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Chemical characterisation and histamine-forming bacteria in salted mullet roe products

Hsien-Feng Kung [a,e], Liang-Tan Chien [b], Hsuan-Jung Liao [c], Chung-Saint Lin [d], Ean-Tun Liaw [e], Wen-Chieh Chen [f], Yung-Hsiang Tsai [f,*]

[a] *Department of Food Science and Technology, Tajen University, Pingtung, Taiwan, ROC*
[b] *Bureau of Standards, Metrology and Inspection, Ministry of Economic Affairs, Keelung, Taiwan, ROC*
[c] *Department of Food Science, China Institute of Technology, Taipei, Taiwan, ROC*
[d] *Department of Food Science, Yuanpei University, Hsin-Chu, Taiwan, ROC*
[e] *Department of Food Science, National Pintung of Science and Technology, Pingtung, Taiwan, ROC*
[f] *Department of Seafood Science, National Kaohsiung Marine University, No. 142, Hai-Chuan Road, Nan-Tzu, Kaohsiung 811, Taiwan, ROC*

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Abstract

Sixteen salted mullet roe products sold in the retail markets in Taiwan were purchased and tested to determine the occurrence of histamine and histamine-forming bacteria. The levels of pH, salt content, water content, total volatile basic nitrogen (TVBN) and aerobic plate count (APC) in all samples ranged from 5.4 to 5.8, 5.1% to 7.2%, 15.4% to 27.3%, 32.0 to 69.6 mg/100 g and <1.0 to 7.1 log CFU/g, respectively. None of these samples contained total coliform and *Escherichia coli*. The average content of each of the nine biogenic amines in all samples was less than 4 mg/100 g, and only one mullet roe sample had the histamine content (8.18 mg/100 g) greater than the 5.0 mg/100 g allowable limit suggested by the US Food and Drug Administration. Two histamine-producing bacterial strains capable of producing 10.7 ppm and 9.6 ppm of histamine in trypticase soy broth (TSB) supplemented with 1.0% L-histidine (TSBH) were identified as *Staphylococcus carnosus* by 16S rDNA sequencing with PCR amplification, and they were isolated from the sample with higher histamine content (8.18 mg/100 g).
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Keywords: Histamine; Histamine-forming bacteria; *Staphylococcus carnosus*; Salted mullet roe product; 16S rDNA

1. Introduction

Grey mullet (*Mugil cephalus*) is one of the most widely distributed food fish in the world. It is found in coastal waters and estuaries throughout the tropics and subtropics (Hsu & Deng, 1980). Mullet roe is especially popular in Taiwan and Japan where it is processed into a dried and salted product (Hsu & Deng, 1980; Lu, Ma, Williams, & Chung, 1979). The traditional process for mullet roe involves salting, desalting and sun-drying, and the final product is yellowish-brown in colour with about 4% salt content and 20–30% moisture content (Hsu & Deng, 1980). Since 1987, Taiwan has been importing grey mullet from America, Brazil, and Australia because of a reduced ocean catch. However, the browning reaction has been more severe in roe products made from these imported frozen mullet; therefore, cultured mullet was sought as an alternative (Lee, Chiang, & Pan, 1998).

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 effects in humans (Lehane & Olley, 2000; Taylor, 1985). Scombroid fish, such as tuna, mackerel, bonito, and saury, which have high

* Corresponding author. Tel.: +886 7 3617141x3605; fax: +886 7 3640634.
E-mail addresses: yht@mail.tajen.edu.tw, yhtsai01@seed.net.tw (Y.-H. Tsai).

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levels of free histidine in their muscle, are often implicated in histamine 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). Although incidents of histamine poisoning following the consumption of salted mullet roe products have not been reported, they may have occurred but went 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 population 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*, *Enterobacter aerogenes*, *Enterobacter cloacae*, and *Citrobacter freundii*, were also identified to be histamine-formers in albacore fish (Kim et al., 2002). Yatsunami and Echigo (1991, 1992, 1993) identified halotolerant *Staphylococcus* sp., *Vibrio* sp., 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* sp. from salted Spanish anchovies. Recently, our research group isolated histamine-formers, *Pantoea* sp. and *E. cloacae*, from salted mackerel, and *Bacillus* sp. from fermented fish products in Taiwan (Tsai et al., 2005, 2006).

No reports are available on the occurrence of biogenic amines, including histamine and histamine-forming bacteria and related bacteria, in salted mullet roe products in Taiwan. Therefore, this research was undertaken by testing 16 salted mullet roe products sold in the retail markets in Taiwan to better understand their safety, including the contents of aerobic plate count, total coliform, *Escherichia coli*, and total volatile basic nitrogen (TVBN) for the purpose of better protecting the consumers.

2. Materials and methods

2.1. Materials

Nine, three, and four salted mullet roe products, respectively, prepared from wild, cultured and imported mullet fish, sold in the retail markets in Taiwan, were purchased in June to September of 2005. The imported mullet fish prepared for mullet roe products were all imported from Mexico. All salted mullet roe products sold in the retail markets were vacuum-packed in plastic and kept at room temperature. Once purchased, they were immediately transported to the laboratory for analysis.

2.2. pH value, salt content, and water content determination

Samples of salted mullet roe product (10 g) were homogenized in sterile blenders with 10 ml of distilled water to make a thick slurry. The pH of this slurry was then measured using a Corning 145 pH meter (Corning Glass Works, Medfield, MA, USA). The salt content in each sample was determined according to the AOAC procedure (1995) by homogenizing 2 g of roe sample with 18 ml of distilled water. The homogenate was titrated with 0.1 M $AgNO_3$, using 10% w/v K_2CrO_4 solution as an indicator. The water content was determined by the standard gravimetric method; by drying 1–3 g of a test sample at 102.0 ± 2.0 °C under atmospheric pressure for 2 h. Consistency of mass was tested by additional drying steps of 1 h until the difference in mass did not exceed 0.5 mg.

2.3. Microbiological analysis

A 25 g portion of the salted mullet roe sample was homogenized at high speed for 2 min in a sterile blender with 225 ml of sterile potassium phosphate buffer (0.05 M, pH 7.0). The sterile blender was prepared by autoclaving for 15 min at 121 °C. The homogenates were serially diluted with a sterile phosphate buffer, and 1.0 ml aliquots of the dilutes were inoculated into aerobic plate count (APC) agar (Difco, Detroit, MI, USA) containing 0.5% NaCl. Bacterial colonies were counted after the plates were incubated at 35 °C for 48 h. The bacterial numbers in the roe samples were expressed as $\log_{10}$ colony forming units (CFU)/g.

Analyses of total coliform and *E. coli* in these roe samples were conducted using the three tube most probable number (MPN) methods (FDA, 1992). Lauryl sulphate tryptose broth (LST broth) and brilliant green lactose bile (2%) broth (BGLB broth) were used for presumptive and confirmed tests for total coliform, respectively. *E. coli* was determined by using the LST broth and EC broth. Cultures that showed positive production of gas were then confirmed by eosine methylene blue agar (EMBA) and IMViC test.

2.4. Isolation and identification of histamine-forming bacteria

To isolate histamine-forming bacteria, a 0.1 ml aliquot of each of the 16 sample dilutes was spread on histamine-forming bacterium isolation agar (HBI agar) fortified with L-histidine (Niven, Jeffreg, & Corlett, 1981). Following incubation of the differential agar plates for 4 days at 35 °C, colonies with blue or purple colour on the plates were picked and further streaked on trypticase soy agar (TSA) (Difco) to obtain pure cultures. Their ability to produce biogenic amines was determined by inoculating the isolates in trypticase soy broth (TSB) (Difco) supplemented with 1% L-histidine (TSBH) and incubated without shaking

at 35 °C for 24 h. Two millilitres of the culture broth were taken for quantitation of biogenic amines.

The presumptive histamine-forming isolates were identified on the basis of morphology, Gram stain, endospore stain, catalase, and oxidase reaction. The identity of histamine-forming isolates was further confirmed by amplifying and sequencing approximately 1400 bp of the 16S ribosomal DNA (rDNA) for bacteria (Kuhnert, Capaul, Nicolet, & Frey, 1996; Kuhnert, Heyberger-Meyer, Nicolet, & Frey, 2000). Amplification of histamine-forming bacteria was performed using the universal primers, UNI-L (5′-AGAGTTTGATCATGGCTCAG-3′) and UNI-R (5′-GTGTGACGGGCGGTGTGTAC-3′) (Kuhnert et al., 1996, 2000). Bacterial cells were cultured overnight in 2 ml of TSB at 35 °C and then centrifuged at 8000 rpm for 10 min. The cell pellet was washed and resuspended in 0.5 ml of TE-buffer (10 mM Tris–HCl, 1 mM EDTA; pH 8.0), and then lysed by 20% sodium dodecyl sulphate (SDS). After the solution was boiled for 20 min and the cellular debris was discarded, following centrifugation at 13,000*g* for 3 min, the total DNA in the supernatant was precipitated with 70% ethanol and used as template DNA for PCR.

PCR amplification was performed in 20 μl of reaction mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 20 pmol of each primer, a 0.2 mM concentration of each of the four deoxynucleotide triphosphates, 0.5 U of *Taq* DNA polymerase (Applied Biosystems, Foster City, CA, USA), and template DNA (10 ng). Amplifications were carried out for 35 cycles (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s) in a GeneAmp PCR 2400 Thermal Cycler (Applied Biosystems) with an initial denaturation at 94 °C for 4 min and a final extension at 72 °C for 7 min (Kuhnert et al., 1996, 2000). Amplicons were detected by electrophoresis on a 1.5% agarose gel, staining with ethidium bromide. Amplicons were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) eluted in Tris–HCl (10 mM, pH 8.5) prior to sequencing. The amplified DNA was directly sequenced with the ABI TaqDye Deoxy Terminator Cycle sequencing kit and ABI Model 377 automated DNA sequencer (Applied Biosystems). The sequences were analyzed with the BLAST (NCBI) for identification of histamine-forming bacteria.

2.5. Determination of total volatile base nitrogen (TVBN)

The TVBN contents of the salted mullet roe samples were measured by the method of Conway's dish (Cobb, Aoaniz, & Thompson, 1973). Each ground sample (10 g) of mullet roe was extracted with 20 ml of 6% trichloroacetic acid (TCA, Sigma, St. Louis, MO, USA) and filtered. The residue was extracted twice. The TCA extract of the roe sample was absorbed by boric acid and then titrated with 0.02 N HCl. The TVBN content was expressed in mg/100 g fish roe.

2.6. Biogenic amine analysis

Each salted mullet roe sample was ground in a Waring Blender for 3 min. The ground samples (5 g) were transferred to 50 ml centrifuge tubes and homogenized with 20 ml of 6% trichloroacetic acid (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 TCA was added to bring to a final volume of 50 ml. Standard biogenic amine solutions and 2 ml aliquots of the roe extracts were derivatized with benzoyl chloride according to the previously described method (Hwang, Chang, Shiau, & Chai, 1997). Two millilitres of each bacterial culture broth were also benzoylated using the same procedure as for the roe extracts. The benzoyl derivatives were dissolved in 1 ml of methanol, and 20 μl aliquots were used for HPLC injection.

The contents of biogenic amines in standard biogenic amines and 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, Darmstadt, Germany) was used for chromatographic separation. The gradient elution programme 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.

2.7. Statistical analysis

Pearson correlation was carried out to determine relationships between pH, salt content, water content, TVBN, APC, putrescine, cadaverine, spermine and histamine contents in the 16 salted mullet roe samples. All statistical analyses were performed using the Statistical Package for Social Sciences, SPSS Version 9.0 for windows (SPSS Inc., Chicago, IL, USA). A value of $P < 0.05$ was used to indicate significant deviation.

3. Results and discussion

Values of the pH, salt content, total volatile basic nitrogen (TVBN), aerobic plate count (APC), total coliform, and *E. coli* in the salted mullet roe products are presented in Table 1. These results are in agreement with those previously reported by Hsu and Deng (1980) and Pan and Luo (1981) for water content and salt content in commercial salted mullet roe products. Although the averages of pH value, salt content and water content were not significantly different in wild, cultured and imported salted mullet roe

Table 1
Values of the pH, salt content, moisture, total volatile basic nitrogen (TVBN), aerobic plate count (APC), total coliform (TC), and *E. coli* in salted mullet roe products

Samples	No. of samples	pH	Salt content (%)	Water content (%)	TVBN (mg/100 g)	APC (log CFU/g)	TC (MPN/g)	*E. coli* (MPN/g)
Wild mullet roe products	9	5.4–5.8 (5.6 ± 0.1)[a]A	5.2–7.2 (6.1 ± 0.7)A	17.5–27.3 (21.7 ± 4.8)A	32.0–51.8 (43.4 ± 9.1)A	<1.0–6.1 (2.3 ± 2.1)A	<3	<3
Cultured mullet roe products	3	5.5–5.6 (5.5 ± 0.1)A	5.3–6.0 (5.6 ± 0.3)A	15.4–16.6 (16.0 ± 0.8)A	57.8–69.6 (63.7 ± 8.4)C	3.1–4.2 (3.6 ± 0.8)A	<3	<3
Imported mullet roe products	4	5.5–5.8 (5.6 ± 0.1)A	5.1–6.1 (5.6 ± 0.4)A	17.7–24.0 (21.8 ± 4.6)A	53.0–56.5 (54.3 ± 1.9)B	5.1–7.1 (6.2 ± 1.0)B	<3	<3

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

products, the average TVBN value of the cultured samples (63.7 mg/100 g) was significantly ($P < 0.05$) higher than that of the imported samples (54.3 mg/100 g) and wild samples as the lowest (43.4 mg/100 g). In contrast, the average APC levels in the wild and cultured products (2.3 and 3.6 log CFU/g, respectively) were significantly ($P < 0.05$) lower than that of the imported products (6.2 log CFU/g). None of these samples contained total coliform and *E. coli*. The higher salt contents (>5.1%) in these salted mullet roe samples apparently had some inhibitory effect on coliform bacterial growth. Wheaton and Lawson (1990) showed that, as the salt content in fish was increased to above 1%, the bacteria associated with fish spoilage were increasingly stressed. Most of these bacteria would die or at least stop growing as the salt content of the fish was increased from 6% to 8%. TVBN, including trimethylamine (TMA), dimethylamine (DMA) and NH_3, is one of the most widely used indicators of fish quality and spoilage. In general, the 30 mg/100 g level was used as a determination index for fish quality and decomposition (Gill, 1990). In this study, the TVBN content of all samples exceeded the decomposition limit level of 30 mg/100 g for fish quality determination. Although detailed information on the initial fish roe quality and the manufacture procedures used for salted mullet roe production was not available, we believe these factors, together with the storage temperatures and the unclean market environment, could have contributed to the high contents of TVBN in samples.

Table 2 summarizes the contents of biogenic amines in the tested salted mullet roe samples. None of the 16 tested salted mullet roe samples contained tryptamine, spermidine, or tyramine (Table 2). The average content of each of the remaining six biogenic amines in all samples were lower than 4.0 mg/100 g, and one of the wild mullet roe samples had a histamine content of 8.18 mg/100 g which is greater than the 5.0 mg/100 g allowable limit suggested by the US Food and Drug Administration (USFDA, 2001, chap. 7). Therefore, based on the content of histamine in the test products, a 6.25% (1/16) unacceptable rate was obtained with these salted mullet roe samples. However, strong evidence exists that biogenic amines, such as putrescine, cadaverine, spermine, and spermidine, in fish tissue can increase the toxic effects of histamine by inhibiting intestinal histamine-metabolizing enzymes, such as diamine oxidase, thereby increasing histamine uptake and liberating endogenous histamine in intestinal fluids (Flick, Oria, & Douglas, 2001). Pearson correlation was conducted to determine if there existed any relationship among the pH values, salt contents, water content, TVBN, APC, and biogenic amine contents of the tested samples. In general, no correlation existed among the pH values, salt contents, water content, TVBN, APC, putrescine, cadaverine, spermine, and histamine contents in the 16 tested samples.

The salted mullet roe samples produced 10 purple colonies on the differential HBI agar plates. Only two of them (20%) produced histamine in TSBH medium. The

Table 2
Contents of biogenic amines in salted mullet roe products

Samples	No. of samples	Content of biogenic amine (mg/100 g)								
		Put[a]	Cad	Try	Phe	Spd	Spm	His	Tyr	Agm
Wild mullet roe products	9	ND[b] – 0.70 (0.23 ± 0.26)[c]	ND – 9.58 (1.80 ± 3.09)	ND	ND	ND	ND – 4.24 (2.14 ± 1.70)	ND – 8.18 (1.03 ± 3.09)	ND	ND – 4.16 (1.36 ± 1.78)
Cultured mullet roe products	3	ND – 0.1 (0.10 ± 0.95)	ND – 0.33 (0.30 ± 0.04)	ND	ND	ND	1.35–4.29 (3.24 ± 1.80)	ND – 1.50 (0.77 ± 0.25)	ND	ND – 4.67 (1.36 ± 1.18)
Imported mullet roe products	4	ND – 0.80 (0.27 ± 0.46)	ND – 4.01 (2.50 ± 2.19)	ND	ND – 3.94 (1.31 ± 2.27)	ND	ND – 4.27 (3.98 ± 0.83)	ND – 1.30 (0.65 ± 0.90)	ND	ND – 4.90 (2.10 ± 1.42)

[a] Put, putrescine; Cad, cadaverine; Try, tryptamine; Phe, 2-phenylethylamine; Spd, spermidine; Spm, spermine; His, histamine; Tyr, tyramine; and Agm, agmatine.
[b] ND, Not detected (amine level less than 0.1 mg/100 g).
[c] Means ± SD.

remaining eight isolates were false-positive histamine-formers. Lopez-Sabater, Rodriguez-Jerez, Hernandez-Herrero, Roig-Sagues, and Mora-Ventura (1996) also found that 63.1% of the potential histamine-producers that were isolated from Niven's medium were actually false-positives when grown in a histidine-supplemented culture broth. Table 3 lists the main characteristics of these two histamine-forming bacteria, as determined by 16S rDNA sequencing, followed by comparison to the reference strains using NCBI database analysis. The PCR amplicons of strains M45-1 and M45-2 had a 99% homology with *S. carnosus*. In addition to the substantial amounts of histamine (10.7 and 9.6 ppm) in TSBH medium, these two histamine-forming isolates also produced different amounts of cadaverine and 2-phenylethylamine through the actions of their respective decarboxylase enzymes on various amino acids that also existed in the culture medium (Table 3). These two histamine-forming bacteria, the *S. carnosus* strains, M45-1 and M45-2, were only isolated from the salted mullet roe sample that contained the highest amount of histamine at 8.18 mg/100 g.

Although these two *S. carnosus* isolates from the test mullet roe samples were weak histamine-formers, they were the only contributing histamine-producing bacteria found in this study for the sample with a histamine content of 8.18 mg/100 g. *Staphylococcus* sp. were the most frequently reported histamine-formers in fermented salted fish, accounting for nearly 50% of histamine-forming microorganisms (Yatsunami & Echigo, 1991, 1992). They were usually shown to have powerful histamine-forming activity (Yatsunami & Echigo, 1991, 1992). For example, *S. epidermidis* and *S. capitis*, isolated from salted Spanish anchovies, produced more than 1000 ppm and 400 ppm of histamine, respectively (Hernandez-Herrero et al., 1999). The *S. capitis* organisms recently isolated from mustard pickle and douchi products in Taiwan were potent histamine-formers, capable of producing more than 500 ppm of histamine in TSBH in the presence of 0.5–10% NaCl (Kung et al., 2006; Tsai, Kung, Chang, Lee, & Wei, 2007). However, the recently isolated *S. pasteuri* organisms from miso and natto products in Taiwan were identified as weak histamine-forming bacteria (Kung, Tsai, & Wei, 2007; Tsai, Chang, & Kung, 2007). Similarly, all of the histamine-forming bacteria strains, e.g., *Staphylococcus* sp. isolated from suspected swordfish fillets implicated in a food poisoning, were also weak histamine-formers and produced only between 12.7 ppm and 33.0 ppm of histamine in TSBH (Chang, Kung, Chen, Lin, & Tsai, 2008). Since staphylococci are one of the major microbial groups inhabiting human skin, we postulate that the salted mullet roes could be subjected to considerable human contact during preparation and processing.

Table 3
Characterization of histamine-forming bacteria isolated from a salted mullet roe sample that contains 8.18 mg/100 g of histamine by 16S rDNA, based on the output results from NCBI database analysis, and their production of histamine and other biogenic amines (ppm) in culture broth

Strain	Organism identified	Percentage identity (%)	GenBank Accession Number	His[a] (ppm)	Cad (ppm)	2-Phe (ppm)
M45-1	*Staphylococcus carnosus*	99	AB009934.1	10.7	2.6	11.3
M45-2	*S. carnosus*	99	AB009934.1	9.6	4.4	ND[b]

[a] His, histamine; Cad, cadaverine; 2-Phe, 2-phenylethylamine.
[b] ND, Not detected (amine level less than 1 ppm).

4. Conclusion

This study, to determine the safety of 16 salted mullet roe products sold in Taiwan, showed that they had satisfactory bacterial quality, with no total coliform and *E. coli*. However, the TVBN content in all samples exceeded the 30 mg/100 g decomposition limit. The average content of each of the nine tested biogenic amines in these samples was less than 4 mg/100 g, although one of them had a histamine content of 8.18 mg/100 g. The two *S. carnosus* strains isolated from this test sample were found to produce low levels of histamine in culture broth.

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