Isolation and identification of hyper-ammonia-producing bacteria from commercial fermented skates (*Raja kenoei*)

Chang-Cheng Zhao¹ · Jong-Bang Eun¹

Revised: 19 September 2018 / Accepted: 24 September 2018 / Published online: 20 October 2018
© Association of Food Scientists & Technologists (India) 2018

Abstract Ammonia-producing bacteria were isolated and identified from five commercial fermented skates (A1, A2, A3, A4, and A5). In addition, the pH, ammonia nitrogen, total volatile nitrogen (TVBN), trimethylamine nitrogen (TMAN), and amino nitrogen contents of skate samples were also determined. A total of 88 strains of ammonia-producing bacteria was isolated and seven hyper-ammonia-producing bacteria isolates (A2-2, A2-3, A2-12, A2-18, A2-20, A3-6 and A3-14) were selected based on ammonia nitrogen producing ability. Those isolates were identified as *Proteus hauseri* (three strains), *Providencia rustigianii* (three strains), and *Kurthia gibsonii*. The pH and ammonia nitrogen content in skate samples were ranged from 8.63 to 9.03, and 4.86 to 7.31 g/kg, respectively. No significant difference of pH values was observed in skate samples A2, A3, A4 and A5. While, skate samples A3, A4 and A5 showed similar level of TVBN and TMAN content. Skate sample A2 showed the highest amino nitrogen content among all samples, which indicated the highest degree of protein degradation of skate muscle during fermentation. Bivariate cluster analysis showed that skate samples A3, A4, and A5 clustered together at a relatively high level, implying a similar microbial environment during fermentation. The cluster analysis allowed different commercial fermented skates to be clearly differentiated based on the

characteristics determined in this study. This study can provide important information for investigating the mechanisms underlying ammonia flavor formation in skate muscle during fermentation.

Keywords Fermented skate · Ammonia producing bacteria · Isolation · Identification · Cluster analysis

Introduction

Skate (*Raja kenoei*) is a typical elasmobranch fish that is widely consumed in China, Korea, and Japan (Lee et al. 2016). Skate is traditionally fermented in a ceramic jar without any additive for 1 week at room temperature (25 °C) (Mizuta et al. 2003; Jang et al. 2017). Fermented skate is popular in South Korea, especially in the Jeollanam-do Province, due to its elastic texture, ammonia-like flavor, and other functional benefits (Hwang et al. 2007). It has been reported that high concentrations of nitrogenous compounds are produced in skate muscle during fermentation, giving skate products a unique ammonia-like flavor (Choi et al. 2005; Kim et al. 2010). Based on previous studies, the formation of ammonia flavor may be related to the degradation of urea and trimethylamine oxide (TMAO) into volatile bases, such as ammonia, mediated by microorganisms (Laxson et al. 2011; Weiner et al. 2014). Production of these chemicals is the result of microbial activity, although little was known about the types of microorganisms responsible for their production in fermented skate until recently.

Previous studies on bacterial communities in fermented skate show that most of the bacteria present in fermented skate are strains closely related to the genera *Pseudomonas*, *Stenotrophomonas*, and *Psychrobacter* (Cho et al. 2004;

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13197-018-3447-9>) contains supplementary material, which is available to authorized users.

✉ Jong-Bang Eun
jbeun@jnu.ac.kr; jongbang@hotmail.com

¹ Department of Food Science and Technology and BK 21 Plus Program, Graduate School of Chonnam National University, Gwangju 61186, South Korea

Lee et al. 2010). In the study on bacterial succession during the fermentation process of skate, Gammaproteobacteria comprised the majority (91%), and Firmicutes constituted the minority (Reynisson et al. 2012). Interestingly, Jang et al. (2017) reported that marine bacteria belonging to the taxa Lactobacillales and Clostridia are more important in the alkaline fermentation of skates. However, we believe that their bacteria community analysis merely provided basic information on the development of the microbiota and the population dynamics during skate fermentation. It is difficult to identify and isolate the exact microorganisms responsible for ammonia production with non-selective culture procedures.

Previous studies on the ruminal ecosystem have indicated the existence of bacteria capable of producing large amounts of ammonia, called hyper-ammonia-producing (HAP) bacteria (Attwood et al. 1998; Chen and Russell 1989). These bacteria were present in low concentrations in the rumen but their ammonia-producing activities were sufficient for them to be quantitatively important. Therefore, enrichment and selective techniques should be employed to isolate ammonia-producing bacteria from fermented skates. The objectives of this study were to isolate and identify the HAP bacteria from 5 different commercial fermented skates using enrichment and selective techniques. This is the first-time ammonia-producing bacteria were isolated from fermented skates, providing important information to further elucidate the mechanism underlying ammonia formation in fermented skate, as well as for developing starter cultures with known characteristics for use in the commercial production of fermented skate with stable and consistent quality.

Materials and methods

Samples

Fermented skates (*Raja kenojei*) were purchased from five different fish processing plants in South Korea. The detailed information on the fermented skate samples used in this study are shown in Table 1S (Supplementary materials). All skate samples were placed in sterile zipper bags and transferred to the laboratory on the day of purchase. The samples were stored at 4 °C until they were used for physicochemical and microbiological analyses.

Enrichment and isolation of ammonia-producing bacteria

Ten grams of each collected fermented skate were transferred aseptically into sterile selection/enrichment (SE) liquid medium (per liter): 1.0 g of uric acid, 2.5 g of yeast

extract, 1.0 g of glucose, 100 mg of KH_2PO_4 , 10 mg of CaCl_2 , 30 mg of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg of NaCl, and 1 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (Schefferle 1965). The cultures were incubated at 30 °C with shaking at 160 rpm for 24 h to measure the total ammonia-producing bacteria that grow under aerobic conditions. The distinct colonies were streaked onto SE plates and incubated at 30 °C for 24 h to obtain pure isolates.

Analyses of bacterial growth and ammonia production

All bacterial isolates were measured by determining the optical density at 660 nm. To measure the ammonia production of the isolates, aliquots of cultures were subjected to centrifugation (14,500×g, 5 min). The concentration of ammonia nitrogen in the supernatant was determined through Nessler's reagent spectrophotometer method (Chen et al. 2015).

DNA extraction

Bacterial isolates that produced high levels of ammonia were selected. Aliquots of overnight cultures were centrifuged (14,500×g, 5 min) and the bacterial cell pellet was used for DNA extraction. A FastDNA Spin Kit (MPbio, LLC., Santa Ana, CA, USA) was used according to the manufacturer's instructions combined with the bead-beating method. The extracted DNA was stored at −20 °C until use.

16S rRNA gene sequencing analyses

The genomic DNA of each bacterial isolate was used as template for PCR amplification of a segment of the 16S rRNA gene using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1942R (5'-GGTTACCTTGTTACGACTT-3'). Amplification was performed in a thermal cycler (TP600, TaKaRa Bio Inc, Shiga, Japan) under the conditions described by Liu et al. (2012): initial denaturation at 94 °C for 1 min; 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 1 min; and final extension at 72 °C for 10 min. The PCR products were confirmed by electrophoresis on 1% agarose gels loaded with 1 mL of sample and purified using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). Sequencing analysis of purified products was performed by ChunLab, Inc. (Seoul, South Korea).

Data analysis and species identification

The raw sequencing reads obtained from pyrosequencing were screened for chimeras using BLAST on the NCBI website (<http://www.ncbi.nlm.nih.gov/>). Taxonomic assignment was performed by comparing the sequence reads against the EzTaxon database (<http://eztaxon-e.ezbiocloud.net>). The alignments were analyzed to construct a phylogenetic tree and to compare similarities among the sequences by the neighbor-joining method (Saitou and Nei 1987) using MEGA software version 4.0.

pH and ammonia nitrogen content

The pH of skate muscle was determined using a pH meter (EF-7732, Istek, Seoul, Korea) according to the method of Xu et al. (2010). Ammonia nitrogen content was determined by the distillation method described by Dissara-phong et al. (2006) with a slight modification. Skate muscle (4 g) was homogenized with 100 mL of distilled water and placed in a 400-mL Kjeldahl flask with 3 g MgO. The mixture was distilled and collected in 50 mL of 4% boric acid consisting of the mixed indicator (methyl red: bromocresol green: methylene blue). Finally, the distillate was titrated with 0.05 M H₂SO₄ until endpoint. The ammonia nitrogen content was calculated according to following equation:

$$\text{Ammonia nitrogen content (g/kg)} = \frac{28 \times M \times V}{W} \quad (1)$$

where M: the concentration of H₂SO₄ (M); V: the volume of H₂SO₄ (mL); W: the weight of sample (g).

Total volatile base nitrogen (TVBN) and trimethylamine nitrogen (TMAN) content

TVBN and TMAN contents of skate muscle were measured based on the method described by Ng (1987). Samples of skate muscle (2 g) were homogenized with 8 mL of 4% trichloroacetic acid (TCA). After centrifugation, 1 mL of the supernatant and 1% boric acid contained Conway indicator were placed in the outer and inner ring of the Conway apparatus, respectively. To initiate the reaction, 1 mL of saturated K₂CO₃ solution was added to the outer ring. The Conway apparatus was closed immediately and incubated at 37 °C for 1 h.

TMAN was measured in the same manner, except 1 mL of 10% formaldehyde was added to the sample extract. The inner ring solution was titrated with 0.02 N HCl until the endpoint, in which the solution color turned from green to pink.

Amino nitrogen content

The formal titration method was used to determine the amino nitrogen content of skate muscle. Five grams of skate muscle was added to 95 mL of distilled water and was homogenized at 11,270 × g for 1 min. After centrifugation (1700 × g, 5 min), the supernatant was adjusted to pH 8.4 with 0.1 N NaOH. Subsequently, 10 mL of distilled water and neutral formal were added to the supernatant, and was then titrated with 0.1 N NaOH to reach to pH 8.4. The amino type nitrogen content was calculated according to the following equation:

$$\text{Amino nitrogen content (mg/g)} = \frac{[\text{sample titration (mL)} - \text{blank test (mL)}] \times 1.4 \times F \times D \times 100}{S} \quad (2)$$

where F: factor of 0.1 N NaOH; D: dilution factor; S: sample amount (g).

Results and discussion

Enrichment and isolation of ammonia producing bacteria

Each fermented skate sample was serially diluted, as described in the Materials and Methods, and either plated directly onto SE agar plates. A variety of colony types was noted upon direct isolation plating and upon streaking of enriched cultures; different colony types were picked for further study. In total, 88 bacterial strains were isolated and grown on SE medium.

Growth rate and ammonia producing ability of isolated strains

Previous studies have not made connections between bacterial populations and ammonia production. However, the dominant bacteria in fermented samples may produce small amounts of ammonia (Whitehead and Cotta 2004). We thus analyzed the growth and ammonia production of particular bacterial strains we isolated and selected on SE medium. Our results showed that strains A1-1, A1-13, A1-16, A3-2, A3-16, A4-1, A4-4, A4-9, A4-10, and A4-14 showed high growth rate (OD₆₆₀ > 0.8) but low ammonia production. On the other hand, high ammonia production was observed in strains A2-2, A2-3, A2-12, A2-18, A2-20, A3-6, and A3-14; however, these strains were present in low concentrations (Table 1). Similar results have also been reported in a previous study where in ammonia-producing bacteria were isolated from New Zealand ruminants (Attwood et al. 1998). They examined the ammonia production of the

Table 1 Growth rate (OD₆₆₀) and ammonia production by fermented skate isolates on selection medium after 24-h incubation

Isolates	OD ₆₆₀	Ammonia production (mg/mL)
A1-1	0.8590 ± 0.065	0.0401 ± 0.0019
A1-2	0.2370 ± 0.0168	0.0342 ± 0.0060
A1-3	0.2447 ± 0.0107	0.0277 ± 0.0039
A1-4	0.2240 ± 0.0062	0.0277 ± 0.0015
A1-5	0.2237 ± 0.0097	0.0353 ± 0.0067
A1-6	0.4857 ± 0.0081	0.0342 ± 0.0039
A1-7	0.2213 ± 0.0067	0.0332 ± 0.0035
A1-8	0.2693 ± 0.0021	0.0291 ± 0.0008
A1-9	0.2307 ± 0.0049	0.0342 ± 0.0056
A1-10	0.2263 ± 0.0119	0.0420 ± 0.0031
A1-11	0.4927 ± 0.0040	0.0349 ± 0.0020
A1-12	0.4190 ± 0.0079	0.0247 ± 0.0028
A1-13	0.8377 ± 0.0057	0.0399 ± 0.0001
A1-14	0.2517 ± 0.0090	0.0062 ± 0.0002
A1-15	0.1637 ± 0.0049	0.0307 ± 0.0046
A1-16	0.8040 ± 0.1179	0.0418 ± 0.0030
A1-17	0.2097 ± 0.0095	0.0377 ± 0.0021
A1-18	0.2270 ± 0.0070	0.0328 ± 0.0022
A1-19	0.2313 ± 0.0103	0.0321 ± 0.0003
A1-20	0.2433 ± 0.0067	0.0216 ± 0.0026
A2-1	0.4387 ± 0.0247	0.0718 ± 0.0025
A2-2	0.4460 ± 0.0149	0.0827 ± 0.0023
A2-3	0.1520 ± 0.0017	0.0854 ± 0.0009
A2-4	0.3583 ± 0.0029	0.0760 ± 0.0004
A2-5	0.6790 ± 0.1100	0.0793 ± 0.0006
A2-6	0.4603 ± 0.0031	0.0775 ± 0.0008
A2-7	0.3103 ± 0.0006	0.0564 ± 0.0003
A2-8	0.4907 ± 0.0031	0.0794 ± 0.0006
A2-9	0.3580 ± 0.0020	0.0777 ± 0.0004
A2-10	0.6687 ± 0.0032	0.0768 ± 0.0007
A2-11	0.1393 ± 0.0168	0.0572 ± 0.0003
A2-12	0.4613 ± 0.0021	0.0814 ± 0.0005
A2-13	0.4680 ± 0.0035	0.0635 ± 0.0006
A2-14	0.4663 ± 0.0074	0.0777 ± 0.0002
A2-15	0.3597 ± 0.0123	0.0586 ± 0.0008
A2-16	0.2533 ± 0.0057	0.0619 ± 0.0004
A2-17	0.4573 ± 0.0021	0.0793 ± 0.0001
A2-18	0.3647 ± 0.0095	0.0844 ± 0.0003
A2-19	0.7133 ± 0.0025	0.0765 ± 0.0006
A2-20	0.3483 ± 0.0064	0.0802 ± 0.0005
A2-21	0.1373 ± 0.100	0.0603 ± 0.0010
A2-22	0.4350 ± 0.0111	0.0691 ± 0.0003
A2-23	0.6860 ± 0.0087	0.0789 ± 0.0004
A2-24	0.4230 ± 0.0115	0.0642 ± 0.0002
A3-1	0.7755 ± 0.0106	0.0727 ± 0.0001
A3-2	0.8075 ± 0.0403	0.0759 ± 0.0002
A3-3	0.4790 ± 0.0226	0.0778 ± 0.0007
A3-4	0.2850 ± 0.0057	0.0573 ± 0.0007

Table 1 continued

Isolates	OD ₆₆₀	Ammonia production (mg/mL)
A3-5	0.2530 ± 0.0057	0.0557 ± 0.0002
A3-6	0.3985 ± 0.0007	0.0799 ± 0.0002
A3-7	0.6680 ± 0.0057	0.0698 ± 0.0002
A3-8	0.3695 ± 0.0389	0.0595 ± 0.0001
A3-9	0.3600 ± 0.0325	0.0715 ± 0.0000
A3-10	0.7190 ± 0.0042	0.0686 ± 0.0003
A3-11	0.4660 ± 0.0014	0.0778 ± 0.0008
A3-12	0.6805 ± 0.0035	0.0727 ± 0.0002
A3-13	0.4665 ± 0.0049	0.0786 ± 0.0005
A3-14	0.4995 ± 0.0021	0.0832 ± 0.0020
A3-15	0.4390 ± 0.0226	0.0791 ± 0.0004
A3-16	1.1800 ± 0.0099	0.0682 ± 0.0001
A3-17	0.5280 ± 0.0141	0.0683 ± 0.0005
A3-18	0.3150 ± 0.0028	0.0766 ± 0.0007
A4-1	0.8263 ± 0.0283	0.0675 ± 0.0016
A4-2	0.3980 ± 0.0040	0.0501 ± 0.0004
A4-3	0.6783 ± 0.0136	0.0688 ± 0.0016
A4-4	0.9213 ± 0.0096	0.0795 ± 0.0099
A4-5	0.7270 ± 0.0396	0.0686 ± 0.0004
A4-6	0.4120 ± 0.0056	0.0642 ± 0.0006
A4-7	0.4727 ± 0.0051	0.0611 ± 0.0006
A4-8	0.4447 ± 0.0035	0.0594 ± 0.0001
A4-9	1.0353 ± 0.0171	0.0713 ± 0.0002
A4-10	1.0187 ± 0.0117	0.0744 ± 0.0001
A4-11	0.7837 ± 0.0143	0.0678 ± 0.0013
A4-12	0.2210 ± 0.0121	0.0574 ± 0.0024
A4-13	0.1883 ± 0.0006	0.0601 ± 0.0010
A4-14	0.8257 ± 0.0110	0.0716 ± 0.0008
A5-1	0.2930 ± 0.0017	0.0561 ± 0.0007
A5-2	0.1537 ± 0.0042	0.0548 ± 0.0004
A5-3	0.5770 ± 0.011	0.0540 ± 0.0007
A5-4	0.1757 ± 0.0085	0.0545 ± 0.0004
A5-5	0.1557 ± 0.0023	0.0551 ± 0.0008
A5-6	0.1857 ± 0.0104	0.0560 ± 0.0003
A5-7	0.2200 ± 0.0056	0.0533 ± 0.0008
A5-8	0.2160 ± 0.0082	0.0470 ± 0.0008
A5-9	0.5690 ± 0.0053	0.0583 ± 0.0004
A5-10	0.2207 ± 0.0093	0.0615 ± 0.0006
A5-11	0.2170 ± 0.0036	0.0580 ± 0.0001
A5-12	0.6550 ± 0.0046	0.0601 ± 0.0001

isolates grown in HAP liquid medium in their study. Though some isolates grew quickly, they produced relatively small amounts of ammonia, which was mainly produced during stationary phase of growth (Attwood et al.

1998). The results of this study confirmed the presence of an ammonia-producing metabolic group of bacteria. Despite their low abundance, the activity of such bacteria contributes significantly to the production of ammonia in skates during fermentation. These results prompted us to determine whether protein- and/or amino acid-fermenting bacteria are present in fermented skates at lower concentrations than those determined through previous isolation studies. There are a number of reports describing bacteria capable of producing large amounts of ammonia and present in low concentrations when isolated from the rumens of cattle around the world (Attwood et al. 1998; Eschenlauer et al. 2002).

Phylogenetic analysis of hyper-ammonia producing bacteria strain

The HAP bacteria isolated from commercial fermented skates were selected based on ammonia production, and these included the isolates A2-2, A2-3, A2-12, A2-18, A2-20, A3-6, and A3-14. The 16S rRNA genes of these isolates were subjected to sequencing and phylogenetic analysis (Fig. 1). Sequences similarity searches indicated that these HAP bacteria isolated from fermented skates were phylogenetically distinct from other HAP bacteria reported in previous researches (Chen et al. 2013; Ferme et al. 2004). Strains A2-2, A2-12, and A3-14 were identified to be *Proteus hauseri*; strain A2-3 was identified to be *Kurthia gibsonii*; and strains A2-18, A2-20, and A3-6 were identified to be *Providencia rustigianii* (Fig. 1). It has been reported that ammonia in the large gut is thought to be derived either from the hydrolysis of urea by bacterial, and possibly mucosal, ureases, or from the deamination of proteins and other nitrogenous substrates (Wolpert et al. 1970). *Proteus* spp., for example, produce ureases during skate fermentation, which breaks down urea in the urine and releases ammonia. O'Grady (1966) showed that the most active producers of ammonia among the intestinal organisms are gram-negative aerobic bacilli such as *Escherichia coli*, *Klebsiella* spp., *Proteus* spp., and *Pseudomonas* spp. Similar results have also been reported by Terence et al. (2011), such that most of the HAP bacteria isolated from swine manure storage pits were found to be most closely related to *Proteus* spp, as well as to the genus *Providencia*, which belongs to the tribe Proteae under the family Enterobacteriaceae. A characteristic feature that distinguishes tribe Proteae from other members of Enterobacteriaceae is the presence of the enzyme phenylalanine-deaminase, which converts phenylalanine to phenylpyruvic acid (PPA reaction) (Mohammed et al. 2016). The strains of the genus *Providencia* are non-lactose-fermenting, methyl red- and PPA-positive bacilli that are motile due to their peritrichous flagella. They also

produce a powerful urease enzyme that rapidly hydrolyses urea to ammonia (El Khatib et al. 2017). *Kurthia* spp. is a gram-positive genus of bacteria from the Planococcaceae family, which are commonly found in various meats, milk, and soils. It has been reported that this genus of bacteria produces 1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase to hydrolyze ACC (ethylene precursor) into ammonia and α -ketobutyrate to use as their sources of carbon and nitrogen (Glick et al. 1995). Interestingly, in the study of Sahadevan et al. (2016), it was also revealed that they could also produce other mycolytic enzymes such as protease, cellulase, and chitinase. The presence of HAP bacteria and other similar strains in fermented skates may prove to be an important factor in the digestion and fermentation of proteinaceous materials, especially in the production of ammonia and other compounds.

pH and ammonia nitrogen content

Figure 2 shows the pH and ammonia nitrogen content of commercial fermented skates. The lowest pH value was observed in fermented skate sample A1, whereas the pH values of the four other skate samples were not significantly different from each other. The observed pH was perhaps related to the accumulation of ammonia compounds, amines, and other basic substances produced from the degradation of proteins (Duan et al. 2010). A shorter fermentation time and/or lower enzyme activity may have caused the lower pH observed in the skate sample A1. The ammonia nitrogen content in the skate samples ranged from 4.86 g/kg (A1) to 7.31 g/kg (A4). As shown in Fig. 2, the lowest ammonia nitrogen content was observed in the skate sample A1, whereas the skate samples A4 and A5 exhibited significantly higher values (7.31 and 7.25 g/kg, respectively). The high ammonia nitrogen content can be explained by the higher concentration of nitrogen compounds such as trimethylamine oxide (TMAO) and urea produced due to the activity of microbial and endogenous enzymes (Xu et al. 2008). Furthermore, a relatively high concentration of urea and TMAO in skate muscle tissue for counterbalancing osmotic pressure in the deep sea—also responsible for this unique ammonia formation (Laxson et al. 2011). Consequently, in our study, the higher ammonia nitrogen content reflected the high pH observed, as well as the degradation of small peptides and amino acids due to enzymatic activity.

TVBN and TMAN content

The TVBN and TMAN contents were considered as the yields of nitrogenous materials produced due to microbial and endogenous enzymes. Both values are the most widely used quality indices in different fresh and fermented fish

Phylogenetic Tree

Fig. 1 Phylogenetic analysis of 16S rRNA gene sequences from hyper-ammonia-producing (HAP) bacterial strains (A2-2, A2-3, A2-12, A2-18, A2-20, A3-6 and A3-14). Bacterial species indicated are sequences obtained from Genbank

Fig. 2 pH (a) and ammonia nitrogen content (b) in different commercial fermented skate samples. Error bars represent standard deviation from three independent experiments; $a-cP < 0.05$ indicates statistical significance

products (Kilincceker et al. 2009). The TVBN and TMAN contents of the skate samples are shown in Fig. 3. Similar results were found for TVBN content as the ammonia nitrogen content that was measured in skate samples. The skate sample A1 showed the lowest TVBN content, while no significant differences were found between skate samples A4 and A5. In general, TVBN includes the nitrogen from ammonia, trimethylamine (TMA), and amine from the degradation of proteins and non-protein nitrogen compounds. Interestingly, skate sample A2 showed the highest TMAN content among the skate samples. TMA was produced from the decomposition of TMAO by bacteria, and is considered one of the primary ingredients responsible for spoilage of fish and the typical fishy odor (Kostaki et al. 2009). In the present study, the high TMAN content in skate sample A2 may be attributed to *Kurthia* spp., which was only found in that particular sample. However, the mechanism underlying TMAN production by *Kurthia* spp. remains unclear. The TVBN and TMAN

Fig. 3 Total volatile base nitrogen (TVBN) (a) and trimethylamine nitrogen (TMAN) (b) content in different commercial fermented skate samples. Error bars represent standard deviation from three independent experiments; $a-dP < 0.05$ indicates statistical significance

contents of fermented skates in the present study were consistent with those in the study by Lee et al. (2008), wherein the TVBN content of ready-to-eat skate products purchased from different markets of different locations was evaluated; they observed that the TVBN content of fermented skates was higher than that of other fermented fish products, such as sardines, tuna viscera, and Yu-lu. The high TVBN and TMAN contents in fermented skates may be directly attributed to the urea content present in the raw material and to the ammonia nitrogen produced during skate fermentation.

Amino nitrogen content

Measured amino nitrogen content consists of low molecular weight peptides and amino acids and is usually used as an indicator for degree of fermentation (Dissaraphong et al. 2006). Figure 4 shows the amino nitrogen contents in different skate samples. It was observed that skate sample A2 showed the highest amino nitrogen content, followed by skate samples A3 and A5. The high amino nitrogen content can be attributed to autolysis and microbial degradation of

Fig. 4 Amino nitrogen content in different commercial fermented skate samples. Error bars represent standard deviation from three independent experiments; ^{a–d} $P < 0.05$ indicates statistical significance

fish muscle (Ijong and Ohta 1996). Furthermore, the peptides and amino acids produced due to proteolytic activities are said to be responsible for the flavor and aroma of the fish products (Zhao et al. 2016). In line with this, perhaps the highest degree of protein degradation may have occurred in skate sample A2, as it had the highest TVBN and TMAN contents.

Bivariate cluster analysis

Bivariate cluster analysis can provide an effective graphical means of discriminating different source types and characteristics (Carslaw and Beevers 2013). In general, this form of cluster analysis is very efficient, though it tends to create small clusters. Thus, it was performed to further classify elements of different sources based on the similarities of their chemical properties. Figure 5 displays three

Fig. 5 Cluster analysis of different commercial fermented skate samples based on chemical characteristics

clusters: (1) A1; (2) A2; and (3) A3, A4, and A5. Two components explain 95.79% of the point variability. Additionally, A3, A4, and A5 were observed to have joined together at a relatively high level, implying similar fermentation conditions and/or bacterial community structures. The groups A1 and A2 were markedly different from the other elements in terms of their Euclidian distances in the cluster analysis. The cluster analysis allowed different commercial fermented skates to be clearly differentiated based on the characteristics determined in this study.

Conclusion

In the present study, enrichment and selective techniques were employed to isolate ammonia-producing bacteria from fermented skates. Seven isolates were selected based on their ammonia production and were identified to be *Proteus hauseri* (three strains), *Providencia rustigianii* (three strains), and *Kurthia gibsonii* (one strain). Although these species of bacteria were present in low concentrations, their ammonia-producing activities were sufficient to be quantitatively important. Further research is necessary to reveal the key enzymes associated with ammonia production and the underlying mechanisms during skate fermentation.

Acknowledgements This work was supported by a National Research Foundation of Korea Grant Funded by the South Korean Government (NRF 2017R1D1A1B03035782).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Attwood GT, Klieve AV, Ouwerkerk D, Patel BKC (1998) Ammonia-hyperproducing bacteria from New Zealand ruminants. *Appl Environ Microbiol* 64:1796–1804
- Carslaw DC, Beevers SD (2013) Characterising and understanding emission sources using bivariate polar plots and k-means clustering. *Environ Model Softw* 40:325–329
- Chen G, Russell JB (1989) More monensin-sensitive, ammonia-producing bacteria from the rumen. *Appl Environ Microbiol* 55:1052–1057
- Chen LL, Wang JL, Hu Y, Qian BJ, Yao XM, Wang JF, Zhang JH (2013) Computational design of glutamate dehydrogenase in *Bacillus subtilis* natto. *J Mol Model* 19:1919–1927
- Chen Z, Zhang WJ, Wang DS, Ma T, Bai RY (2015) Enhancement of activated sludge dewatering performance by combined composite enzymatic lysis and chemical re-flocculation with inorganic coagulants: kinetics of enzymatic reaction and re-flocculation morphology. *Water Res* 83(15):367–376

- Cho SH, Jahncke ML, Eun JB (2004) Nutritional composition and microflora of the fresh and fermented skate (*Raja kenoei*) skins. *Int J Food Sci Nutr* 55:45–51
- Choi JH, Woo JW, Lee YB, Kim SB (2005) Changes in an ammonia-like odor and chondroitin sulfate contents of enzymatic hydrolysates from longnose skate (*Rasa rhina*) cartilage as affected by pretreatment methods. *Food Sci Biotechnol* 14(5):645–650
- Dissaraphong S, Benjakul S, Visessanguan W, Kishimura H (2006) The influence of storage conditions of tuna viscera before fermentation on the chemical, physical and microbiological changes in fish sauce during fermentation. *Bioresour Technol* 97:2032–2040
- Duan JY, Jiang Y, Cherian G, Zhao YY (2010) Effect of combined chitosan-krill oil coating and modified atmosphere packaging on the storability of cold-stored lingcod (*Ophiodon elongates*) fillets. *Food Chem* 122:1035–1042
- El Khatib M, Tran QT, Nasrallah C, Lopes J, Bolla JM, Vivaudou M, Pagès JM, Colletier JP (2017) *Providencia stuartii* form biofilms and floating communities of cells that display high resistance to environmental insults. *PLoS ONE* 12(3):0174213
- Eschenlauer SCP, McKain N, Walker ND, McEwan NR, Newbold CJ, Wallace RJ (2002) Ammonia production by ruminal microorganisms and enumeration, isolation, and characterization of bacteria capable of growth on peptides and amino acids from the sheep rumen. *Appl Environ Microbiol* 68:4925–4931
- Ferre D, Banjac M, Calsamiglia S, Busquet M, Kamel C, Avgustin G (2004) The effects of plant extracts on microbial community structure in a rumen-simulating continuous-culture system as revealed by molecular profiling. *Folia Malacol* 49:151–155
- Glick BR, Karaturovic DM, Newell PC (1995) A novel procedure for rapid isolation of plant growth promoting pseudomonads. *Can J Microbiol* 41(6):533–536
- Hwang JH, Mizuta SS, Yokoyama Y, Yoshinaka R (2007) Purification and characterization of molecular species of collagen in the skin of skate (*Raja kenoei*). *Food Chem* 100(3):921–925
- Ijong FG, Ohta Y (1996) Physicochemical and microbiological changes associated with Bakasang processing—a traditional Indonesian fermented fish sauce. *J Sci Food Agric* 71:69–74
- Jang GI, Kim GH, Hwang CY, Cho BC (2017) Prokaryotic community composition in alkaline-fermented skate (*Raja pulchra*). *Food Microbiol* 61:72–82
- Kilincceker O, Dogan IS, Kucukoner E (2009) Effect of edible coatings on the quality of frozen fish fillets. *LWT Food Sci Technol* 42:868–873
- Kim HJ, Eo JH, Kim SJ, Eun JB (2010) Physicochemical changes in fermented skate (*Raja kenoei*) treated with organic acids during storage. *Korean J Food Sci Technol* 42(4):438–444
- Kostaki M, Giatrakou V, Savvaidis IN, Kontominas MG (2009) Combined effect of MAP and thyme essential oil on the microbiological, chemical and sensory attributes of organically aquaculture sea bass (*Dicentrarchus labrax*) fillets. *Food Microbiol* 26(5):475–482
- Laxson CJ, Condon NE, Drazen JC, Yancey PH (2011) Decreasing urea: trimethylamine N-oxide ratios with depth in chondrichthyes: A physiological depth limit? *Physiol Biochem Zool* 84:494–505
- Lee EJ, Seo JE, Lee JK, Oh SW, Kim YJ (2008) Microbial and chemical properties of ready-to-eat skate in Korean market. *J Food Hyg Saf* 23:137–141
- Lee EJ, Kim TH, Kim HK, Lee JK, Kwak HS, Lee JS (2010) Analysis of bacterial diversity in fermented skate using culture-dependent and culture-independent approaches. *Korean Soc Microbiol Biotechnol* 38:322–328
- Lee GY, Lee SY, Shin HS (2016) Evaluation of gas freshness indicator for determination of skate (*Raja kenoei*) quality during storage. *Food Sci Biotechnol* 25(5):1497–1500
- Liu WJ, Bao QH, Qing MJ, Chen X, Sun T, Li MH, Zhang JC, Yu J, Bilige MH, Sun TS, Zhang HP (2012) Isolation and identification of lactic acid bacteria from Tarag in Eastern Inner Mongolia of China by 16S rRNA sequences and DGGE analysis. *Microbiol Res* 167:110–115
- Mizuta S, Hwang JH, Yoshinaka R (2003) Molecular species of collagen in pectoral fin cartilage of skate (*Raja kenoei*). *Food Chem* 80:1–7
- Mohammed GJ, Kadhim MJ, Hameed IH (2016) *Proteus* species: characterization and herbal antibacterial: a review. *Int J Res Phytochem Pharmacol* 8(11):1844–1854
- Ng CS (1987) Determination of trimethylamine oxide (TMAO-N), trimethylamine (TMA-N), total volatile basic nitrogen (TVB-N) by Conway's method. In Hasegawa H (ed) *Laboratory manual on analytical methods and procedures for fish and fish products*, pp 1–8
- O'Grady FW (1966) Differences in ammonia production by faecal bacteria of patients with hepatic encephalopathy. *Proc R Soc Med* 59:1246–1251
- Reynisson E, Marteinsson VT, Jonsdottir R, Magnússon SH, Hreggvidsson GO (2012) Bacterial succession during curing process of a skate (*Dipturus batis*) and isolation of novel strains. *J Appl Microbiol* 113:329–338
- Sahadevan N, Mathew J, Radhakrishnan EK (2016) Potential of *Kurthia gibsonii* MB 126 as a biocontrol agent against fusarium oxysporum wilt disease of tomato plants. *Bharata Mata J Multidiscip Stud* 3(1):1–15
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
- Schefferle HE (1965) The decomposition of uric acid in built up poultry litter. *J Appl Bacteriol* 28(3):412–420
- Terence RW, Michael AC, Enevold F, Edward M, Paul AL (2011) *Peptostreptococcus russellii* sp. nov., isolated from a swine-manure storage pit. *Int J Syst Evol Microbiol* 61:1875–1879
- Weiner D, Mitch WE, Sands JM (2014) Urea and ammonia metabolism and the control of renal nitrogen excretion. *Clin J Am Soc Nephrol* 10(8):1444–1458
- Whitehead TR, Cotta MA (2004) Isolation and identification of hyper-ammonia producing bacteria from swine manure storage pits. *Curr Microbiol* 48:20–26
- Wolpert E, Phillips SF, Summerskill WH (1970) Ammonia production in the human colon: effects of cleansing, neomycin and acetoxyroxic acid. *N Engl J Med* 283:159–164
- Xu W, Yu G, Xue C, Xue Y, Ren R (2008) Biochemical changes associated with fast fermentation of squid processing by-products for low salt fish sauce. *Food Chem* 107:1597–1604
- Xu YS, Xia WS, Yang F, Nie XH (2010) Physical and chemical changes of silver carp sausages during fermentation with *Pediococcus pentosaceus*. *Food Chem* 122:633–637
- Zhao CJ, Schieber A, Gänzle MJ (2016) Formation of taste-active amino acids, amino acid derivatives and peptides in food fermentations—a review. *Food Res Int* 89:39–47