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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.

electrophoresis. For making amplicons for pyrosequencing, PCR was performed in a 20 μl volume containing 0.1 μl of cDNA using the same procedures and conditions employed for bacterial PCR described above.

2.5. Pyrosequencing of barcoded amplicons, taxonomic assignment, and diversity analyses

Bacterial and archaeal PCR amplicons with different sample-specific barcode sequences were pooled and prepared for pyrosequencing in 1/8 region of the PicoTiterPlate device. Pyrosequencing for bacteria and archaea was performed by the Macrogen Corporation (Korea) using the 454 GS FLX + system (Roche) and 454 GS FLX Titanium system (Roche), respectively. For samples FS-S1 and S-S1, bacterial and archaeal amplicons were pooled, and pyrosequencing was performed in 1/2 region of the PicoTiterPlate device on the 454 GS FLX Titanium system. The pyrosequencing data have been submitted to NCBI's SRA database under accession No. SRP061579.

Pyrosequenced amplicons were denoised using the commands 'shhh.flows' and 'shhh.seqs' in Mothur v.1.32.1 (Schloss et al., 2009). Denoised sequence reads were sorted to each sample based on their unique barcodes, and the barcodes were then removed. After screening for the presence of intact PCR primer sequences, sequences with one or more N bases or that were <250 bp in length were removed using the FUZZNUC program from the EMBOSS package (Rice et al., 2000). Chimera check, clustering of sequences into operational taxonomic units (OTUs), estimates of species richness (i.e., Chao1 and ACE), and taxonomical assignments of sequences in each sample were performed as described in Cho and Jang (2014), except using the updated software versions and normalization of reads data using a randomly subsampling method (Supplementary Information). Multidimensional scaling (MDS) analysis was performed using PRIMER 6 (Clarke and Warwick, 2001) based on the Bray-Curtis similarity obtained from bacterial community compositions. The analysis of similarities (ANOSIM) in PRIMER 6 was performed to test for differences in bacterial communities between fresh and fermented skates.

2.6. Total prokaryotic abundance, isolation of bacterial strains, enzyme activity, and quantitative PCR (q-PCR)

To measure total prokaryotic abundance, 5 g of the homogenized samples were transferred into 10 ml of distilled water and then vortexed at 3000 rpm for 10 min. After centrifugation at 1000 rpm for 10 min, the supernatants (5 μl) were placed in 1 ml of 0.2-μm filtrate of distilled water, stained with DAPI, filtered through 13 mm diameter, 0.22 μm pore-size black polycarbonate membranes (Millipore), and then enumerated using an epifluorescence microscope (BX60; Olympus). The presence of bacteria in the 0.2-μm filtrate of distilled water was assessed.

To obtain isolates, 5 g of the homogenized samples was transferred to 10 ml of sterile dilution buffer (0.1% (w/v) peptone, 2% (w/v) NaCl, pH 8.0). After vortexing at 3000 rpm for 10 min, an aliquot (200 μl) of serially diluted samples was spread for the purpose of isolating archaea, alkalitolerant bacteria, LAB, and heterotrophic marine bacteria onto medium for Archaea, CSA, GYPB, and Marine agar (Difco), respectively, as well as onto the same media modified for salt content and pH (Supplementary Information). After about 3 weeks of incubation at different conditions (4, 25, and 37 °C under aerobic condition and 25 °C under anaerobic condition), morphologically distinct colonies (n = 166) were picked up from the selective media and re-streaked on the same medium. The isolates were identified by sequencing of the 16S rRNA gene as described in Choi et al. (2007). Urease activity in isolates was assessed by the color change after growth in urease-indicating urea broth (Supplementary Information).

To assess the relative importance of bacterial and archaeal abundance, total copy numbers of bacterial and archaeal 16S rDNA in DNA extracted from homogenated skate samples were estimated by q-PCR using standard curves generated from the genomic DNA of *Escherichia coli* (for *Bacteria*) and *Haloarcula marismortui* (for *Archaea*), according to Einen et al. (2008). For more details, see Supplementary Information.

3. Results

3.1. Chemical properties of skate and fermented skate

Compared to the pH of the grinded skates (7.7–7.9), the pH of the three fermented skates (8.4–8.9) were increased (Table 2). The salt content of the grinded skates were 0.7–0.9%, but that of the fermented skates were 1.6–6.6%. Urea concentrations in the skate samples were 6.5–8.7 $\times$ 10^2 mg N per 100 g and > 9-fold higher than those of fermented skates (<0.9 $\times$ 10^2 mg N per 100 g). The concentration of ammoniacal nitrogen was much higher in fermented skate samples (26 $\times$ 10^2 mg N per 100 g) than in skates (5 $\times$ 10^2 mg N per 100 g). Also, the concentrations of TMA and TVB-N were >2.3-folds higher in fermented skates than in skates.

3.2. Total prokaryotic abundance

Prokaryotic abundance measured by epifluorescence microscopy ranged from 4.4 to 8.3 $\times$ 10^7 cells g^{-1} in fermented skates, and was 6–69-fold higher than that in skate samples (12–76 $\times$ 10^5 cells g^{-1}, Table 2). Viable counts were 16 $\times$ 10^5 CFUs g^{-1} and 360 $\times$ 10^5 CFUs g^{-1}, respectively, in a skate and fermented skate sample. Archaea comprised a very small fraction (<1.4 $\times$ 10^{-4}) of the total copy of the 16S rDNA in skates and fermented skates (data not shown). The 16S rDNA copies of archaea in all samples were below the q-PCR detection limits (<7 cells g^{-1}).

3.3. Diversity and composition of prokaryotes determined by pyrosequencing

A total of 126,025 (including 19,383 reads for cDNA) reads for bacterial PCR amplicons were obtained (Table S2). After removing low quality reads and chimera reads (3123 reads), 33,713 (including 2958 reads for cDNA) reads were retained from the 6 samples. The average lengths of reads obtained by GS-FLX Plus and GS-FLX Titanium were 744 ± 124 bp (n = 6488) and 430 ± 65 bp (n = 27,225), respectively.

The number of bacterial OTUs detected in the two skate samples was much lower than that in the fermented skate samples (417 vs. 922 OTUs for Naju samples and 2178 vs. 2894 OTUs for Seoul samples; Table S2). The number of OTUs unique to fermented skates and skates ranged from 329 to 2854 depending on the sample, and those common to both fermented skates and skates represented a small fraction (<0.11) of the OTUs in fermented skates (Fig. S1). Estimates of species richness (i.e., Chao1 and ACE) demonstrated the highest species richness values in the FS-S1 sample, followed by S-S1, FS-N, FS-S2, and S-N samples when equal number of sequences per sample was used (Table S2). Chao1 and ACE richness prediction curves for FS-S2, FS-N, and S-N samples approached an asymptote (Fig. S2).

Predominant bacterial groups differed between fresh skate samples. In the S-N skate sample, *Gammaproteobacteria*, mainly *Moraxellaceae*, and *Bacilli*, mainly *Carnobacteriaceae*, were predominant, whereas the predominant groups in the S-S1 skate

Table 2
Microbiological and chemical properties of skate samples.

Sample[a]	Total prokaryotic abundance[b] ($x10^5$ cells/g)/Viable count ($x10^5$ CFU/g)	pH	Salt concentration (%)	Urea nitrogen (10^2 mg N/100 g)	Ammoniacal nitrogen (10^2 mg N/100 g)	Total volatile basic nitrogen (10^2 mg N/100 g)	Trimethylamine (10 mg N/100 g)
S-N	76 ± 0.5/(16 ± 3.5)[c]	7.7 ± 0.0	0.9 ± 0.0	8.7 ± 0.2	5.1 ± 1.3	0.9 ± 0.07	0.6 ± 0.1
FS-N	440 ± 17/(360 ± 14)[d]	8.9 ± 0.0	1.6 ± 0.1	0.9 ± 0.04	26.0 ± 0.8	3.7 ± 0.3	1.4 ± 0.0
S-S1	12 ± 0.7	7.9 ± 0.4	0.7 ± 0.0	6.5 ± 1.3	5.2 ± 1.2	0.9 ± 0.09	0.3 ± 0.1
FS-S1	830 ± 30	8.4 ± 0.0	1.7 ± 0.1	0.004 ± 0.0	25.8 ± 1.8	2.2 ± 0.2	2.6 ± 0.1
FS-S2	549 ± 18	8.9 ± 0.0	6.6 ± 0.1	–	–	–	–

[a] S-N, fresh skate purchased from Naju; FS-N, fermented skate purchased from Naju; S-S1, fresh skate purchased from Seoul; FS-S1 and FS-S2, fermented skate purchased from Seoul.
[b] Values measured using epifluorescence microscopy.
[c] CFU values obtained at 25 °C from CSA medium (3% salts and pH 9.5) which gave highest value among tested media.
[d] CFU values obtained at 25 °C and anaerobic condition from MA medium (3% salts and pH 9.5) which gave highest value among tested media.

sample were *Gammaproteobacteria* consisting mainly of *Vibrionaceae* and *Moraxellaceae* (Table 3; for details at the level of OTU, see Tables S3 and S4). Furthermore, the three fermented skate samples showed different compositions of major bacterial groups from those in skates (Fig. 1). In the FS-N sample, *Bacilli*, mainly of *Carnobacteriaceae* and *Planococcaceae*, were predominant (72.4%) and *Gammaproteobacteria* were relatively low (25.0%). In the FS-S1 sample, *Gammaproteobacteria* (47.0%) mainly of unclassified *Gammaproteobacteria* and *Clostridia* (38.0%) mainly of unclassified *Clostridia* and *Tissierella* comprised a high percentage of bacterial composition. In the FS-S2 sample, *Bacilli* (69.7%) mainly of *Carnobacteriaceae* were predominant and *Clostridia* (24.9%) mainly of unclassified *Clostridia* were dominant. The major groups observed in the three fermented skates comprised markedly different proportions in each fermented skate sample. The active bacterial community consisted mostly of *Bacilli* (50.0%) and *Clostridia* (39.0%) groups (Table 3). *Atopostipes* (*Carnobacteriaceae*, 46.8%) and *Tissierella* (*Tissierellaceae*, 26.8%) were the major active genera. Active members of *Gammaproteobacteria*, which were undetected in total bacterial composition, comprised the minority (<4.3%), and other classes of *Proteobacteria* and *Bacteroidetes* were undetected in fermented skate. The MDS diagram showed that the active and total bacterial community compositions of fermented skate were most closely associated, and that bacterial community compositions (BCCs) of fermented skates differentiated from those of fresh skates (Fig. 2). The ANOSIM test also demonstrated differences (Global $R = 0.71$) at a level of probability of 0.067 in BCCs between fresh and fermented skate samples.

3.4. Compositions of Archaea determined by pyrosequencing

In skates, *Thaumarchaeota* were generally predominant and *Halobacteria* were often a major group (Table 4). In two out of three fermented skates, *Halobacteria* were a major archaeal group, but in the other fermented skate (sample FS-S1), *Thaumarchaeota* was the major group.

3.5. Predominant OTUs

In sample FS-S2, OTUs belonging to *Atopostipes* genus comprised 40.5% of the reads, but with variable occurrence in other fermented skates (Table S5). In sample S-N, OTUs belonging to *Psychrobacter* genus comprised 17.7% of the reads, but with reduced occurrence in other skate sample and undetected level in fermented skates.

3.6. Compositions of cultured bacteria

Using various types of media and culture conditions, a total of 166 bacterial strains (105 from fresh and 61 from fermented skates) were isolated and identified (Table 5). The majority of isolated bacterial strains belonged to the *Gammaproteobacteria*, i.e., *Moraxellaceae*, *Enterobacteriaceae*, *Pseudomonadaceae*, *Pseudoalteromonadaceae*, and *Aeromonadaceae* families, and to the *Firmicutes*, i.e., *Carnobacteriaceae*, *Staphylococcaceae*, and *Enterococcaceae* families. Notably, 18 isolates showed 16S rRNA gene sequence similarity less than 97% with the closest bacteria. Approximately 1/3 of them belong to the order *Lactobacillales*. No archaea were isolated.

Bacterial isolates affiliated with the genera *Psychrobacter* and *Pseudomonas* were consistently abundant in pyrosequencing reads, respectively, in skates and fermented skates (Tables 5 and S3). However, for other isolates, such consistency between isolates and reads was unclear.

4. Discussion

The most pronounced features of our study were an observation of the dominance (46%) to the predominance (74–99%) of OTUs belonging to the phylum *Firmicutes*, and the absence of OTUs belonging to the order *Vibrionales* in fermented skates. The other dominant group was *Gammaproteobacteria*, which comprised 25–47% in >14-d-old fermented skates. It seems that alkaline fermentation of skates selected for *Lactobacillales* and/or *Clostridia* in their microbiomes. The selection of *Clostridia* in alkaline-fermented skates is notable because *Clostridia* comprise a minor fraction of bacterial compositions in fermented seafood with acidic or neutral pH (Roh et al., 2010; Kiyohara et al., 2012; Marui et al., 2014). An interesting question arises as to whether the dominant groups in *Firmicutes* in fermented skates are LAB. In acidic or neutral pH fermented seafood, occurrences of LAB genera (*Lactobacillus* or *Lactococcus* and *Weissella*) as major components of bacteria have been reported (Roh et al., 2010; Marui et al., 2014). Similarly, in *narezushi*, which is manufactured by the fermentation of salted fish with rice, the *Lactobacillus* population was predominant (88.8% of reads) and lactococci (*Lactococcus* and *Leuconostoc*) comprised 2.5% of reads after 41 days of fermentation (Kiyohara et al., 2012). Furthermore, some highly halotolerant and mostly alkaliphilic marine LAB belonging to the genera *Alkalibacterium*, *Halolactibacillus*, and *Marinilactibacillus* have been isolated from marine environments, marine organisms, and salted foods (Ishikawa et al., 2005, 2009; Toffin et al., 2005), and halotolerant and slightly alkalitolerant *Trichococcus patagoniensis* from penguin guano (Pikuta et al., 2006). Thus, we expected that bacterial populations in fermented skates might contain such known marine LAB. However, OTUs close to *Alkalibacterium*, *Halolactibacillus*, and *Marinilactibacillus* were not detected in the fermented skate samples examined. Moreover, sequences close to the core genera of LAB (i.e., *Lactobacillus*, *Pediococcus*, *Lactococcus*, *Streptococcus*, and

Table 3
Relative abundance of bacterial taxa in fresh and fermented skate samples. Data were normalized by randomly subsampling to 2000 reads in each sample 100 times and average values were used to calculate relative abundances. The relative abundance of each bacterial taxon which was identified using the RDP classifier (80% confidence) was calculated by dividing the numbers of reads assigned to each group by 2000 per sample. Phylum, class, and order level classifications are highlighted in bold and their remaining taxa are classified to the level of family or genus. –: not detected. The description of sample IDs is the same as in Table 2.

Groups		FS-N	S-N	FS-S1	S-S1	FS-S2	FS-S2 (cDNA)
	Unclassified Bacteria	**0.48**	**0.14**	**7.33**	**0.14**	**1.00**	**1.12**
Phylum	**Proteobacteria**	**25.13**	**57.72**	**46.97**	**86.24**	–	**4.26**
	Unclassified Proteobacteria	–	–	0.01	0.02	–	–
Class	**Gammaproteobacteria**	**24.97**	**56.96**	**46.96**	**85.78**	–	**4.26**
	Unclassified Gammaproteobacteria	1.75	0.29	42.36	2.41	–	0.24
	Vibrionales	–	**4.05**	–	**34.43**	–	–
	Vibrionaceae	–	4.05	–	34.43	–	–
	Unclassified Vibrionaceae	–	–	–	1.00	–	–
	Photobacterium	–	3.81	–	14.68	–	–
	Aliivibrio	–	0.24	–	18.75	–	–
	Pseudomonadales	**19.09**	**46.62**	**4.54**	**25.31**	–	**3.99**
	Unclassified Pseudomonadales	0.27	0.24	0.43	0.05	–	0.10
	Moraxellaceae	6.45	42.18	0.76	23.18	–	–
	Unclassified Moraxellaceae	0.55	1.43	0.01	0.72	–	–
	Psychrobacter	5.90	40.75	0.75	22.47	–	–
	Pseudomonadaceae	12.37	4.19	3.36	2.08	–	3.89
	Unclassified Pseudomonadaceae	4.58	1.53	0.79	0.33	–	0.27
	Pseudomonas	7.79	2.67	2.57	1.75	–	3.62
	Xanthomonadales	–	**0.19**	**0.02**	**0.58**	–	–
	Xanthomonadaceae	–	0.19	0.02	0.58	–	–
	Unclassified Xanthomonadaceae	–	–	–	0.08	–	–
	Ignatzschineria	–	0.19	0.02	0.50	–	–
	Alteromonadales	**0.30**	**0.95**	**0.02**	**7.32**	–	–
	Unclassified Alteromonadales	0.09	0.81	0.01	0.58	–	–
	Pseudoalteromonadaceae	0.21	0.14	0.01	6.74	–	–
	Unclassified Pseudoalteromonadaceae	–	–	–	0.01	–	–
	Pseudoalteromonas	0.21	0.14	0.01	6.73	–	–
	Oceanospirillales	**0.05**	–	–	**0.01**	–	–
	Aeromonadales (Aeromonadaceae)	**3.76**	**1.29**	**0.02**	**15.54**	–	**0.03**
	Enterobacteriales (Enterobacteriaceae)	**0.02**	**3.57**	**0.01**	**0.04**	–	–
	Chromatiales (Rheinheimera)	–	–	–	**0.12**	–	–
Class	**Alphaproteobacteria**	**0.05**	**0.29**	–	**0.03**	–	–
Class	**Betaproteobacteria**	**0.11**	**0.48**	–	**0.39**	–	–
Class	**Epsilonproteobacteria** (Campylobacteraceae)	–	–	–	**0.01**	–	–
Class	**Deltaproteobacteria** (Desulfobulbaceae)	–	–	–	**0.01**	–	–
Phylum	**Firmicutes**	**74.37**	**40.99**	**45.67**	**5.26**	**98.91**	**94.59**
	Unclassified Firmicutes	0.11	–	0.58	0.01	3.83	5.34
Class	**Bacilli**	**72.35**	**40.90**	**6.99**	**5.18**	**69.74**	**50.00**
	Unclassified Bacilli	2.14	0.19	0.14	0.04	0.29	0.24
	Lactobacillales	**53.64**	**38.51**	**4.09**	**4.84**	**69.23**	**49.66**
	Unclassified Lactobacillales	0.59	0.67	–	0.32	0.58	0.44
	Carnobacteriaceae	52.30	29.84	4.08	2.22	68.65	49.05
	Unclassified Carnobacteriaceae	2.78	2.86	0.16	0.74	3.99	2.10
	Carnobacterium	12.03	16.59	–	1.45	0.71	0.20
	Atopostipes	37.13	4.43	3.92	0.03	63.95	46.75
	Trichococcus*	0.36	5.96	–	–	–	–
	Enterococcaceae	0.75	8.01	0.02	2.30	–	0.14
	Unclassified Enterococcaceae	–	0.10	–	0.17	–	–
	Vagococcus	0.75	7.91	0.02	1.76	–	0.14
	Enterococcus	–	–	–	0.37	–	–
	Leuconostocaceae	–	–	–	–	–	0.03**
	Bacillales	**16.56**	**2.19**	**2.76**	**0.33**	**0.23**	**0.10**
	Unclassified Bacillales	1.21	1.14	0.04	0.04	0.06	0.03
	Planococcaceae	15.35	1.05	2.71	0.29	0.16	0.07
	Unclassified Planococcaceae	9.48	0.81	1.98	0.23	0.03	–
	Sporosarcina	3.64	0.05	0.11	0.02	0.10	0.07
	Filibacter	2.21	–	0.63	0.01	0.03	–
	Planococcus	0.02	0.19	–	0.02	–	–
	Kurthia	–	–	–	0.01	–	–
Class	**Clostridia**	**1.87**	**0.10**	**38.04**	**0.06**	**24.86**	**39.01**
	Unclassified Clostridia	0.52	0.05	22.93	–	14.53	12.20
	Tissierellales (Tissierella)	**1.23**	**0.05**	**15.03**	**0.03**	**10.23**	**26.81**
	Clostridiales (Clostridium)	**0.11**	–	**0.08**	**0.03**	**0.10**	–
Class	**Erysipelotrichia (Erysipelotrichaceae)**	**0.05**	–	**0.05**	**0.01**	**0.48**	**0.24**
Phylum	**Bacteroidetes**	–	**1.14**	**0.04**	**8.29**	**0.10**	–

Table 3 (*continued*)

Groups		FS-N	S-N	FS-S1	S-S1	FS-S2	FS-S2 (cDNA)
	Unclassified Bacteroidetes	–	–	0.01	0.15	0.03	–
	Flavobacteriaceae	–	1.10	0.03	8.13	0.06	–
	Unclassified Flavobacteriaceae	–	0.91	0.02	0.93	0.06	–
	Myroides	–	0.19	0.01	7.20	–	–
Phylum	**Cyanobacteria**	–	–	–	**0.01**	–	–
Phylum	**Actinobacteria**	**0.02**	–	–	**0.06**	–	**0.03**

*Phylogenetic tree analysis indicates OTUs clustered with *Trichococcus* spp. might be a novel genus (see Fig. S3d).
**A single read closely related to *Leuconostoc* was detected (see Fig. S3a).

Weissella) in acidic- or neutral-fermented seafood were not found and a single *Leuconostoc*-like read was detected in fermented skates (Table 3 and Fig. S3a). Some genera (i.e., *Aerococcus*, *Enterococcus*, *Trichococcus*, and *Vagococcus*) important in acidic or neutral pH fermentation of seafood were rare (<0.8%) in fermented skates examined. Possibly, those genera important in acidic or neutral pH fermentation of seafood may be affected by alkaline pH during fermentation.

The most noteworthy OTUs found in fermented skate samples were *Atopostipes*-, *Tissierella*- and *Carnobacterium*-like OTUs, and OTUs belonging to *Planococcaceae*. The most abundant OTUs in the FS-S2 and FS-N samples (41% and 5%, respectively) are associated with *Atopostipes suicloacale*, probably representing a novel genus in *Lactobacillales* (Fig. S3b). Similarly, in one study it was shown that clonal sequences closest to the genus *Atopostipes* comprised 19% of the clone library in fully fermented skate (Reynisson et al., 2012). Currently, the genus *Atopostipes* (*Carnobacteriaceae*) has a single species isolated from pig manure, and it is not known whether the genus *Atopostipes* includes LAB or marine isolates.

Other dominant OTUs in the fermented skates were closest to *Tissierella*. All known *Tissierella* species grow anaerobically and are from non-marine environments (Table S6). Recently, Alauzet et al. (2014) reported some members of the genus *Tissierella* produce lactic acid. The *Tissierella*-like OTUs possibly representing novel *Tissierella* species (Fig. S3c) might have developed adaptations to saline and alkaline conditions. These dominant taxa (i.e., *Atopostipes*- and *Tissierella*-like OTUs) were major active groups in fermented skate, as confirmed by cDNA synthesis and sequencing. Their dominance and highly increased total bacterial abundance in fermented skates indicate that they actively grew during fermentation. It seemed that those major groups might be important in the alkaline-fermentation of skates. Occasionally, *Carnobacterium*-like sequences and sequences closest to *Planococcaceae* comprised the dominant fraction of reads (12% and 15%, respectively) in the FS-N sample (Table 3). Similarly, 24 isolates out of total 166 strains isolated from fermented skates were *Carnobacterium* spp., but none belonged to *Planococcaceae*. Reynisson et al. (2012) also observed a similar trend in which isolates belonging to *Carnobacterium* spp. represented 8 out of a total of 116 but no isolates belonging to *Planococcaceae* were obtained during skate fermentation. Overall, it seems that bacterial isolates are not always representatives in the community level (Ward et al., 1990). Together with the occurrences of uncultured *Atopostipes*, *Tissierella*, *Carnobacterium*, and *Planococcaceae* OTUs, the fact that about 1/3 of presumable novel isolates

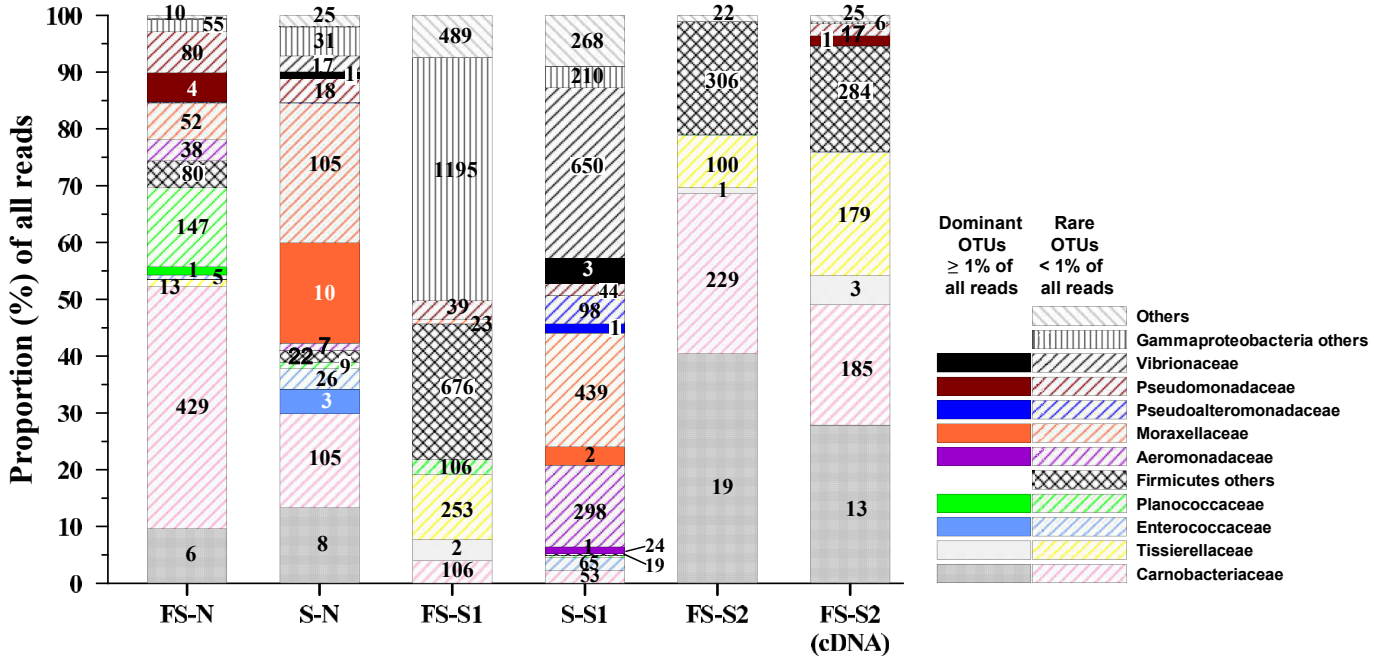

Fig. 1. Distribution of major bacterial groups detected by pyrosequencing in skate and fermented skate samples. Data were normalized by randomly subsampling to 2000 reads in each sample 100 times and average values were shown in percentage. Solid and hatched bars having the same color represent, respectively, dominant (≥1% of total) and rare (<1% of total) bacterial operational taxonomic units (OTUs) affiliated to the same family. The actual number of OTUs for each group in each sample is shown in each bar. For descriptions of the sample IDs, see Table 1. Column FS-S2_cDNA represents the active bacterial community composition of the FS-S2 sample.

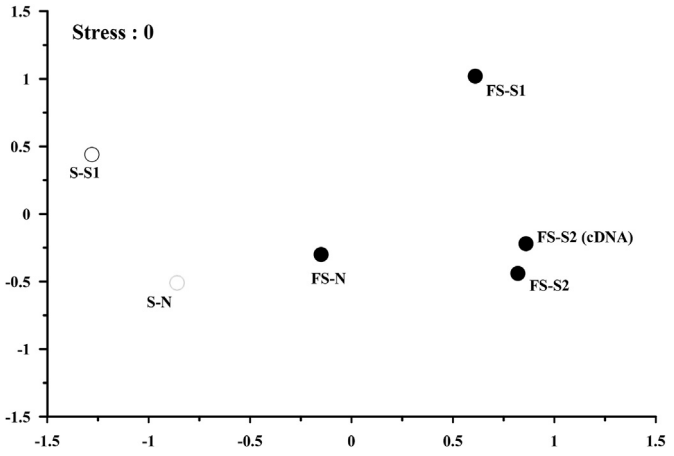

Fig. 2. Multidimensional scaling diagram showing the degree of similarity among skate samples. The description of sample IDs is the same as in Fig. 1.

belonged to the order *Lactobacillales*, a representative group including LAB, indicates that fermented skates might be a useful source for finding novel bacteria and possibly marine LAB.

Fermented seafood products often have non-LAB as the predominant members: *Proteobacteria* dominated in one *jeotgal* (Roh et al., 2010) and one *nukadoko* sample (Sakamoto et al., 2011) and *Actinobacteria* in one *nukadoko* sample (Sakamoto et al., 2011). Non-LAB were also abundant (25–47%), and consisted of mostly *Gammaproteobacteria* (mainly *Pseudomonadaceae* and unclassified *Gammaproteobacteria*) in the two fermented skate samples (FS-N and FS-S1). However, the non-LAB putrefactive bacteria, *Citrobacter* and *Clostridium*, were respectively undetected and undetected/rare (<0.2%) in fresh and fermented skates in this study. Our pyrosequencing data are consistent with a recent study in which those non-LAB putrefactive bacteria did not exceed 6% of the bacterial reads in *narezushi* (Kiyohara et al., 2012). It was also shown that food-poisoning pathogens such as *E. coli*, *Listeria monocytogenes*, *Salmonella* spp. and *Vibrio parahaemolyticus* were not detected in fermented skate products, except *Staphylococcus aureus* which occurred in 2 out of 18 samples examined (Lee et al., 2008). But, OTUs closest to the above-mentioned five pathogens and *Clostridium perfringens* were not detected in fermented skates in this study (data not shown). In acidic fermented foods, LAB are known to inhibit the growth of spoilage bacteria by producing lactic acid or bacteriocin (Jeevaratnam et al., 2005), whereas little is known about the role of *Lactobacillales* and marine LAB in alkaline fermentation of seafood. However, distributions of *Vibrionales*, *Gammaproteobacteria*, and *Firmicutes*, as well as prokaryotic abundance in skates and fermented skates suggest elimination of *Vibrionales* and suppression of *Gammaproteobacteria* by growth of *Firmicutes* in fermented skates. Some strains of the dominant taxa in fermented skates might have potential value as probiotic bacteria.

Interestingly, fermented skates had higher bacterial species richness compared to skates (Table S2). The Venn diagrams suggest that only a small fraction (0.02–0.21) of OTUs in fresh skates survived during fermentation (Fig. S1). This could be largely due to growth of unique OTUs, which were initially undetectable. Furthermore, bacterial species richness (Chao1) in alkaline-fermented skates (1230–6862; Table S2) was much higher than that found in other fermented seafood (11–88; Roh et al., 2010), as determined by pyrosequencing. Archaea have been suggested to play important roles in seafood fermentation such as in *jeotgals* (Roh et al., 2010). However, q-PCR showed that Archaea comprised a very low portion (<0.001%) of the prokaryotic community after fermentation of skate, indicating an overwhelming role for bacteria in skate fermentation. Archaea are probably not as competitive as *Bacilli* and *Clostridia* in the alkaline pH conditions developed during the fermentation of skate.

The MDS plot seems to suggest that the compositions of prokaryotes in fermented skates are influenced by the different fermentation condition (e.g., incubation time) employed among manufacturers and probably the source of skates. It is interesting to note that fermentation of different species of skate (i.e., *Dipturus*

Table 4
Relative abundance of archaeal taxa in fresh and fermented skate samples. Data were normalized by randomly subsampling to 170 reads in each sample 100 times and average values were used to calculate relative abundances. The relative abundance of each archaeal taxon which was identified using the RDP classifier (80% confidence) was calculated by dividing the numbers of reads assigned to each group by 170 per sample. Phylum, class, and order level classifications are highlighted in bold and their remaining taxa are classified to the level of family or genus. –: not detected. The description of sample IDs is the same as in Table 2.

	Groups	FS-N	S-N	FS-S1	S-S1	FS-S2
	Unclassified Archaea	–	–	**0.26**	**1.02**	**0.17**
Phylum	**Thaumarchaeota**	–	**100**	**89.49**	**52.16**	**9.88**
	Nitrosopumilales	–	100	89.49	52.16	9.88
	Nitrosopumilaceae	–	100	89.49	52.16	9.88
	Nitrosopumilus	–	100	89.49	52.16	9.88
Phylum	**Euryarchaeota**	**100**	–	**10.25**	**46.82**	**89.95**
	Unclassified Euryarchaeota	–	–	0.28	0.48	–
Class	**Halobacteria**	**100**	–	**9.95**	**38.05**	**89.95**
	Unclassified Halobacteria	–	–	–	–	0.17
	Haloferacales	**100**	–	**1.68**	**0.12**	**0.52**
	Haloferacaceae	100	–	1.68	0.12	0.52
	Halolamina	100	–	1.68	0.12	0.52
	Halobacteriales	–	–	**8.27**	**37.93**	**89.25**
	Halobacteriaceae	–	–	8.27	37.93	89.25
	Unclassified Halobacteriaceae	–	–	0.09	0.06	–
	Haloarcula	–	–	8.09	0.60	89.25
	Halococcus	–	–	0.09	37.27	–
Class	**Methanomicrobia**	–	–	**0.02**	**8.30**	–
	Methanosarcinales	–	–	**0.02**	**8.30**	–
	Methanosarcinaceae	–	–	0.02	8.30	–
	Unclassified Methanosarcinaceae	–	–	0.02	0.75	–
	Methanolobus	–	–	–	3.00	–
	Methanomethylovorans	–	–	–	4.55	–

Table 5
Bacterial strains isolated from skate and fermented skate samples. Identified closest validly-published species with their accession No, isolate's similarity to the species, No of isolates and isolation conditions are shown. Actino: Actinobacteria, Beta: Beta-Proteobacteria, Gamma: Gamma-Proteobacteria, Lacto: Lactobacillales, A: Archaea medium, C: CSA medium, G: GYPB medium, M: Marine agar (Difco). For references for the composition of media, see Supplemental materials and methods.

Samples[a]	Closest validly-published species	Group (Family name)	Accession #	Similarity (%)	Number of strains isolated (from fermented skates)	Urease activity (no. of positive results/tested samples)	Isolation condition				References[d]
							Medium	Seasalts (%)	pH	Temp (°C)	
S-N/FS-N/ S-S1/FS-S1	*Psychrobacter maritimus* Pi2-20(T)	Gamma (Moraxellaceae)	AJ609272	99.0–100	20 (14)	12/19	A/M/G	1/3/6	7.5/9.5	4/25/37	(1)
FS-N/S-S1/ FS-S1	*Pseudomonas caeni* HY-14(T)	Gamma (Pseudomonadaceae)	EU620679	94.8–99.8	17 (15)	3/16	A/M/G	1/3/6	7.5/9.5	4/25	(2)
S-N/FS-N/ FS-S1	*Carnobacterium mobile* DSM 4848(T)	Lacto (Carnobacteriaceae)	AB083414	96.9–99.9	10 (5)	0/10	A/M/G	1/3	7.5/9.5	4/25[b]/37	(3)
	Vagococcus salmoninarum CCUG 33394(T)	Lacto (Enterococcaceae)	Y18097	99.8–100	7 (5)	0/7	A/M/G	1/3	7.5/9.5	4/25[b]	(4)
S-N/S-S1	*Psychrobacter cibarius* JG-219(T)	Gamma (Moraxellaceae)	AY639871	99.8–100	9	1/9	A/M	3/6	7.5/9.5	4/25	(5)
	Psychrobacter faecalis Iso-46(T)	Gamma (Moraxellaceae)	AJ421528	99.7–99.8	7	1/5	A/M	1/3	7.5/9.5	4/25/37	(6)
	Oceanisphaera ostreae T-w6(T)	Gamma (Aeromonadaceae)	HQ340607	98.0–99.8	4	4/4	A/M	3/6	7.5/9.5	4/25/37	(7)
S-N/FS-N	*Trichococcus pasteurii* KoTa2(T)	Lacto (Carnobacteriaceae)	X87150	94.1–94.6	4 (3)	0/3	A/M/G	3/6	7.5	25/37	(8)
FS-N/S-S1	*Carnobacterium funditum* pf3(T)[c]	Lacto (Carnobacteriaceae)	S86170	99.2–99.7	5 (2)	0/5	A/M/G	3/6	7.5	4/25	(9)
	Facklamia tabacinasalis CCUG30090(T)	Lacto (Aerococcaceae)	Y17820	92.4–94	2	0/2	A/G	1/3	9.5	25	(10)
S-N	*Arthrobacter soli* SYB2(T)	Actino (Micrococcaceae)	EF660748	100	1	0/1	A/G	3	7.5/9.5	25/37	(11)
	Bacillus oceanisediminis H2(T)	Bacillales (Bacillaceae)	GQ292772	99.8	1	0/1	M	3	9.5	25	(12)
	Psychrobacillus insolitus DSM 5(T)	Bacillales (Bacillaceae)	AM980508	93.4	1	0/1	M/G	1/3	7.5/9.5	25/37	(13)
	Brochothrix thermosphacta DSMZ 20171(T)	Bacillales (Listeriaceae)	AY543023	99.8	1	0/1	G	1/6	7.5	25	(14)
	Planococcus halocryophilus Or1(T)	Bacillales (Planococcaceae)	ANBV01000012	99.5	1	0/1	A	6	9.5	25	(15)
	Staphylococcus equorum subsp. *equorum* ATCC 43958(T)	Bacillales (Staphylococcaceae)	AB009939	100	1	1/1	G	6	9.5	25	(16)
	Staphylococcus lentus ATCC 29070(T)	Bacillales (Staphylococcaceae)	D83370	100	12	0/8	A/C/M	3/6	7.5/9.5	25/37	(17)
	Staphylococcus sciuri subsp. *sciuri* DSM 20345(T)	Bacillales (Staphylococcaceae)	AJ421446	100	1	0/0	C	3	9.5	25	(18)
	Staphylococcus xylosus ATCC 29971(T)	Bacillales (Staphylococcaceae)	D83374	99.9–100	5	3/4	A/M/G	3/6	7.5/9.5	25/37	(19)
	Myroides phaeus MY15(T)	Bacteroidetes (Flavobacteriaceae)	GU253339	99.9	1	0/1	A	3	7.5	37	(20)
	Alcaligenes faecalis subsp. *faecalis* IAM12369(T)	Beta (Alcaligenaceae)	D88008	99.2	1	0/0	G	3	9.5	25	(21)
	Enterobacter cancerogenus LMG 2693(T)	Gamma (Enterobacteriaceae)	Z96078	99.5–99.6	2	0/1	A/C	3/6	7.5/9.5	25[b]/37	(22)
	Enterobacter turicensis 508/05(T)	Gamma (Enterobacteriaceae)	DQ273681	99.0	1	0/0	C	6	9.5	25	(23)
			AB273740	99.9	1	0/0	A/C	3	7.5/9.5	25/37	(24)

(*continued on next page*)

Table 5 (*continued*)

Samples[a]	Closest validly-published species	Group (Family name)	Accession #	Similarity (%)	Number of strains isolated (from fermented skates)	Urease activity (no. of positive results/tested samples)	Isolation condition				References[d]
							Medium	Seasalts (%)	pH	Temp (°C)	
	Leclercia adecarboxylata GTC 1267(T)	Gamma (Enterobacteriaceae)									
	Providencia vermicola OP1(T)	Gamma (Enterobacteriaceae)	AM040495	99.8	1	0/0	C/G	3/6	7.5/9.5	25[b]/37	(25)
	Psychrobacter alimentarius JG-100(T)	Gamma (Moraxellaceae)	AY513645	99.4	1	0/1	G	6	9.5	4/25	(26)
	Psychrobacter celer SW-238(T)	Gamma (Moraxellaceae)	AY842259	99.2	1	0/1	A	3	9.5	37	(27)
	Psychrobacter fozii NF23(T)	Gamma (Moraxellaceae)	AJ430827	98.9–99.9	1	0/1	A	6	9.5	25	(28)
	Psychrobacter fulvigenes KC 40(T)	Gamma (Moraxellaceae)	AB438958	99.6–100	3	1/3	A	3/6	7.5/9.5	25/37	(29)
	Psychrobacter sanguinis 13983(T)	Gamma (Moraxellaceae)	HM212668	99.6	1	0/0	G	6	7.5	37	(30)
	Pseudoalteromonas nigrifaciens NCIMB 8614(T)	Gamma (Pseudoalteromonadaceae)	X82146	100	9	2/5	A/C/M	3/6	7.5/9.5	4/25	(31)
	Shewanella baltica NCTC 10735(T)	Gamma (Shewanellaceae)	AJ000214	98.4–99.0	2	0/1	A/G	1/3	7.5/9.5	4/25	(32)
	Aerococcus urinaeequi IFO 12173(T)	Lacto (Aerococcaceae)	D87677	99.9	1	0/1	G	6	9.5	25	(33)
	Vagococcus lutrae CCUG 39187(T)	Lacto (Enterococcaceae)	Y17152	99.9	1	0/1	A	3	9.5	37	(34)
FS-N	*Kytococcus sedentarius* DSM 20547(T)	Actino (Dermacoccaceae)	CP001686	99.6	1	0/1	A	3	7.5	37	(35)
	Micrococcus yunnanensis YIM 65004(T)	Actino (Micrococcaceae)	FJ214355	99.9	1	0/1	A	3	7.5	37	(36)
	Carnobacterium iners LMG 26642(T)	Lacto (Carnobacteriaceae)	HE583595	99.5–100	3	0/2	A/M/G	3/6	7.5	25	(37)
	Carnobacterium inhibens K1(T)	Lacto (Carnobacteriaceae)	Z73313	98.9	1	0/0	G	6	9.5	25	(38)
	Carnobacterium jeotgali MS3(T)	Lacto (Carnobacteriaceae)	EU817500	99.2–100	16 (16)	0/12	A/C/M/G	3/6	7.5/9.5	4/25[b]	(39)
	Carnobacterium maltaromaticum BA (T)	Lacto (Carnobacteriaceae)	AF184247	99.9	1 (1)	0/1	G	1/3/6	7.5/9.5	4/25[b]	(40)
	Trichococcus palustris DSM 9172(T)	Lacto (Carnobacteriaceae)	AJ296179	95.3–95.9	3	0/2	M/G	3/6	7.5/9.5	25	(41)
S-S1	*Vagococcus fluvialis* CCUG 32704(T)	Lacto (Enterococcaceae)	Y18098	99.7	1	0/1	M	3	9.5	25	(42)
	Carnobacterium viridans MPL-11(T)	Lacto (Carnobacteriaceae)	AF425608	99.0	1	0/1	M	3	9.5	25	(43)
	Chryseobacterium carnis NCTC 13525(T)	Bacteroidetes (Flavobacteriaceae)	JX100817	99.0	1	0/1	M	3	7.5	25	(44)
FS-S1	*Psychrobacter urativorans* DSM 14009(T)	Gamma (Moraxellaceae)	AJ609555	98.6	1	1/1	A	3	7.5	25	(45)

[a] S-N, fresh skate purchased from Naju; FS-N, fermented skate purchased from Naju; S-S1, fresh skate purchased from Seoul; FS-S1, fermented skate purchased from Seoul.
[b] The strain(s) was isolated under both aerobic and anaerobic condition at 25 °C.
[c] Strain of *Carnobacterium funditum* seemed to be alkalotolerant at least up to pH 8.9. Type species of *C. funditum* is a known marine lactic acid bacteria and reported to be neutrophilic (9)[d].
[d] References are listed in Supplementary information.

batis) exhibited distinguished bacterial compositions composed predominantly of the *Gammaproteobacteria* group (91%), and the phylum *Firmicutes* (~8%), *Bacteroidetes* (1%), and *Tenericutes* (0.2%) (Reynisson et al., 2012). In fermented skate (*Raja kenojei*) skins, *Photobacterium* sp. and *Vibrio* spp. (*Gammaproteobacteria*) were the predominant microflora (Cho et al., 2004). The composition of LAB found in African alkaline-fermented foods also vary considerably according to the raw material (Ouoba et al., 2010). The occurrence of bacterial OTUs uniquely present in fresh skates may reflect the bacteria in raw materials and the manufacturing environment. Further, the predominance of *Moraxellaceae* in fresh skates might be partially due to storage conditions of low temperature (ca. 0–4 °C) for 3–5 days between fishing and selling; this procedure would favor cold-adapted bacteria like *Psychrobacter* (Bowman, 2006) pre-dominating the family *Moraxellaceae*. The observed changes in BCCs in fermented skates correlated with pronounced changes in the chemical composition of fermented skates, i.e., conspicuous production of ammonia and decomposition of urea. Interestingly, all of the isolated lactobacilli strains did not show urease activity, whereas this was observed in some strains of *Gammaproteobacteria* (Table 5). To date, urease has not been reported in known marine LAB. Presumably, *Gammaproteobacteria* are important in decomposition of urea during the fermentation of skates. High rates of urea decomposition during skate fermentation are deduced on the basis of conservative estimates of urea decomposition based on final bacterial abundance, the fermentation time for the FS-S1 and FS-N samples (i.e., 0.17–0.24 pmol urea $cell^{-1}$ d^{-1}), and the reported maximum per-cell urease activity of 0.09 pmol $cell^{-1}$ d^{-1} in the seas (Cho and Azam, 1995; Cho et al., 1996). Decomposition of urea in skate (equivalent to ammonia production of 715 mg N per 100 g) alone was not sufficient to explain the observed production of ammonia. The urea:methylamine ratio in shallow-dwelling Chondrichthyes is approximately 2:1 (Laxson et al., 2011). Assuming that all methylamine was deaminated (i.e., 358 mg N per 100 g), approximately 1500 mg N ammonia per 100 g must be produced from other processes (e.g., deamination of amino acids). In our study, involvement of yeast or moulds in the fermentation process was not analyzed. However, since yeasts or moulds are present as a rarity or minority in abundance of microorganisms in slightly acidic pH fermented fish product (Rapsang and Joshi, 2012), they might be present and significant in alkaline fermentation of skates and it remains to be seen. Accordingly, the per-cell urease activities in this study might be somewhat overestimated due to the presence of fungi in fermented skates. Finally, it needs to be mentioned that bacterial abundance in contamination controls determined by q-PCR was negligible (<0.004%) compared to that in samples (data not shown), and contamination controls had considerably different compositions from skate samples (Table S7). Thus, profiles of major compositions of bacteria in skate samples would not be affected.

5. Conclusions

The development of unique bacterial communities in fermented skates seems to be associated with anaerobic and alkaline conditions formed during the fermentation of skates. The identified important prokaryotic groups in alkaline-fermented skates were *Atopostipes*- and *Tissierella*-like OTUs, which might aid in the selection of possible starter bacteria for improving the quality of fermented skates. Understanding the role of *Lactobacillales* of marine origin in alkaline-fermented seafood is a promising research area. Our study demonstrates that alkaline-fermented skates are useful reservoirs of undiscovered marine bacteria and possibly of marine LAB and novel antimicrobial substances.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2016.08.008.

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