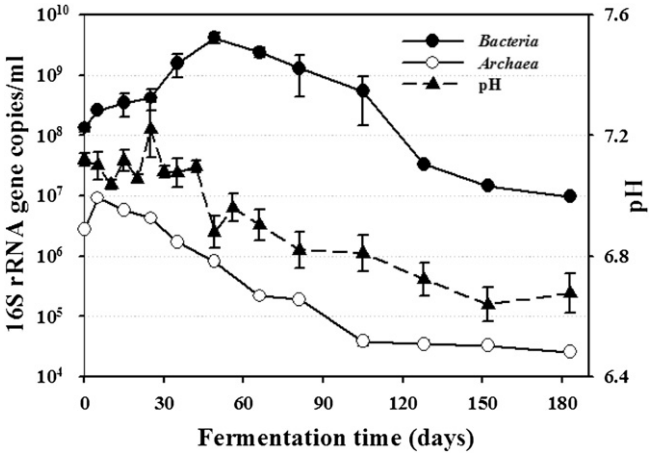

Fig. 1. Changes in pH and 16S rRNA gene copy number of bacteria and archaea during the entire saeu-jeot fermentation process. Measurements of the pH and 16S rRNA gene copy number were performed in triplicate and the error bars represent the standard deviations.

relatively well amplified for the early fermentation samples (S0–S49), but less archaeal 16S rRNA gene amplicons were produced in the case of the late fermentation samples (S66–S183), which might be due to the smaller amount of archaeal populations during the late saeu-jeot fermentation stage (Fig. 1). Therefore, repeated PCR amplifications for the late fermentation saeu-jeot samples were performed in order to obtain enough archaeal PCR amplicons.

A total of 48,715 sequencing reads were generated from a single run with twenty-four bacterial and archaeal PCR amplicons. After the removal of the low-quality and chimera reads and the trimming of the PCR primers, 30,798 high quality sequencing reads with an average sequence length of approximately 492 bp and an average of more than 1283 sequences were obtained and the statistical microbial diversities for each sample were calculated (Table 1). Interestingly, although the statistic diversity indices such as OTUs, Chao1, and Shannon observed were a function of the number of sequencing reads obtained, the diversity indices clearly showed that bacterial diversities increased during the early saeu-jeot fermentation stage (Table 1), which was supported by rarefaction curves shown in Fig. 2. Failure to approach an asymptote in the rarefaction curves indicated that the bacterial communities of the saeu-jeot samples were highly diverse during the early fermentation stage and even 2171 bacterial sequences of S15 were not sufficient to describe the entire diversity of the early saeu-jeot bacterial community. The number of bacterial species detected in the saeu-jeot samples decreased dramatically after 25 days of fermentation. Asymptotes were almost reached the rarefaction curves produced from the late saeu-jeot fermentation samples. Rarefaction curves of the archaeal sequencing reads showed that the archaeal communities of the saeu-jeot samples were also highly diverse and the number of archaeal species (OTU) detected in the saeu-jeot samples slowly increased (Fig. 2B), while the archaeal populations steadily decreased (Fig. 1) over the saeu-jeot fermentation process.

Table 1
Statistical analysis of the barcoded pyrosequencing data sets from the saeu-jeot fermentation samples.

Sample (day)	Total reads	Clean reads	OTUs[a]	Shannon–Weaver[a]	Chao1[a]	Evenness[a]
Bacteria						
0	2510	1804	144	3.07	216.73	0.62
5	1487	1096	134	2.63	359.71	0.54
15	4475	2171	200	3.16	366.79	0.60
25	1513	1065	111	2.78	229	0.59
35	1900	1194	66	1.90	103.71	0.45
49	1641	998	38	1.93	55.14	0.53
66	1735	947	40	1.83	50.11	0.50
81	1460	745	29	1.60	33.67	0.48
105	1802	869	31	1.66	47.50	0.48
128	1653	874	37	1.67	82.50	0.46
152	1780	1065	36	1.49	49.20	0.42
183	2340	1401	41	1.39	71.60	0.37
Archaea						
0	3647	2581	82	3.32	111.55	0.75
5	4338	2997	100	3.39	151	0.74
15	3112	2226	81	3.20	104.40	0.73
25	2599	1878	79	3.13	184.60	0.72
35	2216	1679	79	3.17	102.63	0.73
49	1445	1021	69	3.14	100.5	0.74
66	241	158	35	2.92	55	0.82
81	507	307	51	3.11	76.67	0.79
105	576	337	42	3.12	53	0.83
128	3848	2335	114	3.24	155	0.68
152	1316	732	77	3.18	121	0.73
183	574	318	47	3.13	81.20	0.81

Abbreviations: OTUs, operational taxonomic units.
[a] Diversity indices of the microbial communities were calculated using the RDP pyrosequencing pipeline based on the 16S rRNA gene sequencing reads.

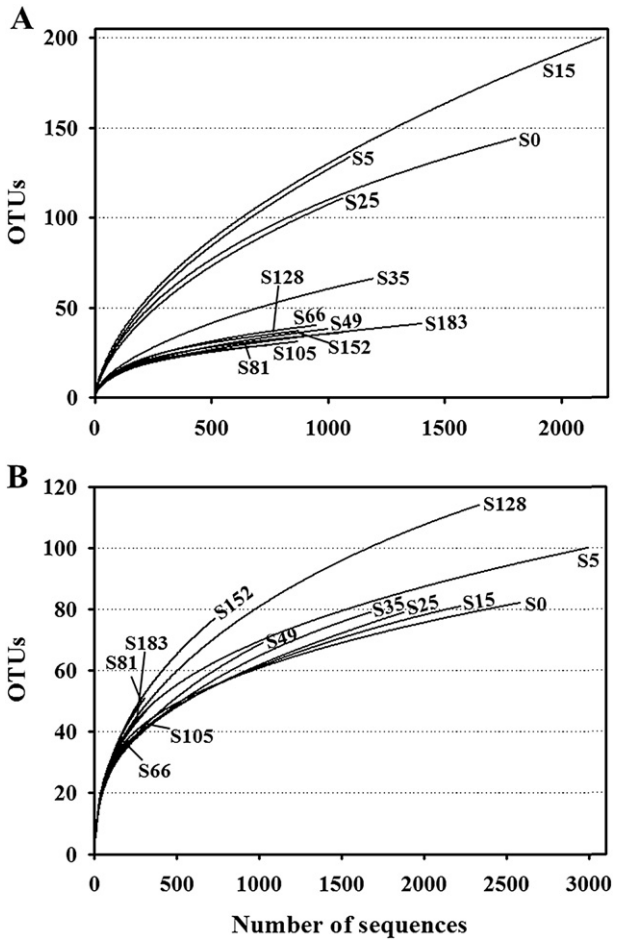

Fig. 2. Rarefaction analysis of 16S rRNA gene sequencing reads for the analysis of bacterial (A) and archaeal (B) diversity during the entire saeu-jeot fermentation period. The rarefaction curves were constructed by the RDP pipeline with a 97% OTU cutoff of the 16S rRNA gene sequences.

3.3. Microbial community succession during saeu-jeot fermentation

The 16S rRNA gene sequencing reads of bacteria and archaea were classified at both the phylum and genus levels in order to investigate the microbial community succession of saeu-jeot during the fermentation process. At the phylum level of bacteria, only two bacterial phyla, *Proteobacteria* and *Firmicutes*, were identified as the predominant major phyla during the entire saeu-jeot fermentation period. The phylum *Proteobacteria* was predominant during the early fermentation stage, but its abundance decreased dramatically with the increase in the members of the phylum *Firmicutes* as the fermentation progressed. *Firmicutes* was the predominant phylum (approximately 98%) after 49 days of fermentation (Fig. 3A). The genus level analysis of the bacterial sequencing reads showed that the bacterial community succession was significant during the saeu-jeot fermentation (Fig. 3B). At the beginning of the fermentation (day 0), the dominant genera were *Pseudoalteromonas*, *Photobacterium*, *Enterovibrio*, *Aliivibrio*, *Vibrio*, and *Psychrobacter* belonging to the phylum *Proteobacteria*, which presumably originated from the raw materials (shrimp and solar salts). The abundances of most of the initially dominant genera, except for *Pseudoalteromonas* and *Psychrobacter*, decreased rapidly during the early fermentation stage. Members of the genus *Pseudoalteromonas* that were the most abundant during the early fermentation stage increased for a short time during the early

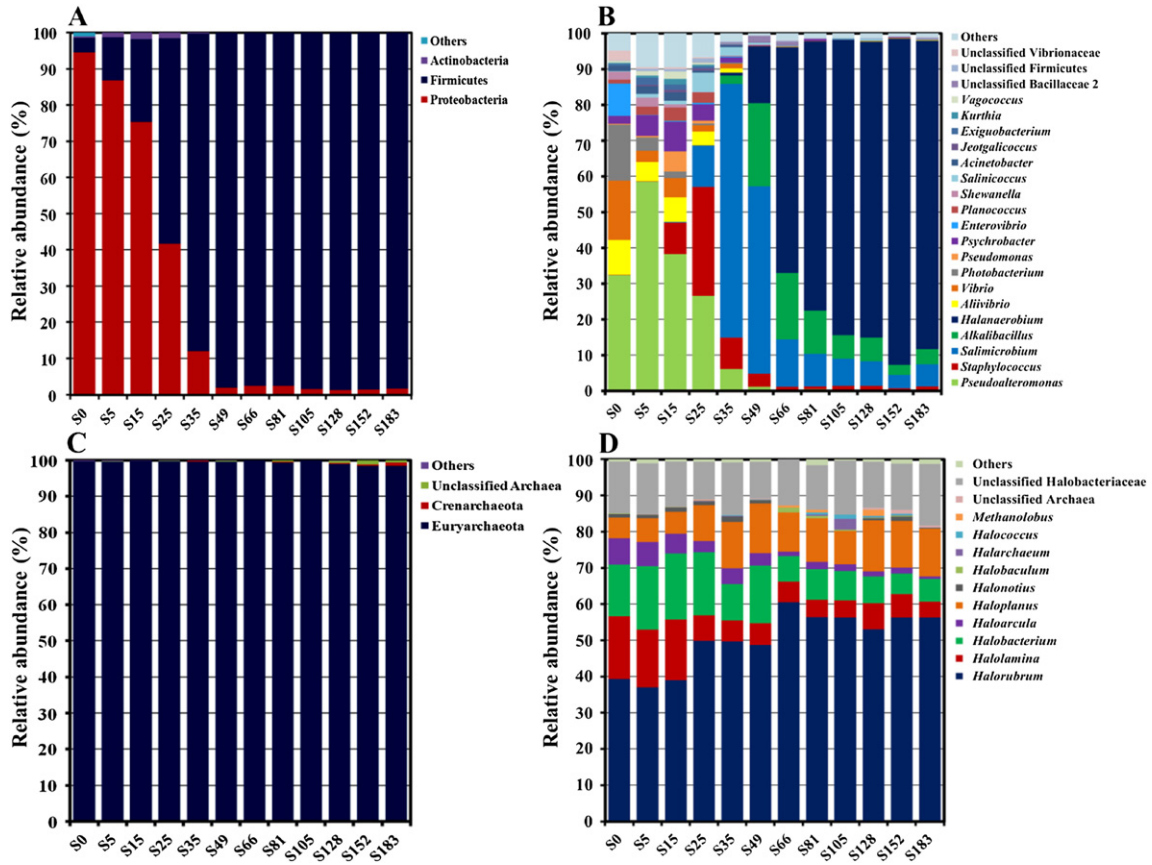

Fig. 3. Bacterial (A and B) and archaeal (C and D) taxonomic compositions showing the microbial successions during the entire saeu-jeot fermentation period. Data portray phylum- (A and C) and genus- (B and D) levels of 16S rRNA pyrotagged gene sequences found in saeu-jeot samples. The 16S rRNA gene sequences with more than 300 bp were classified using the RDP naïve Bayesian rRNA Classifier at an 80% confidence threshold. The others in both panels are composed of the phyla or the genera each showing a percentage of reads <1.0% of the total reads in all of the samples.

fermentation (5 days), but they began to rapidly decrease and became a minor population after 49 days of fermentation. Members of the genus *Psychrobacter* also slowly increased during the early fermentation stage and they decreased rapidly after 15 days of fermentation. Interestingly, as the genera belonging to the phylum *Proteobacteria* gradually decreased, the genera *Staphylococcus*, *Salimicrobium*, and *Alkalibacillus* belonging to the phylum *Firmicutes* constituted trivial populations during the early fermentation, sequentially appeared as major populations and then decreased to minor populations again as the fermentation progressed. Members of the genus *Halanaerobium* appeared as one of dominant populations after 49 days of fermentation and eventually they predominated in the saeu-jeot microbial community after 66 days of fermentation (Fig. 3B).

The classifications of the archaeal sequences showed that almost all of the archaeal reads retrieved from all of the saeu-jeot samples fell into the phylum *Euryarchaeota* (Fig. 3C). At the genus level, the archaeal 16S rRNA gene sequences were dominantly affiliated with the genera *halorubrum*, *haloamina*, *halobacterium*, *haloarcula*, and *haloplanus*, but their abundances were relatively stable unlike the bacterial succession over the entire saeu-jeot fermentation process (Fig. 3D). Our qPCR analysis showed that members of archaea were much less abundant than those of bacteria throughout the entire fermentation process and their populations actually decreased as the saeu-jeot fermentation progressed. These results suggested that members of archaea could not grow in 25% salted shrimp and, therefore, they might not be responsible for saeu-jeot fermentation. Therefore, the archaea populations were excluded from next statistical analyses.

3.4. Analysis of metabolites during saeu-jeot fermentation

^{1}H NMR analysis of the saeu-jeot samples allowed for the identification and quantification of diverse metabolites including amino acids, organic acids, and amines during the entire fermentation period. It is well known that the proteolysis of proteins to amino acids in seafood fermentation is an important process that enhances the flavour and taste of fermented seafood (Kim, 1988; Mok et al., 2000). Analysis of the metabolites also showed that amino acids were detected as the major metabolites during the saeu-jeot fermentation. The concentrations of the amino acids rapidly increased during the early fermentation stage, but their concentrations (i.e., arginine, asparagines, glutamine, lysine, leucine, and tyrosine) were relatively constant or gradually decreased after 49–66 days as the fermentation processed (Fig. 4).

Glucose and glycerol were identified as the primary organic compounds, and lactate, acetate, and butyrate were identified as the primary organic acids in the saeu-jeot samples (Fig. 5A). The concentrations of the organic compounds (glucose and glycerol) increased during the early fermentation stage, but their concentrations gradually decreased as the fermentation processed after 49–66 days like some of the amino acids. On the other hand, the lactate concentration was relatively constant over the entire

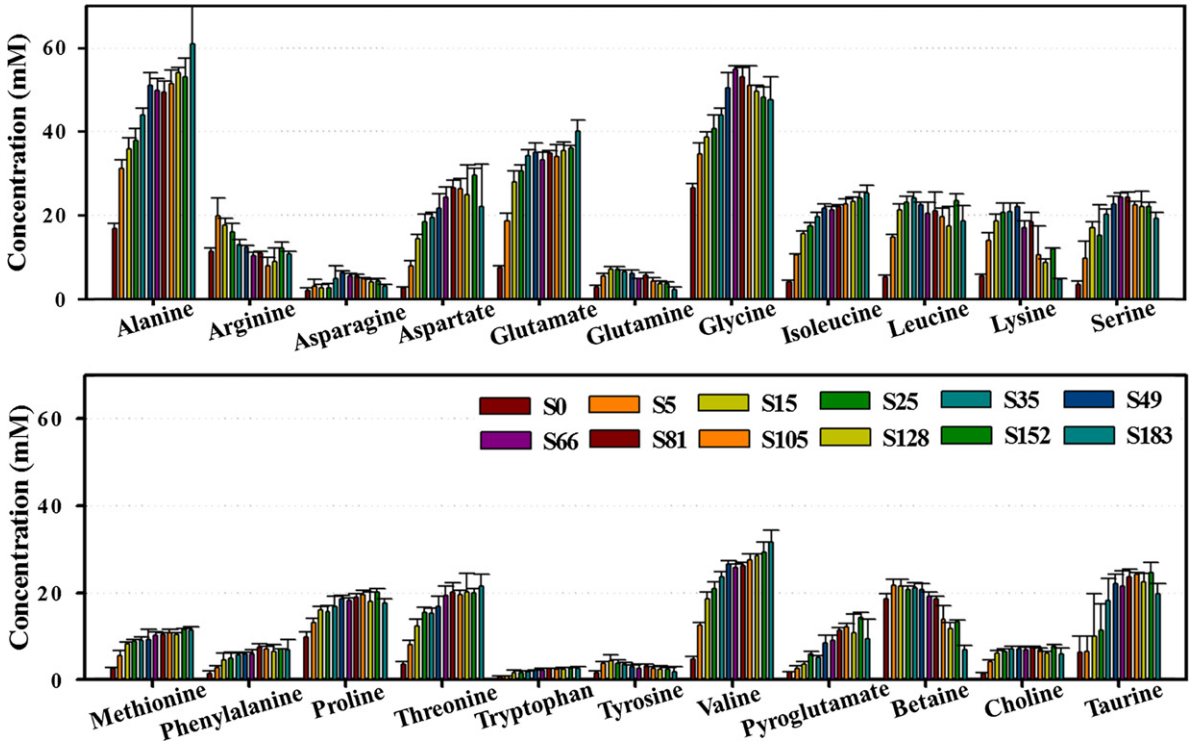

Fig. 4. Changes in the major amino acids and nitrogen compounds identified from the supernatants during the entire saeu-jeot fermentation period. Data are given as the average values ± standard deviations, measured in triplicate. The quantification was determined using Chenomx NMR suite (ver. 6.1) with 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as the internal standard.

fermentation, but the concentrations of acetate and butyrate rapidly increased after approximately 49 days of fermentation, which might cause the decrease in the pH level of the fermented saeu-jeot (Fig. 1). Trimethylamine (TMA) and dimethylamine (DMA), the unique odour of fish, which are produced by the reduction or/and demethylation of trimethylamine N-oxide (TMAO), an osmolyte in saltwater fish, increased with a decrease of the TMAO after approximately 49 days of fermentation (Fig. 5B).

3.5. Multivariate statistical analysis

The triplot showed that the bacterial communities were distributed into the PC1 and PC2 regions over the fermentation period. As shown in Fig. 3, the saeu-jeot bacterial communities were primarily represented by *Pseudoalteromonas*, *Photobacterium*, *Aliivibrio*, *Vibrio*, and *Psychrobacter* during the early fermentation stage. *Pseudoalteromonas* was the most influential group among them during the early fermentation stage. The statistical RDA triplot analysis clearly showed that *Staphylococcus*, *Salimicrobium*, and *Alkalibacillus* became the dominant bacterial groups sequentially during the saeu-jeot fermentation and, finally, *Halanaerobium* was the most influential group during the late fermentation stage, which was in agreement with the bacterial community data of Fig. 3 (Fig. 6). The triplot analysis also demonstrated that members of *Halanaerobium* were clearly related to the production of acetate, butyrate, and methylamines during the end stage of fermentation (Fig. 6).

4. Discussion

Spontaneous seafood fermentation leads to the growth of various microorganisms, which have made it difficult to produce commercially fermented seafood with uniform and good quality in Korea. Therefore, studies on the microbial communities and associated metabolites in seafood are very important to achieve a greater understanding of spontaneous seafood fermentation processes. The majority of previous studies were performed to obtain an understanding of the microbial communities of fermented seafood based on culture-dependent approaches (Sands and Crisan, 1974; Chung and Lee, 1976; Cha et al., 1983; An and Lee, 2011; Guan et al., 2011). However, only limited success has been achieved, because culture-dependent approaches have many limitations in terms of irreproducibility and unculturability of marine-derived seafood microorganisms (Giovannonni, 2004; Robertson et al., 2005). Chung and Lee (1976) reported that members of *Achromobacter*, *Pseudomonas*, and *Micrococcus* were the dominant species isolated from the initial saeu-jeot fermentation stage, while bacterial species belonging to *Halobacterium*, *Pediococcus*, *Sarcina*, and *Micrococcus*, and yeasts belonging to *Saccharomyces* and *Torulopsis* were isolated from samples after 40 days of fermentation. An and Lee (2011) reported that members of *Staphylococcus*, *Salinicoccus*, and *Salimicrobium* were primarily isolated from fermented saeu-jeot, and Guan et al. (2011) reported that members of *Bacillus* and *Staphylococcus* were the major organisms associated with seafood fermentation. However, culture-independent approaches based on 16S rRNA gene sequences have recently been developed to explore the microbial communities more accurately without the culture limitations (Torsvik and Øvreås, 2002). In particular, a high-throughput pyrosequencing strategy technique has emerged as a powerful technique to unveil complex microbial communities (Huber et al., 2007; Costello et al., 2009). The pyrosequencing technique was only applied once to fermented seafood to analyze microbial communities (Roh et al., 2010), which showed that, surprisingly, most of the bacterial pyrosequencing reads from fermented saeu-jeot belonged to the order *Lactobacillales* including the genera *Weissella* and *Lactobacillus*. However, previous taxonomic analyses regardless of culture-dependent or culture-independent approaches were performed as

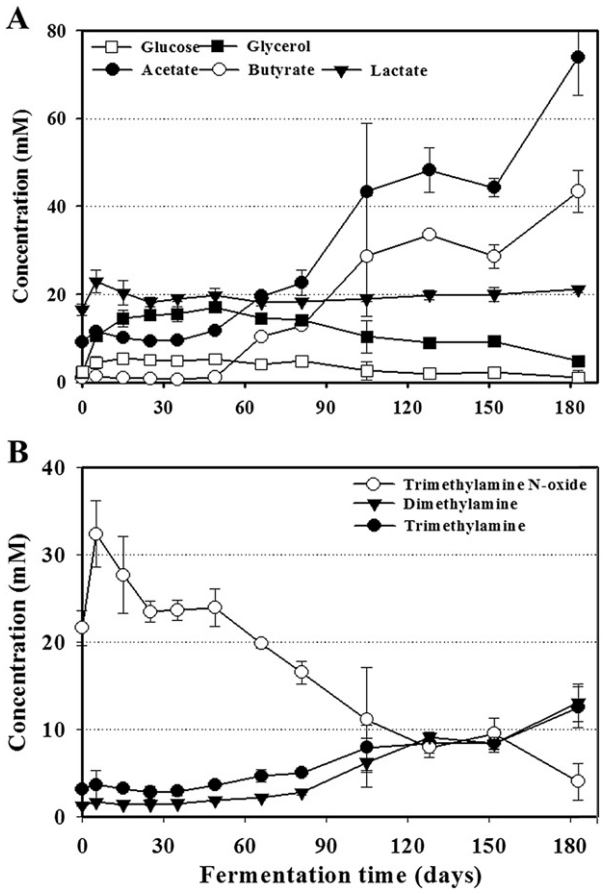

Fig. 5. Changes in some of the organic compounds (A) and amines (B) from the supernatants during the entire saeu-jeot fermentation period. Data are given as the average values ± standard deviations, measured in triplicate. Quantification was determined using Chenomx NMR suite (ver. 6.1) with 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as the internal standard.

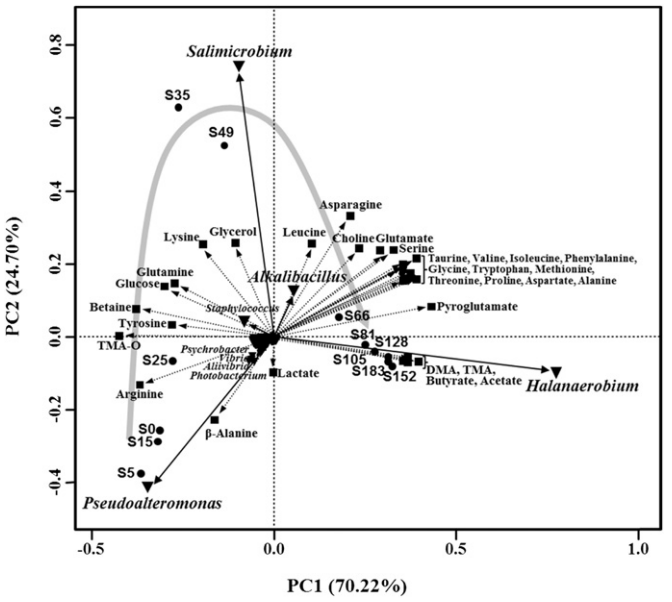

Fig. 6. A triplot produced by a multivariate statistical redundancy analysis (RDA) based on the saeu-jeot samples, the relative abundances of the bacterial communities, and the metabolite concentrations during the saeu-jeot fermentation. The directions of the straight and dotted arrows indicate the relative loadings of the genera and the metabolites on the first and second principal components and their lengths are proportional to their influences on the bacterial communities and the metabolite profiles, respectively. The directions of the curved arrows indicate the route of the bacterial community and metabolite changes in the triplot during the saeu-jeot fermentation.

a snapshot at particular times using fermented seafood samples purchased from markets without detailed sample information about fermented seafood preparation (salt concentration and raw materials) and fermentation method (fermentation temperature and time). Therefore, we prepared saeu-jeot samples directly in triplicate in this study using a traditional manufacturing method and monitored microbial communities and metabolites periodically for 183 days.

At the beginning of the fermentation time (day 0), the saeu-jeot sample contained *Pseudoalteromonas*, *Vibrio*, *Photobacterium*, *Aliivibrio*, *Enterovibrio*, and *Psychrobacter* as the dominant genera (Fig. 3B), which might be derived from raw materials (shrimp or solar salts). The genera *Vibrio*, *Photobacterium*, *Aliivibrio*, and *Enterovibrio* that may include some pathogenic strains (López et al., 2012) disappeared rapidly during the early fermentation stage, which suggests that the fermentation process of salted seafood may contribute to the production of safe seafood. Interestingly, members of *Pseudoalteromonas*, *Staphylococcus*, *Salimicrobium*, and *Alkalibacillus* were sequentially dominant as the fermentation progressed and *Halanaerobium* predominated during the late fermentation stage (Fig. 3B), which indicates that these dominant genera might be primarily responsible for the saeu-jeot fermentation. However, these results were significantly different from previous results (Cha et al., 1983; Roh et al., 2010; An and Lee, 2011) and these differences were presumably caused by culture-derived bias and the seafood samples used. Surprisingly, our analysis showed that members of *Staphylococcus* appeared after 15 days of fermentation and increased to approximately 28% of the total bacterial population at 25 days (Fig. 3B). However, members of *Staphylococcus* rapidly disappeared after 35 days as the fermentation progressed. Previous culture-dependent approaches also have shown that members of *Staphylococcus* were predominant in fermented seafood (An and Lee, 2011; Guan et al., 2011), which might be due to the better culturability of *Staphylococcus* species compared to other seafood bacteria. On the other hand, previous results using the same pyrosequencing method did not show the presence of *Staphylococcus* in fermented saeu-jeot (Roh et al., 2010). However, this was assumed to be the result of the fact that the saeu-jeot sample was accidentally taken at a sampling time that did not contain the *Staphylococcus* species as shown in Fig. 3B. Some members of the genus *Staphylococcus* have been described as common food pathogens (Normanno et al., 2005), but *Staphylococcus* species such as *Staphylococcus equorum* or *Staphylococcus succinus* have been frequently isolated from many fermented meats that are considered to be safe foods (Søndergaard and Stahnke, 2002; Fontana et al., 2005) and they have even been considered as starters for the productions of standardized fermented food (Yongsawatdigul et al., 2007; Talon et al., 2008; Mah and Hwang, 2009). In our study, the taxonomic analysis of respective 16S rRNA gene sequences that were classified as *Staphylococcus* species showed that the *Staphylococcus* sequences were most closely related to the 16S rRNA gene sequence of *S. equorum*. Therefore, it was not surprising that *Staphylococcus* species was identified as a dominant genus in our saeu-jeot samples and it was presumably concluded that the *Staphylococcus* species present in saeu-jeot may not have pathogenic properties and it may play important roles in seafood fermentation.

It has generally been thought that bacteria, as well as archaea, may play important roles in salted seafood fermentation, because of the very high salt concentrations (approximately 25%) of seafood

(Roh et al., 2007, 2010; Tapingkae et al., 2010). However, studies on the population densities of bacteria and archaea in fermented seafood have not been carried out until now. Our qPCR analysis for the enumerations of bacteria and archaea based on 16S rRNA gene copies showed that archaeal populations were much lower than bacterial populations over the entire saeu-jeot fermentation period, which was not the result we expected (Fig. 1), suggesting that archaeal populations may not play important roles in fermentation of salted seafood.

Kim (1996) reported that members of *Vibrio*, *Achromobacter*, and *Bacteroides* might be related to abnormal seafood fermentation or spoilage. However, in the work presented herein, members of *Vibrio*-related species (*Vibrio*, *Aliivibrio*, *Enterovibrio*, and *Photobacterium*) were detected only during the early fermentation stage, and they rapidly disappeared as the fermentation progressed (Fig. 3B). Our bacterial community analysis demonstrated that members of the genus *Halanaerobium* appeared as one of the dominant populations at 49 days of fermentation and eventually they predominated during the late fermentation stage (after 66 days) (Fig. 3B). Members of the genus *Halanaerobium* are known as obligately anaerobic halophiles capable of fermenting monosaccharaides, amino acids, and glycerol, and also capable of producing butyrate, acetate, propionate, H_2, and CO_2 as the primarily fermentation products (Zeikus et al., 1983; Cayol et al., 1995). Our metabolite analysis showed that acetate and butyrate gradually increased after members of the genus *Halanaerobium* appeared (49 days), which was reversely correlated with the decrease in glycerol, glucose, and some of the amino acids and nitrogen compounds (Figs. 3B, 4 and 5A). The increase in the TMA and DMA and the decrease in the TMAO were also associated with the dominance of *Halanaerobium* (Figs. 3B and 5B). Organic acids and amines in fermented seafood can be volatile flavour components, but butyric acid, TMA, and DMA in particular can cause off-flavours and taste changes at high concentrations. We could also smell off-odours during the end fermentation stage (after 100 days of fermentation). The relationships between members of *Halanaerobium* and the production of acetate, butyrate, and methylamines were confirmed statistically using a multivariate statistical redundancy analysis (RDA) based on the saeu-jeot samples, the relative abundances of the bacterial communities, and the metabolite concentrations (Fig. 6). The triplot analysis showed that members of *Halanaerobium* were clearly related to the production of acetate, butyrate, and methylamines during the end of the fermentation period (Fig. 6). Therefore, it was concluded that members of *Halanaerobium* may be responsible for abnormal seafood fermentation or spoilage through the production of acetate, butyrate, and methylamines during seafood fermentation, which meant that the presence of *Halanaerobium* and its products could be a potential indicator to decide over-fermentation of seafood.

This study is the first to reveal microbial community succession and metabolite changes that occur during entire salted seafood fermentation, but further studies regarding the effects of raw materials, salt concentrations, and fermentation temperatures are required for better understanding of seafood microbial communities and metabolites. Eventually, studies on relationships between microbial communities and metabolites and sensory characteristics (seafood taste, flavours, and food safety) are necessary, which will provide a good foundation for the production of safe and high-quality fermented seafood.

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Appendix A

Supplementary Table 1
List of adaptor and barcode sequences in the PCR primer sets used in this study

Name	Sequence (5′–3′)	Reference
Adaptor sequences		
A adaptor	CCATCTCATCCCTGCGTGTCTCCGACTCAG	Jung et al., 2012
B adaptor	CCTATCCCCTGTGTGCCTTGGCAGTCTCAG	Jung et al., 2012
Bacterial barcode sequences		
S0	ACTGAGT	This study
S5	TCAGATG	This study
S15	CTGTGAT	This study
S25	ATGCTGAG	This study
S35	TACAGCAG	This study
S49	ATCGTGTG	This study
S66	CGTGTACTG	This study
S81	CTGTCTACG	This study
S105	AGTCACTAG	This study
S128	CACACGATAG	This study
S152	ATGTGTCTAG	This study
S183	ATGTACGATG	This study
Archaeal barcode sequences		
S0	CATGCTC	This study
S5	TGTCAGC	This study
S15	TCTCGACT	This study
S25	ATCAGCAC	This study
S35	CTGATGCT	This study
S49	CGCATATC	This study
S66	CTAGTGCT	This study
S81	TGACTCGAC	This study
S105	TGACGACGT	This study
S128	TGAGTGACGC	This study
S152	TCTGTCTCGC	This study
S183	TCGCAGACAC	This study

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