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Prokaryotic community composition in alkaline-fermented skate (*Raja pulchra*)

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ABSTRACT

Prokaryotes were extracted from skates and fermented skates purchased from fish markets and a local manufacturer in South Korea. The prokaryotic community composition of skates and fermented skates was investigated using 16S rRNA pyrosequencing. The ranges for pH and salinity of the grinded tissue extract from fermented skates were 8.4–8.9 and 1.6–6.6%, respectively. Urea and ammonia concentrations were markedly low and high, respectively, in fermented skates compared to skates. Species richness was increased in fermented skates compared to skates. Dominant and predominant bacterial groups present in the fermented skates belonged to the phylum *Firmicutes*, whereas those in skates belonged to *Gammaproteobacteria*. The major taxa found in *Firmicutes* were *Atopostipes* (*Carnobacteriaceae*, *Lactobacillales*) and/or *Tissierella* (*Tissierellaceae*, *Tissierellales*). A combination of RT-PCR and pyrosequencing for active bacterial composition showed that the dominant taxa i.e., *Atopostipes* and *Tissierella*, were active in fermented skate. Those dominant taxa are possibly marine lactic acid bacteria. Marine bacteria of the taxa *Lactobacillales* and/or *Clostridia* seem to be important in alkaline fermentation of skates.

1. Introduction

Alkaline-fermented seafood is unique and favored in a few countries like Iceland, the Philippines, Thailand, and South Korea. In particular, alkaline-fermented skate is popular traditional fermented seafood in South Korea. Gutted skates are fermented without additives for more than a week usually at low temperature. Skate retains urea and trimethylamine *N*-oxide (TMAO) in their muscle tissue as organic osmolytes at relatively high concentrations (urea at 292–369 mmol/kg wet mass and TMAO at 85–168 mmol/kg wet mass; Laxson et al., 2011). During anaerobic respiration, fermented skate becomes alkaline, producing a unique odor due to ammonia and trimethylamine (TMA) produced from urea and TMAO, respectively (Reynisson et al., 2012).

Detailed information outlining the prokaryotic or bacterial diversity in various acidic and neutral pH fermented seafood has been demonstrated using next-generation sequencing (Roh et al., 2010; Kiyohara et al., 2012), and *Archaea* has been suggested to be important in those fermented seafood (Roh et al., 2010). However, little is known about the prokaryotic diversity in alkaline-fermented seafood. Previous studies on fermented skate focused on its functionality such as its nutritional composition and quality improvement (Lee et al., 2008; Kim et al., 2010). Limited information based on cloning, denaturing gradient gel electrophoresis, and the culture method is available on the bacterial diversity of fermented skate (Lee et al., 2010; Reynisson et al., 2012). The phylotypes detected in fermented skate by cloning were *Gammaproteobacteria*, and most of them were closest to the genera *Pseudomonas*, *Stenotrophomonas*, and *Psychrobacter* (Lee et al., 2010). In a study of bacterial succession during the fermentation process of skate, *Gammaproteobacteria* were the overwhelming (91%) clones detected, and *Firmicutes* constituted the minority (Reynisson et al., 2012). These findings were intriguing because lactic acid bacteria (LAB) were not the major bacteria in alkaline-fermented skates. In fermented seafood with acidic or neutral pH, LAB (e.g., genera *Lactobacillus*, *Lactococcus,* and *Weissella*) are generally the major bacteria present (Roh et al., 2010; Kiyohara et al., 2012; Marui et al., 2014). Recently, traditional alkaline-fermented plant foods were reported to contain diverse genera of LAB (Ouoba et al., 2010). Furthermore, various alkaliphilic/alkalitolerant marine LAB have been reported from marine

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environments, marine organisms, and salted food (Ishikawa et al., 2005, 2009; Toffin et al., 2005; Pikuta et al., 2006). Thus, it can be expected that marine LAB are a significant component of the prokaryotes in alkaline-fermented skate. Alkaline conditions developed during fermentation of skate might select for unique prokaryotic communities including marine LAB. To address this, we investigated the total prokaryotic community composition of both fresh and fermented skates by using barcoded pyrosequencing and active bacterial community composition in fermented skate by pyrosequencing of 16S rDNA amplicons generated from cDNA, and measured the major chemical compositions of fresh and fermented skates.

2. Materials and methods

2.1. Samples

Two fresh and three fermented skates (*Raja pulchra*) were purchased from a local skate restaurant in Naju and fish markets in Seoul, Korea, assuming that difference in microbial communities between fermented and fresh skates may far exceed the variations due to their locality of origin and places of purchase. Skate species identifications were confirmed by sequencing their mitochondrial cytochrome oxidase subunit gene (data not shown). Internal organs were separated from the two fresh skates. The three fermented skates were fermented after evisceration. Detailed information on skate samples, fermentation temperature, and period are shown in Table 1. All skate samples were put in sterile zipper bags and transferred to the laboratory on the day of purchase. Skate samples, except sample FS-S2, were homogenized in whole using a blender (SMX-4000DY; Sinil). For FS-S2, only specific parts (tail: 25 g, wing: 62 g, snout: 20 g, and body: 12 g) were pooled and homogenized to reduce the processing time in order to minimize changes in active bacterial composition. The blender was thoroughly cleaned, then rinsed three times with 10% HCl and three times with Milli-Q water before use, and the washed Milli-Q water served as a contamination control (see Supplementary Information).

2.2. Urea, total volatile basic-nitrogen, trimethylamine, and ammonia measurements

Urea content was measured according to the method of Rahmatullah and Boyde (1980). Total volatile basic-nitrogen (TVB-N) and trimethylamine (TMA) were determined using Conway's micro-diffusion method (Conway and Byrne, 1933). For measurements of ammoniacal nitrogen and salt content, 15 ml of distilled water was added to 5 g of homogenized skate samples and vortexed at 3000 rpm for 10 min. After centrifugation at 3000 × *g* for 20 min, the supernatants were collected and then filtered through a GF/F filter. Using an automated nutrient analyzer (Alliance), ammoniacal nitrogen in the filtrates was measured (Kim and Kim, 2014). The supernatants (10 ml) were used in triplicate for measurements of salinity by T-S bridge (30/10 FT; YSI). The measured values were expressed as percent salt concentration. All chemical analyses were performed in triplicate, and the results are expressed as mg N per 100 g of sample. The sample pH was measured ten times using a pH meter (SP-701; Suntex).

2.3. DNA extraction and PCR amplification for pyrosequencing

Total genomic DNA was extracted from 50 g of each homogenized sample using the bead-beating method with some modifications (see Supplementary Information for details). For *Bacteria*, the V1 to V4 fragment of the bacterial 16S rDNA was amplified from genomic DNA extracted from each sample using primer set 27F-800R or 27F-518R attached with an adapter and sample-specific barcode (Table S1). PCR using primer 27F-800R was performed in a 20 μl volume and PCR for primer 27F-518R was performed in a 50 μl volume containing the same PCR mixture as Cho and Jang (2014) except using 0.1 μl of the DNA extract. PCR for primer 27F-800R was performed as described in Supplementary Information. PCR condition for primer 27F-518R is described in Cho and Jang (2014). For *Archaea*, nested PCR amplification was performed as follows. The first round of PCR was performed using a primer set, 21F–958R (DeLong, 1992), in the same manner as that for bacterial 16S rDNA except using 0.1 or 1 μl of the DNA extract. The temperature cycling for the first round of PCR was performed according to Vissers et al. (2009) with an initial denaturation step of 94 °C for 1 min and a final extension at 72 °C for 8 min. The second round of PCR was performed using the primer set, Parch519f–Arch915r with an adapter and sample-specific barcode (Table S1), in the same manner as for the first round except using 1–5 μl of the product of the first PCR. The temperature cycling for the second PCR was the same as that for the first round PCR. The second PCR's products were analyzed by gel electrophoresis and then visualized by UV transillumination. Approximately 500 μl of the final PCR products from each sample were purified using the AccuPrep® PCR Purification Kit (Bioneer). The purified products were quantified using a Quant-iT™ PicoGreen® dsDNA Reagent Kit (Invitrogen).

2.4. RNA extraction, cDNA synthesis, and RT-PCR

To explore the active bacterial community composition of a fermented skate, RNA-based pyrosequencing was performed for sample FS-S2 as follows. Total RNA was extracted from the homogenized sample using the RNeasy® Plus Mini kit (Qiagen), and cDNA was synthesized using the ThermoScript™ RT-PCR system with random hexamers (Invitrogen), according to the manufacturer's instructions (see Supplementary Information for incubation conditions). The resulting cDNA was then stored at −20 °C until further analysis. To test for residual contaminating DNA in the RNA sample, a PCR assay was performed using RNA as the template. The negative control without RNA was included to check for reagent contamination. The absence of residual DNA was confirmed by gel

Table 1
Detailed information on skate (*Raja pulchra*) samples used in this study. The skates were identified by sequencing mitochondrial COI gene (see Supplementary Information). –: Not fermented.

Sample ID	Size (L × W, cm)	Weight (kg)	Conditions for fermentation		Place of purchase (Korea)	Fishing area	Sex
			Temperature (°C)	Duration (days)			
S-N	72 × 60	4	–	–	A skate restaurant in Naju	Yellow sea	F
FS-N[a]	70 × 64	4	2–5	27			
S-S1	73 × 60	4	–	–	Noryangjin fisheries wholesale market in Seoul	Yellow sea	F
FS-S1[a]	72 × 55	3	3–4	17			
FS-S2[a]	66 × 58	3.8	4–5	14	Noryangjin fisheries wholesale market in Seoul	Yellow sea	M

[a] FS-N, FS-S1 and FS-S2 represent fermented skates after internal organs were removed.