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Histamine contents of fermented fish products in Taiwan and isolation of histamine-forming bacteria

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Abstract

Twenty-seven imported fermented fish products from Southeast Asian countries and sold in the supermarkets in Taiwan, including fish sauce, fish paste and shrimp paste, were tested to determine the occurrence of histamine and histamine-forming bacteria. The levels of pH, salt content, total volatile basic nitrogen, trimethylamine, and aerobic plate count in all samples ranged from 4.8% to 6.5%, 16.2% to 45.3%, 51 to 275 mg/100 g, 5.4 to 53.9 mg/100 g and 1.0 to 4.2 log CFU/g, respectively. The average content for each of eight different biogenic amines in all samples was less than 90 ppm, except for histamine which has an average content of 394 ppm in fish sauce, 263 ppm in fish paste, and 382 ppm in shrimp paste. Most of the tested fermented fish products (92.6%) had histamine levels greater than the FDA guideline of 50 ppm, while seven of them (25.9%) contained >500 ppm of histamine. Although *Bacillus coagulans* and *Bacillus megaterium* were identified as the two histamine-producing bacteria capable of producing 13.7 and 8.1 ppm of histamine, respectively, in trypticase soy broth broth supplemented with 1.0% L-histidine, they were not determined to be the main contributors to histamine accumulation in these fermented fish products.

Keywords: Histamine; *Bacillus* spp.; Histamine-forming bacteria; Fermented fish products

1. Introduction

Fish sauce is one of the most popular fermented fish products used as a condiment in Southeast Asia. It is traditionally produced by mixing whole fish with salt at a ratio of 1:1–3:1, and fermented for 6–12 months or longer. When most of the fish tissue has solubilized, the liquid fish sauce is drained off and filtered to yield a clear amber solution (Saisithi, Kasemsarn, Liston, & Alexander, 1966). Fish sauces contain about 20 g/l nitrogen; 80% of which is in the form of amino acids. Thus, fish sauce is considered as an important source of dietary proteins and amino acids, and has become a necessity in the household in Southeast Asian countries (Sanceda, Kurata, & Arakawa, 1996). Since the fermented fish products, including fish sauce and fish paste, have recently been widely utilized in a variety of processed products in Taiwan, their importation to Taiwan continues to increase year after year.

Biogenic amines are basic nitrogenous compounds occurring in meat, fish, cheese, and wine products, mainly due to amino acid decarboxylation activities of certain microbes (Arnold & Brown, 1978). High levels of histamine in foods can have important vasoactive

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effects in humans (Lehane & Olley, 2000; Taylor, 1985). Scombroid fish, such as tuna, mackerel, bonito, and saury, which contain high levels of free histidine in their muscle are often implicated in scombroid poisoning incidents when not properly processed and stored (Taylor, 1986). Some of the non-scombroid fish, cheese, and other foods have also been implicated in incidents of histamine poisoning (Taylor, 1985). Fermented fish products, such as fish sauce and fish paste, may contain high contents of histamine (Fardiaz & Markakis, 1979). Although incidents of histamine poisoning following the consumption of these fermented fish products have not been reported, they may have occurred and gone unnoticed because symptoms of histamine poisoning closely resemble those of food allergies.

Biogenic amines, including histamine, are formed through the decarboxylation of specific free amino acids by exogenous decarboxylases released from microbial populations associated with the seafood (Rawles, Flick, & Martin, 1996). Although only *Morganella* (*Proteus*) *morganii*, *Klebsiella pneumoniae* and *Hafnia alvei* have been isolated from fish incriminated in scombroid poisoning (Taylor & Speckard, 1983), a variety of other bacterial species including *Proteus vulgaris*, *P. mirabilis*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Serratia fonticola*, *S. liquefaciens* and *Citrobacter freundii*, were also identified to be histamine-formers in fish (Eitenmiller, Wallis, Orr, & Phillips, 1981; Lopez-Sabater, Rodriguez-Jerez, Hernandez-Herrero, Roig-Sagues, & Mora-Ventura, 1996; Middlebrooks, Toom, Douglas, Harrison, & McDowell, 1988; Taylor & Speckard, 1983; Tsai, Kung, Lee, Lin, & Hwang, 2004; Yoshinaga & Frank, 1982). Yatsunami and Echigo (1991, 1992, 1993) identified halotolerant *Staphylococcus* spp., *Vibrio* spp., and *Pseudomonas* III/IV-NH as the histamine-formers from fermented salted sardine and fish products. Hernandez-Herrero, Roig-Sagues, Rodriguez-Jerez, and Mora-Ventura (1999) and Rodriguez-Jerez, Mora-Ventura, Lopez-Sabater, and Hernandez-Herrero (1994) isolated histamine-producing *S. epidermidis, S. xylosus, K. oxytoca, E. cloacae, Pseudomonas cepaciae,* and *Bacillus* spp. from salted Spanish anchovies. Recently, our research group isolated histamine-formers *Pantoea* spp. and *E. cloacae* from salted mackerel in Taiwan (Tsai et al., 2005). Although prolific histamine-forming bacteria have been isolated from temperature abused raw fish, they have rarely been isolated from fermented fish products, such as fish sauce and fish paste (Hernandez-Herrero et al., 1999; Kim et al., 2003). Only a few halophilic bacteria, such as *Tetragenococcus muriaticus*, have been isolated as prolific histamine-formers from fish sauce (Kimura, Konagaya, & Fujii, 2001).

In our previous study to demonstrate the presence of high contents of histamine and other biogenic amines in kimchi products, we proposed the possible sources of the biogenic amines be the added fermented fish products, such as fish sauce or shrimp paste, during kimchi fermentation (Tsai et al., 2005). No reported literature is available on the occurrence of biogenic amines, including histamine, and histamine-forming bacteria and related bacteria in fermented fish products in Taiwan. Therefore, this research was undertaken by testing 27 fermented fish products, including fish sauce, fish paste and shrimp paste, which were sold in the supermarkets in Taiwan so that a better understanding of the safety quality of the products can be accomplished to better protect the health of the consumers.

2. Materials and methods

2.1. Materials

Twenty-seven samples of fermented fish products (12 fish sauce products, 9 fish paste products and 6 shrimp paste products) were purchased from the supermarkets in Taiwan from July to October, 2003. The fermented fish products were all imported from Southeast Asian countries including Philippines, Vietnam and Thailand, and packed in plastic or glass bottles. The fish paste products were mostly the preserved anchovy fish that was immersed in brine or oil. All tested samples were kept at room temperature in the supermarkets. After purchase, they were immediately transported to the laboratory for use.

2.2. pH value and salt content

Samples of fish paste and shrimp paste products (10 g) were homogenized with 10 ml of distilled water to make thick slurry, and the pH of the slurry measured using a Corning 145 pH meter (Corning Glass Works, Medfield, MA). The pH of the fish sauce was determined by direct measurement of the test samples with the pH meter. The salt content of each test sample was determined according to the AOAC procedures (1995).

2.3. Microbiological analysis and isolation of histamine-forming bacteria

A 10-g portion of the fermented fish product was removed from each test sample and homogenized at high speed for 2 min in a sterile blender with 90 ml of sterile potassium phosphate buffer (0.05 M, pH 7.0). The homogenates were serially diluted with a sterile phosphate buffer, and 1.0 ml aliquots of the diluents were poured with aerobic plate count (APC) agar (Difco, Detroit, MI, USA) containing 3% NaCl. Bacterial colonies were counted after the plates were incubated at 35 °C for 48 h. The bacterial numbers in the fermented fish products were expressed as $\log_{10}$ colony forming units (CFU)/g.

To isolate histamine-forming bacteria, a 1-ml aliquot of the sample dilute was taken and mixed with differential plating medium fortified with L-histidine (histamine-forming bacterium isolation agar) (Niven, Jeffreg, & Corlett, 1981). Following incubation of the differential agar plates for 4 days at 35 °C, colonies with blue or purple color were further streaked on trypticase soy agar (TSA) agar (Difco) for pure cultures. Their ability to produce biogenic amines was determined by inoculating the isolates in trypticase soy broth (TSB) supplemented with 1% L-histidine (TSBH) and incubated without shaking at 35 °C for 24 h. Two milliliters of the culture broth were taken for quantitation of biogenic amines. Histamine-forming bacteria were identified on the basis of Gram stain, endospore stain, catalase and oxidase reactions. Further identification of the pure isolates to the species level was accomplished by a variety of biochemical tests using the API 50CHB system (BioMeriex, Marcy-l'Etoile, France).

2.4. Determination of total volatile basic nitrogen and trimethylamine

The total volatile basic nitrogen (TVBN) content of each fermented fish product was measured by the method of Conway's dish (Cobb, Aoaniz, & Thompson, 1973). The TVBN extract of fermented fish sample in 6% trichloroacetic acid (TCA, Sigma, St. Louis, MO, USA) was absorbed by boric acid and then titrated with 0.02 N HCl. The TVBN content was calculated and expressed in mg/100 g sample.

The TMA content of the fermented fish products was determined by the Modified Dyer Picrate method (AOAC, 1995). The TMA extract of the fermented fish product sample in 6% TCA was further extracted in toluene and reacted with 1% picric acid. Absorbance was measured at 410 nm using trimethylamine as a standard. The TMA content was expressed as mg TMA/100 g sample.

2.5. Biogenic amine analysis

Biogenic amines, including tryptamine hydrochloride (Trp), 2-phenylethylamine hydrochloride (Phe), putrescine dihydrochloride (Put), cadaverine dihydrochloride (Cad), spermidine trihydrochloride (Spd), spermine tetrahydrochloride (Spm), histamine dihydrochloride (Him), tyramine hydrochloride (Tyr), and agmatine sulfate (Agm), were obtained from Sigma. Trp (61.4 mg), Phe (65.1 mg), Put (91.5 mg), Cad (85.7 mg), Spd (87.7 mg), Spm (86.0 mg), Him (82.8 mg), Tyr (63.2 mg), and Agm (87.7 mg) were dissolved in 50 ml of 0.1 M HCl and used as the standard stock solutions (each at 1.0 mg/ml). A series of diluted standard solutions were prepared from the standard stock solutions and used to obtain the standard curve for each biogenic amine.

Each fermented fish product sample was ground in a Waring Blender for 3 min. The ground samples (5 g or 5 ml) were transferred to 50-ml centrifuge tubes and homogenized with 20 ml of 6% TCA for 3 min. The homogenates were centrifuged (10,000*g*, 10 min, 4 °C) and filtered through Whatman No. 2 filter paper (Whatman, Maidstone, England). The filtrates were then placed in volumetric flasks, and 6% TCA was added to bring to a final volume of 50 ml. Samples of standard amine solutions and 2-ml aliquots of the fermented fish product extracts were derivatized with benzoyl chloride according to the previously described method (Hwang, Chang, Shiau, & Chai, 1997). Two milliliters of each bacterial culture broth were also benzoylated following the same procedures as the fermented fish product extracts. The benzoyl derivatives were dissolved in 1 ml of methanol, and 20 μl aliquots were used for HPLC analysis.

The contents of biogenic amines in the test samples were determined with a Hitachi liquid chromatograph (Hitachi, Tokyo, Japan) consisting of a Model L-7100 pump, a Rheodyne Model 7125 syringe loading sample injector, a Model L-4000 UV–vis detector (set at 254 nm), and a Model D-2500 Chromato-integrator. A Lichrospher 100 RP-18 reversed-phase column (5 μm, 125 × 4.6 mm, E. Merck, Damstadt, Germany) was used for separation. The gradient elution program began with 50:50 (v/v) methanol:water at a flow rate of 0.8 ml/min for the first 0.5 min, followed by a linear increase to 85:15 methanol:water (0.8 ml/min) during the next 6.5 min. The methanol:water mix was held constant at 85:15 (0.8 ml/min) for 5 min, and then decreased to 50:50 (0.8 ml/min) during the next 2 min.

A set of biogenic amine standards and their mixtures were analyzed together with test samples. During analysis, a standard solution was also injected intermittently between test samples to check chromatographic consistency. Each sample was injected twice. The peak heights of the biogenic amine standard solutions were used to prepare standard curves, and then for determination of the amine concentrations in the test samples.

2.6. Statistical analysis

Pearson correlation was carried out to determine relationships between pH, salt content, APC, TVBN, TMA and histamine contents in fermented fish products. Data were analyzed by t test to detect significant variation between the pH, salt content, APC, TVBN and TMA in different products. Value of $P < 0.05$ was used to indicate significant deviation. All statistical analyses were performed using the Statistical Package for Social Sciences, SPSS Version 9.0 for windows (SPSS Inc., Chicago, Il.).

3. Results and discussion

Values of the pH, salt content, total volatile basic nitrogen (TVBN), trimethylamine (TMA) and aerobic plate count (APC) in the fermented fish products are presented in Table 1. The levels of pH and salt content in all samples ranged from 4.8% to 6.5%, and 16.2% to 45.3%, respectively. The average pH value in different types of products was approximately 5.3, while the average salt content in fish sauce, fish paste and shrimp paste was 33.6%, 26.7% and 34.3%, respectively (Table 1). These results are in agreement with the previously reported data for fermented fish products in Japan, China and Southeast Asian countries (Fujii, Basuki, & Tozawa, 1980; Ijong & Ohta, 1996; Lopetcharat & Park, 2002; Mizutani, Kimizuka, Ruddle, & Ishige, 1992; Ren, Hayashi, Endo, & Watanabe, 1993). The levels of TVBN, TMA, and APC in all samples ranged from 51 to 275 mg/100 g, 5.4 to 53.9 mg/100 g and 1.0 to 4.2 log CFU/g, respectively (Table 1). The levels of salt content, TVBN, and TMA in fish paste products were lower than those in fish sauce and shrimp paste products ($P < 0.05$) (Table 1).

In general, no significant correlation exists among the parameters of the pH values, salt contents, APC, TVBN, TMA and histamine contents in the test samples ($r = 0.02$–0.25) using the Pearson correlation test, except for the salt contents and the TVBN values ($r = 0.64$, $P < 0.05$), and the TVBN and TMA values ($r = 0.74$, $P < 0.05$) in all samples. Hernandez-Herrero, Roig-Sagues, Lopez-Sabater, Rodriguez-Jerez, and Mora-Ventura (1999) proposed that the increase of TVBN value in salted anchovies during ripening was due to bacterial and enzymatic actions, particularly the halophilic bacteria. Since higher levels of NaCl content were found with the fish sauce and shrimp paste products than the fish paste products, the former samples might support better growth of halophilic bacteria which then contributed to the higher contents of TVBN and TMA.

Table 2 summarizes the contents of biogenic amines in the tested fermented fish products. Except for histamine, the average content of various biogenic amines in tested samples was less than 90 ppm. The average histamine content in fish sauce, fish paste, and shrimp paste samples was 394, 263 and 382 ppm, respectively. High contents of histamine were frequently detected in fermented fish products in previous studies (Fardiaz & Markakis, 1979). For example, Sanceda et al. (1996) reported a histamine level of 430 ppm in *nampla* and 1380 ppm in Korean anchovy sauce. Kirschbaum, Rebscher, and Bruckner (2000) also reported histamine at 721 to 757 ppm in anchovy fish sauce. In general, higher levels of putrescine, cadaverine, 2-phenylethylamine, and histamine were detected in fish sauce and shrimp paste samples than the fish paste samples in this study. Hernandez-Herrero et al. (1999) suggested that part of the total histamine and other biogenic amines might have been diffused into the brine with other nitrogen fractions during the ripening process of fish paste production. Dapkevicius, Nout, Rombouts, Houben, and Wymenga (2000) reported that *Lactobacillus sake* strains could cause degradation of histamine in fermented fish pastes. Therefore, the lower contents of histamine and other biogenic amines in the fish paste products observed in this work, when compared to those in fermented fish sauce and shrimp paste products, might be due not only to their diffusion losses into the brine but also by microbial degradation.

Table 3 shows the distribution of histamine contents in the tested fermented fish products, with 25 (92.6%) of them containing greater than 50 ppm of histamine, the allowable limit of the US Food and Drug Administration (FDA) for scombroid fish and/or products (USFDA, 2001, chap. 7). Three fish sauces, two fish pastes, and two shrimp paste products containing greater than 500 ppm of histamine, could be hazardous to the health of consumers based on the data collected from numerous outbreak reports (Taylor, 1989). Hernandez-Herrero et al. (1999) detected 680 ppm of histamine in semipreserved Spanish anchovies that were implicated in an incident of scombroid poisoning. Bartholomev, Berry, Rodhouse, and Gilhouse (1987) demonstrated that histamine at greater than 1000 ppm in the fish would be toxic and unsafe for human consumption. Based on information on the toxicological levels of histamine in various seafood products in causing health hazard, one of the fish sauce products and one of the shrimp paste products could have caused disease symptoms when consumed, mainly due to their greater than 1000 ppm level of histamine content (Table 3). However,

Table 1
Values of the pH, salt content, total volatile basic nitrogen (TVBN), trimethylamine (TMA), and aerobic plate count (APC) in fermented fish products

Samples	No. of samples	pH	Salt content (%)	TVBN (mg/100 g)	TMA (mg/100 g)	APC (log CFU/g)
Fish sauce	12	4.9–5.8 (5.3 ± 0.3)[a]A	22.1–37.0 A (33.6 ± 4.8)A	51–270 (194 ± 65)A	8.4–53.9 (26.9 ± 12.8)A	1.1–3.0 (2.1 ± 0.7)A
Fish paste	9	5.2–5.5 (5.3 ± 0.1)A	17.5–35.4 (26.7 ± 6.5)B	57–138 (92 ± 29)B	5.4–18.6 (13.5 ± 5.2)B	1.1–3.3 (2.1 ± 0.8)A
Shrimp paste	6	4.8–6.5 (5.5 ± 0.7)A	16.2–45.3 (34.3 ± 9.2)A	73–275 (158 ± 45)A	10.3–44.9 (26.7 ± 10.2)A	1.0–4.2 (2.7 ± 1.1)A

[a] Mean ± SD. Values in the same column with different letters are statistically different ($P < 0.05$) from each other.

Table 2
The levels of biogenic amines in various fermented fish products

Samples	No. of samples	Range of amine level (ppm)								
		Put[a]	Cad	Tpm	Phe	Spd	Spm	His	Tym	Agm
Fish sauce	12	2.0–243 (24 ± 18)[c]	ND[b]–243 (89 ± 51)	ND–177 (87 ± 53)	ND–42 (3.8 ± 9.2)	ND–98 (9.0 ± 10)	ND–121 (52 ± 36)	45–1220 (394 ± 380)	ND–42 (9.4 ± 8.7)	ND–85 (18 ± 14)
Fish paste	9	5.0–17 (12 ± 5)	22–107 (58 ± 37)	ND–117 (70 ± 45)	ND	ND–105 (15 ± 10)	ND–127 (60 ± 42)	101–760 (263 ± 221)	ND–32 (8.8 ± 9.5)	ND–72 (15 ± 12)
Shrimp paste	6	5.0–118 (40 ± 27)	ND–162 (80 ± 45)	ND–140 (67 ± 36)	ND–91 (30 ± 22)	ND–91 (36 ± 28)	ND–135 (43 ± 30)	20–1180 (382 ± 402)	ND–19 (3.7 ± 5.1)	ND–56 (14 ± 10)

[a] Put, putrescine; Cad, cadaverine; Tpm, tryptamine; Phe, 2-phenylethylamine; Spd, spermidine; Spm, spermine; His, histamine; Tym, tyramine; Agm, agmatine.
[b] ND, not detected (amine level is less than 1 ppm).
[c] Mean ± SD.

Table 3
Distribution of the histamine contents in 27 various fermented fish products

Histamine content (ppm)	Fish sauce	Fish paste	Shrimp paste
<49	1 (8.3%)	0 (0%)	1 (16.7%)
50–499	8 (66.7%)	7 (77.8%)	3 (50.0%)
500–999	2 (16.7%)	2 (22.2%)	1 (16.7%)
>1000	1 (8.3%)	0 (0%)	1 (16.7%)
Total	12	9	6

histamine is not the only compound responsible for scombrotoxicosis, since ingestion of pure histamine does not automatically cause toxic symptoms (Bjeldanes, Schutz, & Morris, 1978). The toxic effects of histamine are increased by the presence of other amines, such as putrescine and cadaverine, that inhibit histamine-metabolizing enzymes in the small intestine (Bjeldanes et al., 1978; Arnold & Brown, 1978; Lehane & Olley, 2000). Consequently, histamine may act in association with such diamines, but whether they are also present in fish sauces and whether fish sauces with these apparently high and possibly dangerous levels of histamine are toxic has yet to be proved.

The tested fermented fish products produced 16 purple colonies on the differential plating agar medium. Following incubation in TSBH broth, two out of the 16 isolates (12.5%) showed the ability to produce histamine, as determined by HPLC analysis of the cultured TSBH broth. These two histamine-forming bacteria were identified as *Bacillus coagulans* and *Bacillus megaterium* and were capable of producing 13.7 ppm and 8.1 ppm of histamine in TSBH medium, respectively (Table 4). The *B. coagulans* strain V3 also produced 1.3 ppm of spermidine; while *B. megaterium* strain T1 produced 1.9 ppm of cadaverine and 1.0 ppm of putrescine in the culture broth (Table 4).

Potential histamine-forming bacteria that have been isolated from various salted fish products, including salted sardine, salted Spanish anchovies, and fish sauce, were *Staphylococcus* spp., *Vibrio* spp., and *Pseudomonas* III/IV-NH (Yatsunami & Echigo, 1991, 1992), *S. epidermidis, S. xylosus, K. oxytoca, E. cloacae, P. cepaciae,* and *Bacillus* spp. (Hernandez-Herrero et al., 1999; Rodriguez-Jerez et al., 1994), and *T. muriaticus*, a halophilic lactic acid bacterium (Kimura et al., 2001). Only the *Bacillus* genus was isolated as a histamine former

Table 4
Production of histamine and other biogenic amines (ppm) in TSBH medium by histamine-forming bacterial isolates

Strain	Histamine-forming bacteria	His[a]	Put	Cad	Spd
V3	*Bacillus coagulans*	13.7	ND[b]	ND	1.3
T1	*Bacillus megaterium*	8.1	1.0	1.9	ND

[a] His, histamine; Put, putrescine; Cad, cadaverine; Spd, spermidine.
[b] ND: Not detected (amine level is less than 1 ppm).

in this study with the fermented fish products. The *Bacillus* spp. isolates from salted anchovies produced low levels of histamine at 10.5 and 12.4 ppm, respectively (Hernandez-Herrero et al., 1999; Rodriguez-Jerez et al., 1994). Those *Bacillus* spp. isolates that were most frequently detected in canned anchovies also produced negligible amounts of histamine in the culture broth (Kim et al., 2004). The similar findings to show that our two *Bacillus* spp. isolates from the fermented fish products were weak histamine-formers in TSBH medium was indicative that they are not the actual major contributor of histamine accumulation in the test products. Since the major histamine-forming bacteria, such as *M. morganii*, *K. pneumoniae*, and *H. alvei*, that have been frequently identified in other fish species, were not found in our test samples, it is possible that these prolific histamine-formers, after their formation of the high levels of accumulated histamine and other biogenic amines in the fermented fish products, were killed or inhibited due to the high NaCl contents (>16%) in these samples.

Improper storage of raw fish could result in substantial quality deterioration and histamine accumulation in a short period of time (Hernandez-Herrero et al., 1999). When fresh fish was used for ripening, histamine formation in anchovy products did not occur (Hernandez-Herrero et al., 1999). Histamine contents in the fish products only increased from 0.78 ppm in raw fish to 11.6 ppm during the fist 2-4 weeks of ripening due to unsaturation of fish with NaCl (Veciana-Nogues, Alba-la-Hurtado, Marine-Font, & Vidal-Carou, 1996). Yongsawatdigul, Choi, and Udomporn (2004) also reported that the main source of biogenic amines was associated with raw material, rather than the fermentation process of fish sauce. Brillantes, Paknoi, and Totakien (2002) reported that the high contents of histamine in fish sauce could come from the raw fish material or originate during fermentation. Therefore, we postulate that the accumulation of high levels of histamine in the tested fermented fish products results from the use of mishandled or poor quality raw fish for processing.

4. Conclusion

This study showed that the histamine contents in most of the fermented fish products exceeded the 50 ppm USFDA guideline value, and 7.4% (2/27) of the tested samples contained >1000 ppm of histamine. Consumption of these products could lead to scombroid poisoning in consumers. Since only the weak histamine-forming *B. coagulans* and *B. megaterium* were isolated from these samples, it is postulated that the use of poor quality raw fish and improper handling practices could have contributed to the accumulation of histamine and other biogenic amines in these fermented fish products.

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