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Effect of Bacterial Community and Free Amino Acids on the Content of Biogenic Amines During Fermentation of Yu-lu, a Chinese Fermented Fish Sauce

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ABSTRACT

The main objective of this work was to investigate the effects of bacterial community and free amino acids on the content of biogenic amines in Yu-lu during fermentation. Four major biogenic amines (histamine, tyramine, cadaverine, and putrescine) were identified, and the content of tyramine was positively correlated with histamine (R value = 0.9113). Aspartic acid, glutamic acid, histidine, leucine, and lysine were the dominant free amino acids. 16S rRNA sequencing showed that the composition of the bacterial community changed significantly during fermentation of Yu-lu. Principal component analysis revealed the crucial links between microbial community and biogenic amines. For example, *Halanaerobium* was probably associated with the formation of putrescine, while *Halomonas* might be associated with the degradation of biogenic amines at the end of fermentation of Yu-lu. This study provided a detailed evaluation of the Yu-lu fermentation process, enabling development of better strategies for biogenic amine control in fish sauce.

KEYWORDS

Bacterial diversity; *Halanaerobium*; *Halomonas*; putrescine; high-throughput sequencing; biogenic amine; free amino acid; fish sauce

Introduction

Fish sauce, called NamPla in Thailand, Myeolchi-Aekjeot in Korea, Noucnam in Vietnam, Budu in Malaysia, and Yu-lu in China, is a traditional seasoning in Asian countries and is now gradually gaining worldwide acceptance (Gildberg et al., 2007; Sasaki et al., 2015). Asian fish sauces are clear brown liquids manufactured by spontaneous fermentation of various fishes such as anchovy, sardine, menhaden, and mackerel, following addition of salt to 20–30% (w/w). Fermentation is a widespread technique used to prevent spoilage of seafood by decreasing water activity, and it provides a unique flavor for the fish sauce product and an ideal source of essential nutrients. However, long-term fermentation results in the accumulation of biogenic amines, causing potential food safety hazards. Previous studies have demonstrated that high levels of biogenic amines were generated in the production of fish sauce (Jiang et al., 2014; Zaman et al., 2010).

Biogenic amines, including histamine, phenylethylamine, cadaverine, tryptamine, putrescine, spermine, tyramine, and spermidine, are low molecular weight nitrogenous organic compounds with biological activity (Halász et al., 1994; Kielwein et al., 1996; Silla Santos, 1996). Indeed, biogenic amines are widely found in foods containing abundant protein and amino acids, such as aquatic products (Jiang

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et al., 2014), meat products (Kaniou et al., 2001), and cheeses (Shalaby et al., 2016). Studies support the view that appropriate biogenic amines are essential for maintaining the normal function of the body and the immune system, but excessive intake can lead to headache, rash, swelling, diarrhea, vomiting, and other symptoms of poisoning (Rice et al., 1976). The formation of biogenic amines is related to the characteristics of the food, the microorganism involved, and process conditions, all of which affect the amino acid content and the activity of decarboxylase enzymes. Furthermore, biogenic amines in fermented food are mainly produced by microbial decarboxylation of amino acids. Microorganisms with the ability to perform amino acid decarboxylation can secrete decarboxylases to act on free amino acids, thus producing the corresponding biogenic amines (Min et al., 2016).

Microorganisms are known to play significant roles in the content of biogenic amines. Recently, the rapid development of accurate analytical techniques to assess the microbial community has accelerated the identification of the microorganisms present in fermented foods. Culture-independent approaches such as denaturing gradient gel electrophoresis (DGGE) and gene clone libraries can be applied to analyze bacterial communities, including bacteria that cannot be cultured (Ji et al., 2013). However, DGGE and gene clone library approaches are time consuming and produce low depth information, especially for complex bacterial communities involved in the fermentation of seafood. High-throughput sequencing techniques bypass the limitations of isolating and culturing microorganisms and provide a powerful means to quickly determine the types and abundance of microorganisms at the genus level. High-throughput sequencing techniques have been applied to investigate the bacterial community and diversity in various fermented foods such as soybean paste (Min et al., 2016), shrimp sauce (Shan et al., 2016), and glutinous rice wine (Lv et al., 2016). Moreover, previous studies have demonstrated the relationship between the chemical characteristics (salinity, pH, and nitrogen content) and bacterial community of Korean fish sauce (Lee et al., 2016). However, there is a lack of information on the effect of bacterial communities on the content of biogenic amines during fermentation of fish sauce.

In this study, high-throughput sequencing techniques were applied to investigate the bacterial community in Chinese fermented fish sauce. The effects of bacterial community composition and free amino acids on the content of biogenic amines during fermentation were studied by statistical analysis of the results from 16S rRNA sequencing and biochemical measurements of free amino acids and biogenic amines. It is expected to develop better strategies to control biogenic amine content during the fermentation of fish sauce.

Materials and methods

Preparation of Yu-lu samples

The Yu-lu samples produced from salted anchovy were collected from a local Yu-lu processing factory in Guangdong Province, China. Briefly, the fresh anchovies (each approximately 20 cm in length and weighing 100 g) were harvested from the South China Sea and frozen immediately at −20°C. After thawing, whole anchovies were dispensed into fermentation tanks and mixed with 30% (w/w) solar salt to completely cover the anchovies. The salted anchovies were open fermented at 25°C and stirred twice a day. Samples were collected at 0, 1, 3, 6, and 12 months after fermentation began and within 24 h were taken to the laboratory for storage at 4°C. In order to harvest microorganisms, the solid particles from the Yu-lu samples were removed by filtration through three layers of sterile gauze, and the harvested filtrates were centrifuged at 6000 g for 15 min at 4°C. After centrifugation, the pelleted cells were stored at −80°C for use in microbial community analysis. The supernatants were stored at −80°C for measurement of biogenic amines and free amino acids. All samples for analysis were prepared in triplicate.

Determination of biogenic amines

Procedures for the extraction and derivatization of biogenic amines were based on a previously published method (Zhai et al., 2012) with some modifications. Briefly, a 2 mL sample was transferred into a

centrifuge tube and extracted with 10 mL 5% trichloroacetic acid. The mixture was then centrifuged at 8000 g for 3 min at 4°C. One mL of the supernatant was mixed with 200 μL 2 M NaOH and 300 μL saturated sodium bicarbonate. The mixture was then derivatized with 2 mL derivatization reagent (1% dansyl chloride in acetone, prepared daily). The sample was incubated at 40°C for 45 min and 100 μL 25% ammonium hydroxide added to remove residual dansyl chloride. After being derivatized, the mixture was adjusted to 5 mL with acetonitrile and filtered through 0.2 μm filters for high performance liquid chromatography (HPLC) analysis. An Agilent 1100 HPLC equipped with fluorescence detector (G1315B) and HP Chem Station software was used. The derivatized sample was separated by a reverse-phase C_{18} column (250 mm × 4.6 mm, 5 μm) at 40°C, with 0.1 M ammonium acetate, acetonitrile, and distilled water used as mobile phases at a flow rate of 1.0 mL/min. The excitation and emission wavelengths for the detection of biogenic amine concentrations were 350 and 520 nm, respectively. The biogenic amine concentrations were quantified on the basis of retention time and area of the standards.

Determination of free amino acids

The extraction and determination of free amino acids was performed according to a previous method (Jiang et al., 2007) with some modifications. The analysis of amino acids was carried out by an Agilent 1100 HPLC equipped with a fluorescence detector (G1315B). The sample was separated by a ZORBAX Eclipse AAA column (150 mm × 4.6 mm). The column flow rate was 1 mL/min at 40°C. The excitation and emission detection wavelengths for the detection of free amino acids were 266 and 305 nm, respectively. Amino acid standard solution, obtained from Sigma Aldrich (St. Louis, MO, USA), was used for calibrating the concentrations of free amino acid in Yu-lu samples. The concentrations of free amino acids were quantified on the basis of retention time and area of the standards.

Pyrosequencing for bacterial community analysis

The extraction of total genomic DNA from the samples was performed with a Power DNA isolation kit (Omega Bio-Tek, Norcross, GA, USA), according to the manufacturer's instructions. The concentration of DNA was quantified by Qubit Fluorometer, and the sample integrity tested by electrophoresis on 1% agarose gels. The V4 hypervariable regions of bacterial 16S rRNA genes were amplified using the universal primer set (515F, 5′-GTGCCAGCMGCCGCGGTAA-3′; 806R, 5′- GGACTACHVGGGTWTCT AAT-3′), with sample-specific barcode sequences of 10 nucleotides. The polymerase chain reaction (PCR) protocol was as follows: initial denaturation at 98°C for 3 min; 30 cycles of denaturation at 98°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 45 s; and a final extension at 72°C for 7 min. The PCR products were purified with AMPure XP beads (Beckman, Indianapolis, IN, USA) to remove the unspecific products. High-throughput sequencing was performed with Illumina MiSeq PE250 according to the manufacturer's procedure.

The raw data were filtered to eliminate adapter sequences and low quality reads, and then paired end reads with overlap were merged to tags. If the two paired-end reads overlapped, the consensus sequence was generated by FLASH (Fast Length Adjustment of Short reads, v1.2.11) software. Tags were clustered to operational taxonomic unit (OTU) at 97% sequence similarity by USEARCH (v7.0.1090) software. Taxonomic ranks were assigned to OTU representative sequences using RDP (Ribosomal Database Project, v.2.2) software. Finally, alpha diversity, beta diversity, and the different species screening were analyzed based on OTU and taxonomic ranks. The Simpson index and Shannon index were used to calculate the richness and diversity of each microbial sample.

Statistical analysis

All experiments were carried out in triplicate ($n = 3$). Statistical analyses were carried out by the one-way analysis of variance procedure. Significant differences among the five samples were analyzed by Tukey's

highest significant difference test. The relationships between the dominant biogenic amines, free amino acids, and present bacteria were subjected to principal component analysis (PCA). Both the significant differences and PCA were analyzed using SPSS 19.0 for Windows (SPSS, Chicago, IL, USA).

Results and discussion

Changes in biogenic amines during the Yu-lu fermentation

The changes in biogenic amines in Yu-lu during the fermentation process are shown in Table 1. Histamine, tyramine, cadaverine, and putrescine were the dominant biogenic amines in Yu-lu. This was in accordance with Zaman et al., who reported the biogenic amines mentioned above as the major components in their fish sauce samples (Zaman et al., 2010). Tryptamine, phenylethylamine, and spermidine were also detected, while spermine was only detected at 6 months. Additionally, the total content of biogenic amines showed an increase in the initial fermentation period from 0 to 3 months, with the content increasing by 55.33%, but the total biogenic amine level at 12 months was decreased by 28.60% relative to the level at 0 month. There was no significant increase in level of total biogenic amines during the first month of fermentation. The total content of biogenic amines reached the highest quantity after 3 months of fermentation. After that, the total biogenic amines gradually decreased because the environment of the fermentation system may not be suitable for growth of the bacteria producing biogenic amines, and the bacteria that degrade biogenic amines became dominant at the later stages of fermentation.

Different biogenic amines reached the highest quantity at different times during the fermentation period. As shown in Table 1, cadaverine was detected within a range of 109.46–178.86 mg/kg in Yu-lu samples, lower than the results obtained by Jiang et al. (2007). The content of putrescine was within the range of 81.22–230.24 mg/kg. Almost all samples were found to contain a large amount of histamine, ranging from 164.18 to 302.03 mg/kg, and the maximum histamine level was found at 3 months. Histamine is the most toxic biogenic amine, with excessive histamine resulting in headaches, digestive disorders, abnormal blood pressure, and even nerve toxicity (Lehane and Olley, 2000). In the United States, the Food and Drug Administration suggested that the safe limit for histamine in seafood is 50 mg/kg. Fish sauces are mainly used as a condiment, so the intake would not be too much in normal use. The Codex Alimentarius Commission has ruled that the maximum permitted level of histamine in fish sauce should be 400 mg/kg (Jiang et al., 2014). According to this limit, all samples were within the acceptable range. The toxicity of tyramine is second only to that of histamine, and excessive tyramine can also cause headache and hypertension. The tyramine concentrations of the samples ranged from 80.14 to 234.60 mg/kg and reached the highest value at 3 months. Interestingly, it was observed that the level of tyramine was positively correlated to the histamine level with an R value of 0.9113. Therefore, there was most likely a strong relationship between the levels of histamine and tyramine. This observation was in agreement with the conclusion by Bai et al. (2013), who found a significant positive correlation between the content of histamine and tyramine in a black soybean paste. To explain this,

Table 1. Biogenic amine concentrations (mg/kg) in Yu-lu during fermentation.

Biogenic amines	0M	1M	3M	6M	12M
Tryptamine	5.55 ± 0.22^{a}	8.07 ± 0.11^{b}	11.23 ± 0.33^{c}	20.72 ± 0.21^{d}	10.52 ± 0.05^{c}
Phenylethylamine	ND	5.76 ± 0.46^{b}	7.62 ± 0.16^{c}	7.71 ± 0.32^{c}	2.90 ± 0.04^{a}
Putrescine	169.81 ± 2.37^{c}	154.13 ± 3.17^{c}	230.24 ± 8.32^{d}	112.99 ± 5.02^{b}	81.22 ± 3.32^{a}
Spermine	ND	ND	ND	1.97 ± 0.27^{a}	ND
Cadaverine	109.46 ± 2.95^{a}	145.22 ± 5.71^{c}	178.86 ± 1.30^{d}	123.66 ± 2.14^{ab}	133.75 ± 4.14^{bc}
Histamine	194.69 ± 1.08^{c}	164.18 ± 3.16^{b}	302.03 ± 10.59^{e}	238.76 ± 8.22^{d}	121.97 ± 3.19^{a}
Tyramine	139.83 ± 0.69^{b}	80.14 ± 0.18^{a}	234.6 ± 4.88^{d}	189.07 ± 1.21^{c}	93.49 ± 8.85^{a}
Spermidine	20.82 ± 0.45^{b}	56.24 ± 1.27^{e}	29.79 ± 0.22^{c}	45.87 ± 0.18^{d}	13.29 ± 1.27^{a}
Total	640.16 ± 4.42^{b}	614.27 ± 3.67^{b}	994.37 ± 12.44^{d}	740.75 ± 3.51^{c}	457.14 ± 18.22^{a}

Values from triplicate samples are expressed as mean ± standard deviation. Mean values with different superscript letters in the same row are significantly different ($P < 0.05$) by ANOVA and the Tukey's test during fermentation. ND, not detected.

the correlation could occur as a result of the existence of endogenous decarboxylases simultaneously responsible for the accumulation of histamine and tyramine (Bai et al., 2013). Further studies are needed to understand the potential mechanisms for the simultaneous accumulation of the biogenic amines, which would be helpful for reducing the levels of biogenic amines in fish sauces.

Changes in free amino acids during the Yu-lu fermentation

The changes in free amino acids during the fermentation process producing Yu-lu are shown in Table 2. Aspartic acid, glutamic acid, histidine, leucine, and lysine were the most abundant of the 18 detectable free amino acids. The percentage of these free amino acids mainly reflected the characteristic amino acid composition of Yu-lu, and the dominant free amino acids were similar to those reported previously (Jiang et al., 2007). However, these results differed from a report by Park et al. (2001), which determined that lysine, arginine, and aspartate were the dominant free amino acids of fish sauces produced in different Asian countries. These differences may be caused by variation in the fermentation environment and the fish varieties used as raw material. The concentration of total free amino acids showed a significant increasing trend during the fermentation process ($p < 0.05$), ranging from 1460.28 mg/100 g at 0 month to 4363.84 mg/100 g at 12 months. It was reported that extracellular enzymes hydrolyzed the proteins into small peptides and amino acids, which could be utilized by microorganisms and transformed into biogenic amines under suitable conditions (Bai et al., 2013). In this study, the low production of biogenic amines during the initial period (0–1 month) might be due to the slow liberation of free amino acids in the fermentation system.

Amino acids had a great influence on the development of the taste of fish sauce, with division into fresh-tasting amino acids (aspartic acid and glutamic acid), sweet amino acids (glycine, alanine, serine, threonine, proline, and lysine), bitter amino acids (arginine, tryptophan, leucine, isoleucine, methionine, phenylalanine, histidine, tyrosine, and valine), and tasteless amino acids (cysteine; Bermúdez et al., 2014; Domínguez et al., 2016). Except for serine, glycine, alanine, threonine, isoleucine, and proline, the content of the other amino acids exceeded the threshold value for detection by taste (Kato et al., 1989), indicating that most free amino acids had a significant effect on the taste of Yu-lu. Interestingly, the concentrations of aspartic acid and glutamic acid increased notably with the degree of fermentation, and the concentration was greater than the threshold value (Table 2). These increases made a greater contribution to the taste of fish sauces, producing an umami taste. Previous evidence

Table 2. Free amino acids concentration in Yu-lu during fermentation process (mg/100 g).

Amino acids	0M	1M	3M	6M	12M	Threshold
Aspartic acid	254.3 ± 17.55^{a}	394 ± 11.03^{b}	282.87 ± 15.69^{a}	417.69 ± 17.46^{b}	624.62 ± 17.49^{c}	100
Glutamic acid	86.61 ± 5.98^{a}	82.65 ± 2.31^{a}	285.77 ± 15.85^{b}	764.96 ± 31.98^{c}	917.45 ± 87.85^{c}	30
Serine	30.75 ± 2.12^{a}	39.38 ± 1.10^{ab}	33.96 ± 1.88^{ab}	45.38 ± 1.90^{b}	72.34 ± 6.93^{c}	150
Histidine	101.81 ± 7.03^{a}	159.76 ± 4.47^{a}	178.10 ± 9.88^{a}	183.31 ± 7.66^{a}	510.61 ± 48.89^{b}	20
Glycine	49.06 ± 3.39^{a}	56.22 ± 1.57^{a}	61.20 ± 3.40^{a}	63.15 ± 2.64^{a}	102.45 ± 9.81^{b}	130
Threonine	3.96 ± 0.27^{a}	5.88 ± 0.16^{b}	4.99 ± 0.28^{ab}	4.05 ± 0.17^{a}	7.47 ± 0.71^{c}	260
Arginine	97.68 ± 6.74^{a}	141.58 ± 3.97^{ab}	162.32 ± 9.01^{bc}	191.89 ± 8.02^{cd}	237.86 ± 22.78^{d}	50
Alanine	8.70 ± 0.60^{a}	17.41 ± 0.49^{b}	17.93 ± 0.99^{b}	$23.95 \pm 1.00b^{c}$	21.86 ± 2.09^{c}	60
Tyrosine	30.65 ± 2.12^{a}	41.37 ± 1.16^{a}	35.74 ± 1.98^{a}	82.40 ± 3.44^{b}	130.56 ± 12.50^{c}	91
Cysteine	94.43 ± 6.52^{a}	125.74 ± 3.52^{ab}	134.06 ± 7.44^{ab}	163.5 ± 6.84^{b}	286.72 ± 27.46^{c}	
Valine	23.33 ± 1.61^{a}	51.05 ± 1.43^{b}	76.99 ± 4.27^{cd}	73.37 ± 3.07^{c}	96.05 ± 9.20^{d}	40
Methionine	69.43 ± 4.79^{a}	108.43 ± 3.04^{b}	134.30 ± 7.45^{bc}	164.29 ± 6.87^{cd}	170.34 ± 16.31^{d}	30
Phenylalanine	10.64 ± 0.73^{a}	159.05 ± 4.45^{bc}	183.30 ± 10.17^{c}	127.26 ± 5.32^{b}	195.73 ± 18.74^{c}	90
Isoleucine	72.80 ± 5.03^{c}	48.06 ± 1.35^{b}	68.64 ± 3.81^{c}	21.60 ± 0.90^{a}	36.13 ± 3.46^{b}	90
Leucine	168.03 ± 11.60^{a}	230.55 ± 6.46^{ab}	260.45 ± 14.45^{b}	287.75 ± 12.03^{b}	403.38 ± 38.63^{c}	190
Lysine	211.95 ± 14.63^{a}	378.78 ± 10.61^{b}	459.05 ± 25.47^{bc}	518.9 ± 21.70^{c}	481.21 ± 46.08^{bc}	50
Proline	31.24 ± 2.16^{b}	29.15 ± 0.82^{b}	13.05 ± 0.72^{a}	11.11 ± 0.46^{a}	11.30 ± 1.08^{a}	300
Tryptophan	14.93 ± 1.03^{a}	23.40 ± 0.66^{a}	38.21 ± 2.12^{b}	42.24 ± 1.77^{b}	57.75 ± 5.53^{c}	90
Total	1460.28 ± 93.90^{a}	2092.48 ± 58.60^{ab}	2430.93 ± 134.87^{bc}	3186.88 ± 133.24^{c}	4363.84 ± 417.87^{d}	

Values from triplicate samples are expressed as mean ± standard deviation. Mean values with different superscript letters in the same row are significantly different ($P < 0.05$) by ANOVA and the Tukey's test during fermentation.

illustrated a significant role for glutamic acid in regulating the homeostasis of free radicals in the cell and sensing in the gastrointestinal tract (San and Uneyama, 2013). Moreover, different free amino acids showed different patterns of variation during the fermentation process, but most of them exhibited a growth trend. Compared with the initial levels, the concentrations of histidine and leucine increased significantly after 12 months of fermentation ($p < 0.05$). The lysine level showed a progressive increase from 211.95 mg/100 g to 518.96 mg/100 g with fermentation process increasing from 0 to 6 months and then a slight decrease thereafter. The concentrations of threonine, alanine, proline, and tryptophan were relatively low compared to the levels of other amino acids. Eight essential amino acids (tryptophan, isoleucine, threonine, phenylalanine, leucine, lysine, valine, and methionine) cannot be synthesized by the human body and are essential for normal mental function and health of infants and growing children during development (Ponka et al., 2015). In this study, all essential amino acids were found at detectable levels in Yu-lu during fermentation, thus fish sauce could be recommended as a source of essential amino acids.

Changes in the bacterial community of Yu-lu during fermentation

The features of a massively parallel pyrosequencing analysis of the bacterial community during the Yu-lu fermentation are summarized in Supplementary Table S1 and Figure S1. After the removal of chimeric sequences and trimming, a total of 156,304 high-quality sequences were retrieved, with an average read length of 252 bp and more than 31,260 sequences per sample, suggesting that sufficient sequences were obtained for analysis at each time point. Alpha diversity was determined by statistical indexes (Table S1) such as observed species index, Chao index, ACE index, Shannon-Weaver and Simpson index, reflecting species diversity and richness. The Shannon-Weaver indices, representing bacterial diversity, were in the range of 0.51–2.39 (average 1.43) and increased at 6 and 12 months of fermentation. The rarefaction curves showed a similar pattern of increase during fermentation, providing support for the development of highly diverse bacterial communities during fermentation (Supplementary Figure S1). In addition, all rarefaction curves reached the saturation phase, which suggested that the OTUs had basically covered all the species in the sample.

The bacterial taxonomic compositions were determined at the phylum and genus levels. At the phylum level, the bacterial phyla *Firmicutes* and *Proteobacteria* were predominant in all Yu-lu samples during the entire fermentation period, followed by *Fusobacteria* (Figures 1A and 2A), similar to the previous results reported for other types of fermented salted seafood (Kuda et al., 2012; Lee et al., 2014a). The phylum *Firmicutes* was predominant in the initial fermentation period, with 96.78% abundance in the 3-month sample, whereas the abundance of *Firmicutes* decreased dramatically to 51.13% as the fermentation progressed. The relative abundance of *Proteobacteria* increased quickly, with more than 45.96% abundance at 12 months of fermentation. However, these results differed from a previous report by Lee, who showed that the phylum *Proteobacteria* was predominant in the initial fermentation period but was rapidly replaced with the phylum *Firmicutes* at the end of fermentation (Lee et al., 2014b). In this study, the phylum *Fusobacteria* was also identified as a minor group, with relative abundance reaching a maximum of approximately 2.47% at 6 months.

The genus level analysis revealed that the bacterial community was significantly different at the various time points during the Yu-lu fermentation (Figures 1B and 2B). From the sequencing analysis, the most dominant genus was *Halanaerobium*, which has been reported to be present in salted shrimp (Lee et al., 2014a), canned fermented herrings (Kobayashi et al., 2000), and anchovy sauce (Lee et al., 2016). At the initial fermentation period (0 month), the relative abundance of *Halanaerobium* was approximately 90.75%. *Salinvibrio* was observed as a minor group in the early Yu-lu sample. The genera *Fusobacterium, Photobacterium*, and *Tetragenococcus* were found at relatively high abundance after 1 month of fermentation, and their relative abundance was much higher than at later times. After 3 months of fermentation, almost all genera were replaced by *Halanaerobium*, with maximum relative abundance reaching 92.06%. *Halanaerobacter* was also identified as a dominant group at 3 months of fermentation, but its relative abundance rapidly decreased to 0.04% at the end of fermentation.

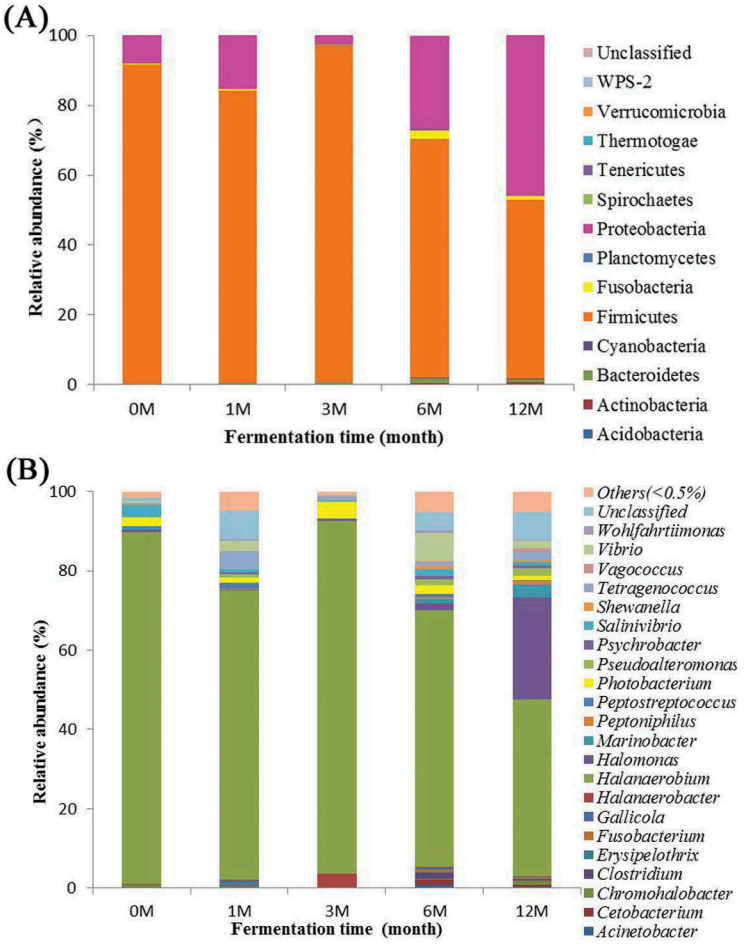

Figure 1. Taxonomic composition of bacterial communities in Yu-lu samples during the fermentation process at the phylum (A) and genus level (B). The relative abundance (%) was estimated from 16S rRNA gene sequences.

Previous research showed that *Halanaerobium* was a potential indicator, reflecting the spoilage or excessive fermentation of aquatic products by the production of butyrate, acetate, and methylamines (Lee et al., 2014b). As the fermentation progressed, the bacterial community became more diverse; but in contrast, the relative abundance of *Halanaerobium* decreased rapidly after 6 months of fermentation, indicating that the fermentation process was conducive to improving the safety of fish sauces. The genus *Tetragenococcus* has been found as the major microbial component in salty fermented foods such as fermented soybean paste (Min et al., 2016) and soy sauces (Tanasupawat et al., 2002). However, in this study *Tetragenococcus* was present at low levels in the microbial communities. The environment of Yu-lu may be unsuitable for the growth of *Tetragenococcus* because of inhibition by the bacteriocins from *Halanaerobium*. In particular, the genus *Halomonas*, known to be nonpathogenic, appeared as one of the detectable genera after 6 months of fermentation and was eventually identified as a dominant group in the Yu-lu microbial community at 12 months of fermentation.

Relationship among dominant biogenic amines, free amino acids, and bacterial composition

The data on the dominant biogenic amines, free amino acids, and bacteria present at the genus level were compared by PCA, an effective mathematical method that reduces the

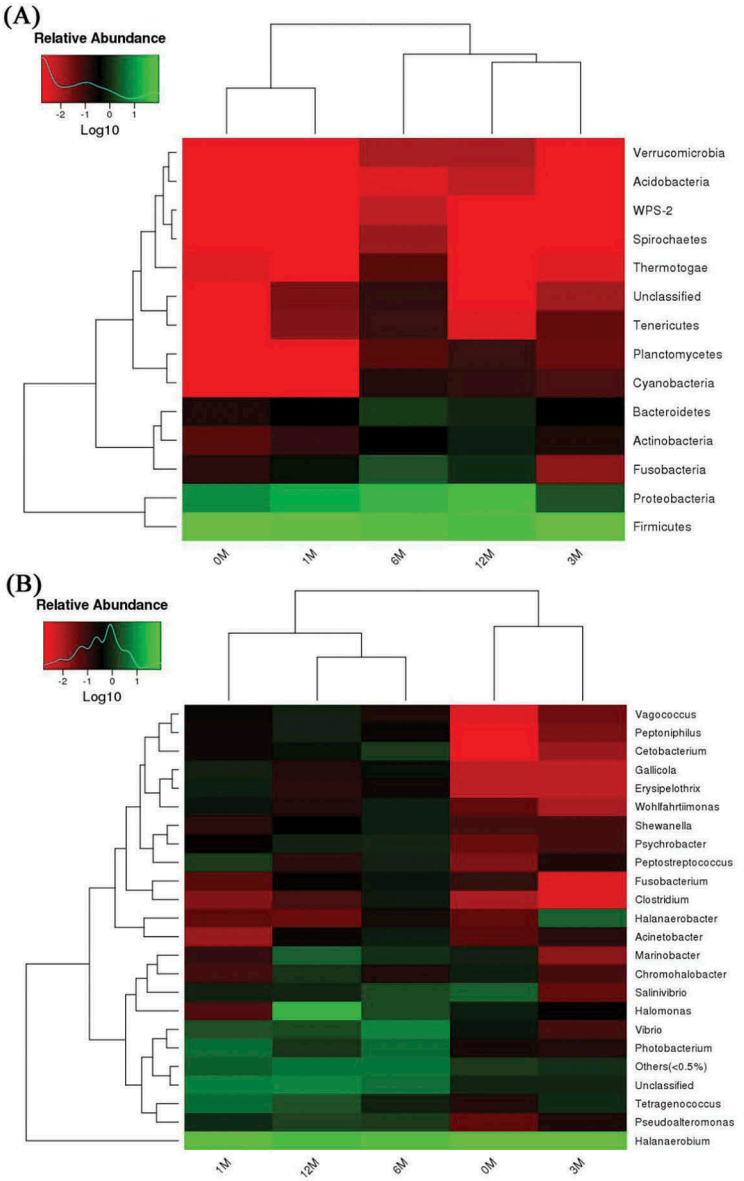

Figure 2. Heat map of bacterial communities in Yu-lu samples during fermentation process at the phylum (A) and genus level (B). The color intensity of each panel is proportional to the operational taxonomic unit abundance.

dimensionality of multivariate data, while transforming original variables to accumulative variables for a better overall idea of relationships. PCA has been applied to understand the relationship between the dominant bacteria community and chemical characteristics in Chinese rice wine (Liu et al., 2016) and Korean salted seafood (Ji et al., 2013). As can be seen in Figure 3, two principal components were extracted, which accounted for 85.87% of the total variance in the data. The principal component 1 (PCl), representing 61.74% of the total variance, was positively related to the amount of aspartic acid, glutamic acid, histidine, leucine, lysine, *Tetragenococcus*, and *Halomonas* and negatively related to the amount of histamine, putrescine, tyramine, cadaverine, *Photobacterium*, and *Halanaerobium*. The principal component 2 (PC2) explained an additional 24.13% of the total variance. Almost all indicators were

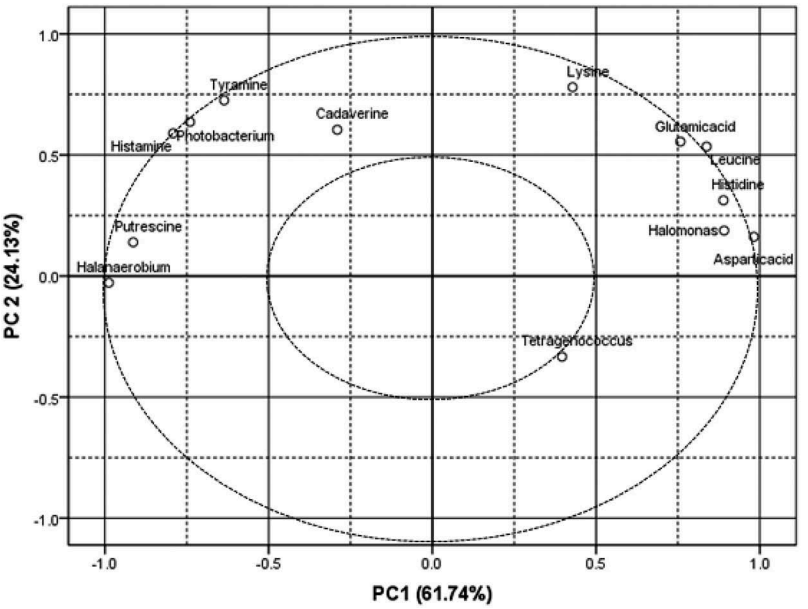

Figure 3. Principal component analysis for relationships among free amino acids, biogenic amines, and bacterial community composition in Yu-lu samples during the fermentation process.

on the positive side of PC2, except for the value of *Tetragenococcus*, which was negatively related to PC2, and the values of cadaverine and *Tetragenococcus* were significantly less than the others.

In the present study, *Halanaerobium* content was highly related to the formation of putrescine. A similar result was reported for putrescine that increased rapidly in Myeolchi-Aekjeot (a Korean traditional fermented fish sauce), which was well correlated with the growth of *Halanaerobium*, indicating that *Halanaerobium* was responsible for the formation of putrescine via the bacterial decarboxylation of ornithine (Lee et al., 2015). In addition, plasmids with ornithine decarboxylase genes have been observed for a *Halanaerobium* species from anaerobic sediments of Soap Lake, and this further indicated that *Halanaerobium* may be responsible for the production of putrescine (Brown et al., 2011). In this study, it was also observed that the formation of histamine was associated with the genus *Photobacterium* but not related to the content of histidine. Histamine in aquatic products was mostly attributed to the activity of gram-negative enteric bacteria, and histamine was formed by the decarboxylation of histidine (Landete et al., 2007). In a previous study on biogenic amine formation and microbial spoilage in chilled garfish, *Photobacterium* could produce above 1000 mg/kg of histamine from fish stored both in air and packaged in a modified atmosphere (Dalgaard et al., 2006). Thus, further insight is needed to understand and control the formation of putrescine and histamine by *Halanaerobium* and *Photobacterium*, respectively.

Interestingly, another bacterial group, *Halomonas*, did not show strong association with biogenic amines but showed high values at the positive side of PC1. A recent study found that *Halomonas shantousis* SWA25 isolated from fish sauce showed effective degradation of tryptamine, tyramine, phenethylamine, histamine, cadaverine, and putrescine (Xu et al., 2016). Therefore, *Halomonas* apparently degraded biogenic amines rather than synthesizing them, which could explain why it was not associated with biogenic amines as were other bacteria in Figure 3. This suggests that *Halomonas* strains may be useful for degrading biogenic amines in fermented food. Aspartic acid, glutamic acid, histidine, and arginine also had high values on the positive side of PC1, but these free amino acid indicators were not grouped closely with dominant biogenic amines and bacteria (Figure 3). This indicated that the concentration of

dominant free amino acids was not linked to the concentration of biogenic amines and bacteria. Similar research reported that the concentration of free amino acids was not associated with histamine content in Yu-lu (Jiang et al., 2007).

Conclusions

The current research represents an exhaustive determination of the effect of bacterial community and free amino acids on the content of biogenic amines during fermentation of Yu-lu. The knowledge of this study will enable further optimization of microbial composition and upgrade the quality of fish sauce product. According to PCA, there were significant links between the microbial community and biogenic amines. *Halanaerobium* was highly related to the formation of putrescine, and the formation of histamine was associated with the genus *Photobacterium* but not related to the histidine content. Therefore, further research needs to control the putrescine and histamine formation by *Halanaerobium* and *Photobacterium* and understand that the endogenous enzymes act on the changes of free amino acid during the fish sauces fermentation. *Halomonas* were not clustered with biogenic amines. *Halomonas* apparently degraded biogenic amines rather than synthesizing them, which could explain why it was not associated with biogenic amines as were other bacteria. This suggests that *Halomonas* strains may improve the safety of the traditional fermented food from a microbial point of view.

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References

Bai, X., Byun, B. Y., and Mah, J. H. 2013. Formation and destruction of biogenic amines in Chunjang (a black soybean paste) and Jajang (a black soybean sauce). Food Chemi. 141(2): 1026–1031. doi:10.1016/j.foodchem.2013.03.054

Bermúdez, R., Franco, D., Carballo, J., Sentandreu, M. Á., and Lorenzo, J. M. 2014. Influence of muscle type on the evolution of free amino acids and sarcoplasmic and myofibrillar proteins through the manufacturing process of Celta dry-cured ham. Food Res. Int. 56(2): 226–235. doi:10.1016/j.foodres.2013.12.023

Brown, S. D., Begemann, M. B., Mormile, M. R., Wall, J. D., Han, C. S., Goodwin, L. A., Pitluck, S., Land, M. L., Hauser, L. J., and Elias, D. A. 2011. Complete genome sequence of the haloalkaliphilic, hydrogen-producing bacterium Halanaerobium hydrogeniformans. J. Bacteriol. 193(14): 3682–3683. doi:10.1128/JB.05209-11

Dalgaard, P., Madsen, H. N., and Emborg, J. 2006. Biogenic amine formation and microbial spoilage in chilled garfish (Belone belone belone) - effect of modified atmosphere packaging and previous frozen storage. J. Appl. Microbio. 101(1): 80–95. doi:10.1111/j.1365-2672.2006.02905.x

Domínguez, R., Munekata, P. E., Agregán, R., and Lorenzo, J. M. 2016. Effect of commercial starter cultures on free amino acid, biogenic amine and free fatty acid contents in dry-cured foal sausage. LWT Food Sci. Tech. 71: 47–53. doi:10.1016/j.lwt.2016.03.016

Gildberg, A., Wichaphon, J., Lertsiri, S., Assavanig, A., Sørensen, N. K., and Thongthai, C. 2007. Chemical and organoleptic comparison of fish sauce made from cold water species and typical thai fish sauce. J. Aquat Food Prod. Tech.16(3): 31–42. doi:10.1300/J030v16n03_04

Halász, A., Baráth, Á., Simon-Sarkadi, L., and Holzapfel, W. 1994. Biogenic amines and their production by microorganisms in food. Trends Food Sci. Tech. 5(94): 42–49. doi:10.1016/0924-2244(94)90070-1

Ji, Y. J., Lee, S. H., Lee, H. J., and Che, O. J. 2013. Microbial succession and metabolite changes during fermentation of saeu-jeot: traditional Korean salted seafood. Food Microbio. 34(2): 360. doi:10.1016/j.fm.2013.01.009

Jiang, J. J., Zeng, Q. X., Zhu, Z. W., and Zhang, L. Y. 2007. Chemical and sensory changes associated Yu-lu fermentation process - A traditional Chinese fish sauce. Food Chem. 104(4): 1629–1634. doi:10.1016/j.foodchem.2007.03.024

Jiang, W., Xu, Y., Li, C., Dong, X., and Wang, D. 2014. Biogenic amines in commercially produced Yulu, a Chinese fermented fish sauce. Food Addit Contam. Part. B Surveill. 7(1): 25–29. doi:10.1080/19393210.2013.831488

Kaniou, I., Samouris, G., Mouratidou, T., Eleftheriadou, A., and Zantopoulos, N. 2001. Determination of biogenic amines in fresh unpacked and vacuum-packed beef during storage at 4°C. Food Chem. 74(4): 515–519. doi:10.1016/S0308-8146(01)00172-8

Kato, H., Rhue, M. R., and Nishimura, T. 1989. Role of Free Amino Acids and Peptides in Food Taste. Washington: American Chemical Society.

Kielwein, G., Treptow, H., Askar, A., Dross, A., and Wittkowski, R. 1996. Biogene Amine in Lebensmitteln. Stuttgart: Verlag Eugen Elmer.

Kobayashi, T., Kimura, B., and Fujii, T. 2000. Strictly anaerobic halophiles isolated from canned Swedish fermented herrings (Surströmming). Int. J. Food Microbio. 54(1–2): 81–89. doi:10.1016/S0168-1605(99)00172-5

Kuda, T., Izawa, Y., Ishii, S., Takahashi, H., Torido, Y., and Kimura, B. 2012. Suppressive effect of Tetragenococcus halophilus, isolated from fish- nukazuke, on histamine accumulation in salted and fermented fish. Food Chem. 130 (3): 569–574. doi:10.1016/j.foodchem.2011.07.074

Landete, J. M., De, L. R. B., Marcobal, A., and Muñoz, R. 2007. Molecular methods for the detection of biogenic amine-producing bacteria on foods. Int. J. Food Microbio. 117(3): 258–269. doi:10.1016/j.ijfoodmicro.2007.05.001

Lee, H. W., Choi, Y. J., Hwang, I. M., Hong, S. W., and Lee, M. A. 2016. Relationship between chemical characteristics and bacterial community of a Korean salted-fermented anchovy sauce, myeolchi-aekjeot. LWT Food Sci. Tech. 73: 251–258. doi:10.1016/j.lwt.2016.06.007

Lee, S. H., Jung, J. Y., and Jeon, C. O. 2014a. Microbial successions and metabolite changes during fermentation of salted shrimp (saeu-jeot) with different salt concentrations. Plos One. 9(2): e90115. doi:10.1371/journal.pone.0090115

Lee, S. H., Jung, J. Y., and Jeon, C. O. 2014b. Effects of temperature on microbial succession and metabolite change during saeu-jeot fermentation. Food Microbio. 38(2): 16–25. doi:10.1016/j.fm.2013.08.004

Lee, S. H., Jung, J. Y., and Jeon, C. O. 2015. Bacterial community dynamics and metabolite changes in myeolchi-aekjeot, a Korean traditional fermented fish sauce, during fermentation. Int. J. Food Microbio. 203: 15–22. doi:10.1016/j.ijfoodmicro.2015.02.031

Lehane, L., and Olley, J. 2000. Histamine fish poisoning revisited. Int. J. Food Microbio. 58(1–2): 1. doi:10.1016/S0168-1605(00)00296-8

Liu, S. P., Yu, J. X., Wei, X. L., Ji, Z. W., Zhou, Z. L., Meng, X. Y., and Mao, J. 2016. Sequencing-based screening of functional microorganism to decrease the formation of biogenic amines in Chinese rice wine. Food Control. 64: 98–104. doi:10.1016/j.foodcont.2015.12.013

Lv, X. C., Jia, R. B., Li, Y., Chen, F., Chen, Z. C., Liu, B., Chen, S. J., Rao, P. F., and Ni, L. 2016. Characterization of the dominant bacterial communities of traditional fermentation starters for Hong Qu glutinous rice wine by means of MALDI-TOF mass spectrometry fingerprinting, 16S rRNA gene sequencing and species-specific PCRs. Food Control. 67: 292–302. doi:10.1016/j.foodcont.2016.03.005

Min, J. K., Han, S. K., Jung, H. Y., and Sang, S. K. 2016. Microbial communities related to sensory attributes in Korean fermented soy bean paste (doenjang). Food Res. Int. 89: 724–732. doi:10.1016/j.foodres.2016.09.032

Park, J. N., Fukumoto, Y., Fujita, E., Tanaka, T., Washio, T., Otsuka, S., Shimizu, T., Watanabe, K., and Abe, H. 2001. Chemical composition of fish sauces produced in Southeast and East Asian Countries. J. Food Comp. Anal. 14(2): 113–125. doi:10.1006/jfca.2000.0963

Ponka, R., Fokou, E., Kansci, G., Beaucher, E., Piot, M., Leonil, J., and Gaucheron, F. 2015. Amino acids, major carotenoids and vitamin A activity of some traditional sauces consumed in the Far North Region of cameroon. J. Food Comp. Anal. 43: 88–95. doi:10.1016/j.jfca.2015.05.005

Rice, S. L., Eitenmiller, R. R., and Koehler, P. E. 1976. Biologically active amines in food: A review. J. Milk Food Tech. 43: 88–95.

San, G. A., and Uneyama, H. 2013. Amino acid sensing in the gastrointestinal tract. Amino Acids. 45(3): 451–461. doi:10.1007/s00726-012-1371-2

Sasaki, T., Araki, R., Michihata, T., Kozawa, M., Tokuda, K., Koyanagi, T., and Enomoto, T. 2015. Removal of cadmium from fish sauce using chelate resin. Food Chem. 173: 375–381. doi:10.1016/j.foodchem.2014.08.134

Shalaby, A. R., Anwar, M. M., Sallam, E. M., and Emam, W. H. 2016. Quality and safety of irradiated food regarding biogenic amines: ras cheese. Int. J. Food Sci. Tech. 51(4): 1048–1054. doi:10.1111/ijfs.13058

Shan, D., Hu, X., Li, M., Miao, J., Du, J., and Wu, R. 2016. Composition and metabolic activities of bacterial community in shrimp sauce at the flavor forming stage of fermentation as revealed by metatranscriptome and 16S rRNA gene sequencing. J. Agri Food Chem. 64: 12.

Silla Santos, M. H. 1996. Biogenic amines: their importance in foods. Int. J. Food Microbio. 29(2–3): 213. doi:10.1016/0168-1605(95)00032-1

Tanasupawat, S., Thongsanit, J., Okada, S., and Komagata, K. 2002. Lactic acid bacteria isolated from soy sauce mash in Thailand. J. Gen. Appl. Microbio. 48(4): 201–209. doi:10.2323/jgam.48.201

Xu, Y., Liu, Y., Xu, B., Wang, D., and Jiang, W. 2016. Characterisation and application of Halomonas shantousis SWA25, a halotolerant bacterium with multiple biogenic amine degradation activity. Food Addit. Contam. Part. Chem. Anal. Control Exposure Risk Assess. 33(4): 674.

Zaman, M. Z., Bakar, F. A., Selamat, J., and Bakar, J. 2010. Occurrence of biogenic amines and amines degrading bacteria in fish sauce. Czech J. Food Sci. 28(5): 440–449. doi:10.17221/312/2009-CJFS

Zhai, H., Yang, X., Li, L., Xia, G., Cen, J., Huang, H., and Hao, S. 2012. Biogenic amines in commercial fish and fish products sold in southern China. Food Control. 25(1): 303–308. doi:10.1016/j.foodcont.2011.10.057