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ScienceDirect
Food Chemistry 105 (2007) 1289–1296
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Determination of histamine and histamine-forming bacteria in dried milkfish (*Chanos chanos*) implicated in a food-borne poisoning

Yung-Hsiang Tsai [a,*], Hsien-Feng Kung [b], Hwi-Chang Chen [c], Shu-Chen Chang [b], Hsiu-Hwa Hsu [d], Cheng-I Wei [e]

[a] *Department of Seafood Science, National Kaohsiung Marine University, Kaohsiung 811, Taiwan, ROC*
[b] *Department of Food Science and Technology, Tajen University, Pingtung, Taiwan, ROC*
[c] *Southern Region Laboratory, Bureau of Foods and Drug Analysis, Department of Health, Executive Yuan, Taiwan, ROC*
[d] *Department of Living Science (Home Economics), Tainan University of Technology, Tainan, Taiwan, ROC*
[e] *College of Agriculture and Natural Resources, 1296 Symons Hall, University of Maryland, College Park, MD 20742, USA*

Received 22 September 2006; received in revised form 21 January 2007; accepted 7 March 2007

Abstract

An incident of food-borne poisoning, causing illness in three victims due to ingestion of dried milkfish, occurred in February, 2006, in Tainan Prefecture, southern Taiwan. The leftovers of the victims' dried milkfish and three other dried milkfish samples, from the same retail store as the leftover fish, were collected and tested to determine the occurrence of histamine and histamine-forming bacteria. The levels of pH, aerobic plate count (APC) and total volatile basic nitrogen (TVBN) in the leftover dried milkfish sample were significantly higher than those of the other three milkfish samples. None of the tested milkfish samples contained total coliform and *Escherichia coli*. Although the histamine contents in the three other milkfish samples were less than 5 mg/100 g, the suspected milkfish sample contained 61.6 mg/100 g of histamine, which is greater than the hazard action level of 50 mg/100 g. Given the allergy-like symptoms of the victims and the high histamine content in the suspected milkfish sample, this food-borne poisoning was strongly suspected to be due to histamine intoxication. Four histamine-producing bacterial strains, capable of producing 11.9–1243 ppm of histamine in trypticase soy broth supplemented with 1.0% L-histidine (TSBH), were identified as *Staphylococcus sciuri* subsp. *sciuri*, *Serratia grimesii*, *Bacillus cereus* and *Raoultella ornithinolytica*, by 16S rDNA sequencing with PCR amplification. *R. ornithinolytica* was a potent histamine-former capable of producing more than 800 ppm of histamine in TSBH in the presence of 1.5% or 3.5% NaCl. Therefore, *R. ornithinolytica* could be the causative bacterium responsible for the production of high content of histamine that eventually led to this dried milkfish poisoning incident.

Keywords: Histamine; Milkfish; Histamine-forming bacteria; *Raoultella ornithinolytica*; Food poisoning

1. Introduction

Histamine is the causative agent of scombroid poisoning, a food-borne chemical hazard. Scombroid poisoning is usually a mild illness with a variety of symptoms, including rash, urticaria, nausea, vomiting, diarrhea, flushing, and tingling and itching of the skin (Taylor, 1986). Severity of the symptoms can vary considerably with the amount of histamine ingested and the individual's sensitivity to histamine (Russell & Maretic, 1986). Scombroid fish, such as tuna, mackerel, bonito, and saury, that contain high levels of free histidine in their muscle, are often implicated in scombroid poisoning incidents (Taylor, 1986). However, several species of nonscombroid fish, such as mahi-mahi, bluefish, herring and sardine, have often been implicated in incidents of scombroid poisoning (Price & Melvin, 1994). In Taiwan, scombroid poisoning occurs occasionally (Chen & Malison, 1987; Murray, Hobbs, & Gilbert, 1982;

* Corresponding author. Tel.: +886 7 3617141 3605; fax: +886 7 3640634.
E-mail address: yhtsai01@seed.net.tw (Y.-H. Tsai).

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doi:10.1016/j.foodchem.2007.03.018

Tsai, Kung et al., 2005), and the fish implicated in these outbreaks are tuna, mackerel, and black marlin. Recently, due to their popularity among Taiwanese people, sailfish and marlin fillets have become the most frequently implicated fish species in scombroid outbreaks in Taiwan (Hwang, Chang, Shiau, & Cheng, 1995; Hwang, Chang, Shiau, & Chai, 1997; Hwang et al., 1999).

Biogenic amines are formed, mainly, through the decarboxylation of specific free amino acids by exogenous decarboxylases released by the microbial species associated with the seafood (Rawles, Flick, & Martin, 1996). Many different bacterial species are known to possess histidine decarboxylase and have the ability to produce histamine (An & Ben-Gigirey, 1998). Although only *Morganella* (*Proteus*) *morganii*, *Klebsiella pneumoniae* and *Hafnia alvei* have been isolated from the fish incriminated in scombroid poisoning (Taylor & Speckard, 1983), several other bacterial species capable of producing histamine have been identified in fish (Eitenmiller, Wallis, Orr, & Phillips, 1981; Middlebrooks, Toom, Douglas, Harrison, & McDowell, 1988; Taylor & Speckard, 1983; Yoshinaga & Frank, 1982). Among them are the enteric bacteria, that include *Proteus vulgaris*, *P. mirabilis*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Serratia fonticola*, *Serratia liquefaciens*, *Raoultella* (formerly *Klebsiella*) *planticola*, *R. ornithinolytica* and *Citrobacter freundii* (Ababouch, Afila, Rhafiri, & Busta, 1991; Kim et al., 2003; Klausen & Huss, 1987; Lopez-Sabater, Rodriguez-Jerez, Roig-Sagues, & Mora-Ventura, 1994; Tsai, Lin et al., 2005). In addition to the enteric bacteria, *Clostridium* spp., *Vibrio alginolyticus*, *Acinetobacter lowffi*, *Plesiomonas shigelloides*, *Pseudomonas putida*, *P. fluorescens*, *Aeromonas* spp., and *Photobacterium* spp. have also been reported as histamine producers (Lopez-Sabater, Rodriguez-Jerez, Hernandez-Herrero, & Mora-Ventura, 1994; Middlebrooks et al., 1988; Okuzumi, Hiraishi, Kobayashi, & Fujii, 1994; Ryser, Marth, & Taylor, 1984; Yatsunami & Echigo, 1991). Recently, we demonstrated the presence of histamine-forming *Proteus*, *Enterobacter*, *Klebsiella*, *Rahnella* and *Acinetobacter* in sailfish fillets in Taiwan, but failed to isolate any of the three above-mentioned major histamine-formers the *H. alvei*, *M. morganii* and *K. pneumoniae* (Tsai, Kung, Lee, Lin, & Hwang, 2004).

Milkfish (*Chanos chanos*) is an important aquacultured fish in the Indo-Pacific region, particularly the Philippines, Indonesia, and Taiwan (Chen, 1990). Histidine, at approximately 441 mg/100 g, is the most prominent free amino acid (FAA) found in the white muscle of milkfish, accounting for 80% of the total FAAs in the fish (Chiou, Shiau, & Chai, 1990). Milkfish has a FAA pattern similar to that of the migratory fish, such as mackerel, tuna, and skipjack, which also possess very high levels of histidine in the white muscle (Konosu & Yamaguchi, 1982; Suzuki, Hirano, & Suyama, 1987). Recently, Tsai, Chang, Kung, Wei, and Hwang (2005) reported that milkfish was a better substrate than was sailfish for histamine formation by bacterial histidine decarboxylation at elevated temperatures (>15 °C). However, unlike mackerel, tuna, skipjack and sailfish histamine poisoning, due to consumption of milkfish, has never been reported in Taiwan, possibly because milkfish is usually consumed within hours of harvest in Taiwan.

Dried milkfish is prepared by soaking the fish in brine for a few hours and drying under cool air for several days. An incident of food-borne poisoning due to ingestion of dried milkfish occurred in Tainan Prefecture, southern Taiwan, in February 2006. The incident caused three victims to become ill. They all suffered from allergy-like symptoms, including rash, nausea, diarrhea, flushing, and tingling and itching of skin, but all recovered within 24 h. To elucidate the causative agent, the suspected dried milkfish was collected from the victims' leftovers. Additionally, three dried milkfish samples were purchased from the same retail store as the suspected sample and processed for determination of biogenic amine levels. In addition, the chemical and microbiological quality, and histamine-forming bacteria in these dried milkfish samples were also investigated.

2. Materials and methods

2.1. Samples

A 95 g portion of leftovers from the victims' dried milkfish was collected. Another three dried milkfish samples were obtained from the same retail store in Tainan prefecture where the victims purchased the suspected product that caused the poisoning. All collected samples were wrapped in aseptic bags, placed in ice, and immediately transported to the laboratory for use within 8 h.

2.2. pH value and salt content determination

Dried milkfish samples (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 procedures (1995) by homogenizing 2 g of dried milkfish 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.

2.3. Microbiological analysis and isolation of histamine-forming bacteria

A 25 g portion of the dried milkfish 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 blender was sterilized 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. Bacterial numbers in the milkfish samples were expressed as $\log_{10}$ colony forming units (CFU)/g.

To isolate histamine-forming bacteria, a 0.1 ml aliquot of the dilute sample 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 d 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.

Analyses of total coliform and *Escherichia coli* in these dried milkfish samples were conducted, using the three-tube most probable number (MPN) methods (FDA, 1992). Lauryl sulfate 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. Identification of histamine-forming isolates

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 5000*g* 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 sulfate (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 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 for 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 content of the dried milkfish sample was measured by the method of Conway's dish (Cobb, Aoaniz, & Thompson, 1973). The TVBN extract of the 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 expressed in mg/100 g fish.

2.6. Biogenic amine analysis

Each dried milkfish 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. Samples of standard biogenic amine solutions and 2 ml aliquots of the dried milkfish extracts were derivatized with benzoyl chloride according to the previously described method (Hwang et al., 1997). Two millilitres of each bacterial culture broth were also benzoylated, using the same procedures as for dried milkfish 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 the dried milkfish 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. Effect of NaCl content on histamine-forming bacteria

The effect of NaCl content on histamine production by histamine-forming bacteria was determined in 50 ml of TSBH medium in flasks containing 1.5%, 3.5%, 7.5%, 10%, 15% or 20% of NaCl. One hundred microlitres of the 18-h-old bacterial cultures in 5 ml of TSBH medium at 35 °C were inoculated into fresh TSBH to obtain an initial concentration of about 5.6 log CFU/ml. Bacterial growth and histamine production in TSBH were determined after incubation at 35 °C for 1, 2, 3 and 4 days.

2.8. Statistical analysis

Microsoft Excel for Windows XP (2002; Microsoft Corp., Redmond, WA, USA) was used to calculate the means and standard deviations for chemical, microbial, and biogenic amines data. A value of $P < 0.05$ was used to indicate significant deviation.

3. Results and discussion

Values of the pH, salt content, APC, TVBN, total coliform and *E. coli* in the suspected dried milkfish implicated in food-borne poisoning, and the three dried milkfish samples from the same retail store as the suspected milkfish, are presented in Table 1. Although the levels of salt content, ranging from 3.38% to 3.70%, were not significantly different among the four milkfish samples, the pH, APC and TVBN values of the suspected milkfish sample (8.53, 7.72 log CFU/g, and 177 mg/100 g, respectively) were significantly higher than the average values of the other three milkfish samples (5.79, 4.00 log CFU/g and 12.4 mg/100 g, respectively) ($p < 0.05$). The APC and TVBN levels of the suspected milkfish sample exceeded the Taiwanese regulatory standard of 6.47 log CFU/g for APC and 25 mg/100 g for TVBN. None of those four milkfish samples contained total coliform and *E. coli* (Table 1).

Of the nine biogenic amines checked, only low levels of putrescine, cadaverine, and spermine were detected in all the tested milkfish samples (Table 2). While the suspected milkfish sample had a histamine content of 61.6 mg/100 g, the other three control milkfish samples had only 2.1–4.8 mg/100 g of histamine. The US Food and Drug Administration (FDA) has established a histamine "hazard action level" of 50 mg/100 g (500 ppm) for fish, based on data collected from numerous outbreaks (Taylor, 1989). It is also observed that histamine at 20 mg/100 g may be sufficient to cause the symptoms of scombroid poisoning (CDC, 2000). Thus, the high level of histamine in this suspected milkfish sample, along with the allergy-like symptoms developed in the victims, supported the conclusion that histamine was the causative agent of this food-borne poisoning incident.

High histamine contents have been found in various types of fish implicated in scombroid poisoning. The marlin implicated in a poisoning incident had a histamine content ranging between 93.5 and 276 mg/100 g (Morrow, Margo-

Table 1
Values of the pH, salt content, aerobic plate count (APC), total volatile basic nitrogen (TVBN), total coliform (TC) and *E. coli* in the dried milkfish implicated in food poisoning, and three other dried milkfish samples from the same retail store as the suspected milkfish

Source and type of dried milkfish	pH	Salt content (%)	APC (log CFU/g)	TVBN (mg/100 g)	TC (MPN/g)	*E. coli* (MPN/g)
Victims' leftover	8.53	3.70	7.72	177	<3	<3
Retail store samples as the suspected milkfish						
No. 1	5.88	3.63	3.44	7.47	<3	<3
No. 2	5.76	3.38	4.65	15.9	<3	<3
No. 3	5.74	3.69	3.92	13.7	<3	<3
Average	5.79 ± 0.08[a]	3.57 ± 0.16	4.00 ± 0.60	12.4 ± 4.39		

[a] Means ± SD for Nos. 1, 2, and 3 milkfish samples collected from the same retail store.

Table 2
The levels of biogenic amines in the dried milkfish implicated in food poisoning and other dried milkfish samples from the same retail store as the suspected milkfish

Source and type of dried milkfish	Levels of biogenic amine (mg/100 g)								
	Put[a]	Cad	Tpm	Phe	Spd	Spm	Hm	Tym	Agm
Victims' leftover	0.9 ± 0.2[b]	1.9 ± 0.4	ND[c]	0.6 ± 0.1	ND	8.1 ± 1.6	61.6 ± 2.8	1.1 ± 0.4	ND
Retail store samples as the suspected milkfish									
No. 1	1.0 ± 0.1	2.6 ± 0.6	0.5 ± 0.1	ND	ND	6.8 ± 1.4	4.8 ± 1.2	1.4 ± 0.9	ND
No. 2	2.1 ± 0.7	1.0 ± 0.3	ND	ND	ND	4.4 ± 0.9	2.1 ± 0.3	ND	ND
No. 3	0.6 ± 0.2	2.7 ± 0.3	1.0 ± 0.2	ND	ND	3.6 ± 0.8	2.6 ± 0.9	ND	ND

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

lies, Rowland, & Robert, 1991); the hot-smoked mackerel implicated in a scombrotoxic incident had a histamine content of 270 mg/100 g (Clifford, Walker, & Wright, 1989); and the canned tuna implicated in a poisoning had a histamine content of 116 mg/100 g, while that of wholesome canned tuna had only 2.74 mg/100 g (Kim & Bjeldanes, 1979). In Taiwan, incidences of scombroid poisoning have only occurred occasionally, and the fish implicated in those occasional outbreaks were tuna, mackerel, and black marlin (Chen & Malison, 1987; Murray et al., 1982; Tsai, Kung et al., 2005). Sailfish and marlin fillets have recently become the most frequently implicated fish species in scombroid outbreaks in Taiwan (Hwang et al., 1995, 1997, 1999). However, food-borne poisoning of histamine by milkfish products has never been reported in Taiwan. To the best of our knowledge, this is the first report in Taiwan to demonstrate that milkfish products could cause histamine intoxication.

Table 3 lists the identity of four histamine-forming bacteria, as determined by 16S rDNA sequences, following comparison to reference strains, using NCBI database analysis. The PCR amplicon from strain MF1 had a 100% homology with *Staphylococcus sciuri* subsp. *sciuri*, while those from strains MF2 and MF3 aligned with *Serratia grimesii* and *Bacillus cereus*, respectively, at 100%. The PCR amplicon from strain MF6 had a homology with *R. ornithinolytica* at 99%. These four histamine-forming isolates produced substantial amounts of histamine (11.9–1243 ppm) in TSBH medium. Some of them also produced different amounts of cadaverine, 2-phenylethylamine and spermine through the action of their respective decarboxylase enzymes on various amino acids that also existed in the culture medium (Table 3).

Staphylococcus spp. were the most frequently reported histamine-formers in fermented salted fish, accounting for nearly 50% of histamine-forming microorganisms. 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, in TSBH broth (Hernandez-Herrero, Roig-Sagues, Rodriguez-Jerez, & Mora-Ventura, 1999). The *S. capitis* recently isolated from mustard pickle products in Taiwan was a potent histamine-former, capable of producing more than 1000 ppm of histamine in TSBH broth (Kung et al., 2006). However, the recently isolated *S. pasteuri* from miso products in Taiwan was a weak histamine-former, producing only 28.1 ppm histamine in TSBH broth (Kung, Tsai, & Wei, 2007). Similarly, the *S. sciuri* subsp. *sciuri*, which was isolated in this study, was also a weak histamine-former, capable of producing only 13.1 ppm of histamine in TSBH (Table 3). Since staphylococci are among the major microbial groups that inhabit human skin, it is reasonable to expect that they would be transferred to food products through considerable human contact during food preparation and processing.

The *S. grimesii* strain MF2 and *B. cereus* strain MF3 that were isolated in this study were weak histamine-formers, and they produced only 11.9 ppm and 16.7 ppm of histamine in TSBH, respectively. *Serratia marcescens*, *Serratia plymuthica* and *S. fonticola* have been isolated from tuna as weak histamine-formers, producing 5.5–135 ppm of histamine in culture broth (Lopez-Sabater, Rodriguez-Jerez, Hernandez-Herrero, Roig-Sagues, & Mora-Ventura, 1996). The *Bacillus* spp. isolates from salted anchovies produced low levels of histamine at 10.5–12.4 ppm (Hernandez-Herrero et al., 1999; Rodriguez-Jerez, Mora-Ventura, Lopez-Sabater, & Hernandez-Herrero, 1994). The *Bacillus* spp. that were most frequently detected in canned anchovies also produced negligible amounts of histamine in culture broth (Kim et al., 2004). The recently isolated *B. coagulans* and *B. megaterium* from fermented fish products in Taiwan were also identified as weak histamine-forming bacteria (Tsai et al., 2006). *B. amyloliquefaciens*, *B. subtilis* and *B. megaterium*, isolated from miso products, and *B. subtilis*, isolated from sufu products in Taiwan, were also shown to be weak histamine-formers (Kung, Tsai et al., 2007; Kung, Lee, Chang, Wei, & Tsai, 2007). Since *B. cereus* is the most important food-borne pathogen among the *Bacillus* spp. (Jay, 1986), the isolation of this species, in the suspected milkfish sample implicated in food-borne poisoning, implies that the dried milkfish had been seriously contaminated during its processing.

R. planticola and *R. ornithinolytica* were isolated from tuna, bonito and sardines. They produced 2610–5250 ppm of histamine in culture broth (Kanki, Yoda, Tsukamoto, & Shibata, 2002). The *R. ornithinolytica* strain MF6, that was isolated in this study, was also a potent histamine-former and produced 1243 ppm of histamine in TSBH (Table 3). In the presence of 1.5% or 3.5% NaCl, histamine production by this bacterial strain in TSBH was accelerated and exceeded over 1600 ppm in one day, along with rapid

Table 3
Identification of histamine-forming bacteria isolated from the dried milkfish implicated in food poisoning 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 (%)	Gene bank accession number	His[a]	Cad	2-Phe	Spm
MF1	*Staphylococcus sciuri* subsp. *sciuri*	100	AJ421446.1	13.1	174	1.1	ND[b]
MF2	*Serratia grimesii*	100	AY789460.1	11.9	148	ND	ND
MF3	*Bacillus cereus*	100	DQ207729.1	16.7	222	ND	18.0
MF6	*Raoultella ornithinolytica*	99	AB004756.2	1243	2.5	ND	ND

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

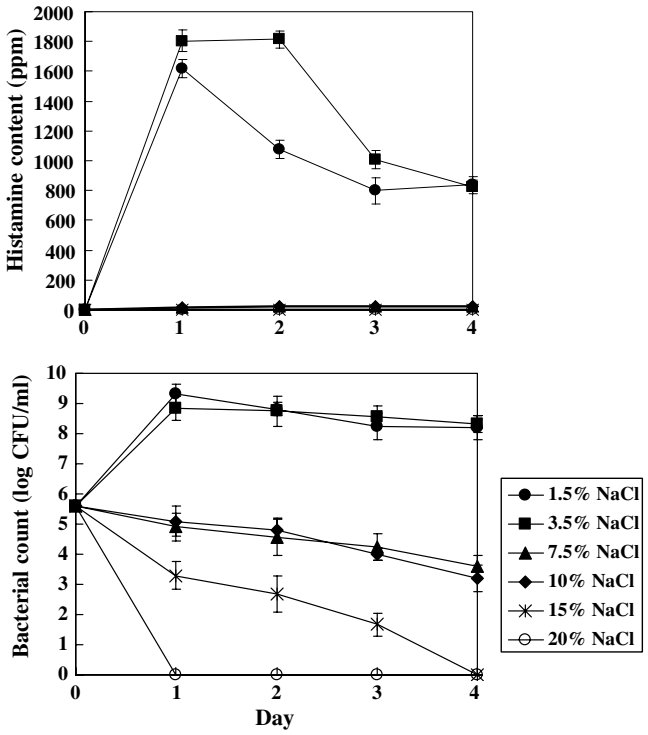

Fig. 1. The growth and histamine production of *R. ornithinolytica* strain MF6 at 35 °C in TSBH medium containing 1.5%, 3.5%, 7.5%, 10%, 15% or 20% of NaCl.

bacterial growth (Fig. 1). Higher levels of histamine were detected in TSBH containing 3.5% NaCl rather than 1.5% NaCl, at each incubation time before three days of incubation. However, as the medium NaCl content was increased to 7.5–15%, bacterial growth was gradually inhibited for four days. Low levels of histamine (at below 25 ppm) were produced by this bacterial strain during the four days of incubation. No bacterial growth and no histamine production occurred when the NaCl content in TSBH was increased to 20%. Taylor and Speckard (1983) reported that NaCl at 0.5–2.0% did not inhibit the growth of *Morganella morganii* and *K. pneumoniae*, and their histamine production. The *Enterobacter cloacae* from salted anchovies (Hernandez-Herrero et al., 1999) and salted mackerel (Tsai, Lin et al., 2005) produced histamine in culture broths that had a NaCl content of 0.5% or 3%. These bacterial isolates failed to produce histamine in a broth containing 10% or 20% of NaCl. Apparently, the decarboxylase activity of enterobacteria is highly sensitive to elevated NaCl content (Rodriguez-Jerez, Lopez-Sabater, Roig-Sagues, & Mora-Ventura, 1994; Rodriguez-Jerez, Mora-Ventura et al., 1994). All these reports supported our finding that higher content of NaCl, at about 7.5%, reduced both the growth and histidine decarboxylase activity of enterobacteria. In our previous study, two *Bacillus* spp. isolates from the fermented fish products (fish sauce, fish paste and shrimp paste) were weak histamine-formers in TSBH medium which was indicative that they are not the actual major contributor of histamine accumulation in the test products (Tsai et al., 2006). On the contrary, the prolific histamine-former, *R. ornithinolytica*, was the main contributor of histamine accumulation in the implicated dried milkfish sample in this study.

It is interesting to note that the dried milkfish samples tested in this study had a salt content of 3.38–3.70% (Table 1), while the optimal NaCl concentration for histamine formation in TSBH for *R. ornithinolytica* strain MF6 was 3.5%. It is very possible that, once the processed milkfish is contaminated with *R. ornithinolytica* during manufacturing, the bacteria will grow rapidly under these conditions with optimal salt content and produce hazardous levels of histamine to cause food-borne poisoning. Therefore, we conclude that *R. ornithinolytica* was the causative bacterium to produce hazardous levels of histamine that in turn were responsible for this dried milkfish poisoning incident. It is also very important for people, especially those from the Indo-Pacific region, such as the Philippines, Indonesia and Taiwan, to be aware that milkfish products could become a hazardous food item, causing histamine poisoning if the fish is contaminated with histamine-forming bacteria, such as *R. ornithinolytica*, and stored at improper holding temperatures.

4. Conclusion

This study showed that the suspected milkfish sample had APC and TVBN levels greater than the Taiwanese regulatory limit of 6.47 log CFU/g and 25 mg/100 g, respectively. The high content of histamine at 61.6 mg/100 g in the suspected milkfish sample could be the etiological factor for this fish-borne poisoning. While the bacterial isolates of *S. sciuri* subsp. *sciuri*, *S. grimesii* and *B. cereus* were identified to be weak histamine-formers, the *R. ornithinolytica* isolate was proven to be a prolific histamine-former with a consistent ability to produce >800 ppm histamine at an elevated NaCl level of 3.5% in TSBH medium. To our knowledge, this is the first report in Taiwan to demonstrate that a milkfish product could cause histamine intoxication and *R. ornithinolytica* was the major histamine-producing bacterium responsible for the high content of histamine in the implicated milkfish sample.

Acknowledgements

The study was partly supported by the National Science Council, ROC (Contract No. NSC 95-2313-B-022-005).

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