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Original Article

# The histamine content of dried flying fish products in Taiwan and the isolation of halotolerant histamine-forming bacteria

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ABSTRACT

Thirty dried flying fish products were purchased from fishing village stores in Taiwan and tested to detect the presence of histamine and histamine-forming bacteria. Except for histamine and cadaverine, the average content of various biogenic amines in the tested samples was less than 3.5 mg/100 g. Eight (26.6%) dried flying fish samples had histamine levels greater than the United States Food and Drug Administration guideline of 5 mg/100 g for scombroid fish and/or scombroid products, whereas four (13.3%) samples contained more than the hazard action level of 50 mg/100 g. One histamine-producing bacterial isolate was identified as *Staphylococcus xylosus* by 16S rDNA sequencing with polymerase chain reaction amplification. This isolate was capable of producing 507.8 ppm of histamine in trypticase soy broth supplemented with 1.0% L-histidine (TSBH). The *S. xylosus* isolate was a halotolerant bacterium that had a consistent ability to produce more than 300 ppm of histamine at 3% sodium chloride concentration in TSBH medium after 72 hours.



## 1. Introduction

Histamine, a biogenic amine, is a causative toxin of scombroid fish poisoning [1]. Scombroid poisoning is usually a mild illness with a variety of symptoms such as rash, urticaria, nausea, vomiting, diarrhea, flushing, and tingling, and itching of the skin [2]. The severity of the symptoms can vary considerably with the amount of histamine ingested and an individual's sensitivity to histamine. Scombroid fish such as tuna, mackerel, bonito, and saury that contain high levels of free histidine in their muscle tissue are often implicated in incidents of scombroid poisoning [2]. However, several species

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of nonscombroid fish such as mahi-mahi, bluefish, herring, and sardine have also been implicated in incidents of scombroid poisoning. In Taiwan, scombroid poisoning occurs occasionally and the fish implicated in these outbreaks are tuna, mackerel, milkfish, swordfish, and marlin [3–8].

Biogenic amines are formed primarily through the decarboxylation of specific free amino acids by exogenous decarboxylases released by the microbial species associated with seafood. Many bacterial species possess histidine decarboxylase and are able to produce histamine [9]. *Morganella morganii*, *Klebsiella pneumoniae*, and *Hafnia alvei* have been isolated from fish incriminated in scombroid poisoning [10], and several species of enteric histamine-producing bacteria have also been isolated from fish [11,12]. These include *Proteus vulgaris*, *Proteus mirabilis*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Serratia fonticola*, *Serratia liquefaciens*, and *Citrobacter freundii* [13,14]. Other than histamine-producing enteric bacteria, *Clostridium* spp., *Vibrio alginolyticus*, *Acinetobacter lwoffii*, *Plesiomonas shigelloides*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Aeromonas* spp., and *Photobacterium* spp. have been reported as histamine producers [11,15]. We also isolated several prolific histamine-forming bacteria such as *Enterobacter*, *Klebsiella*, *Raoultella*, and *Citrobacter* spp. from sailfish fillets, dried milkfish, tuna dumplings, and tuna sandwiches in Taiwan [16–20].

Flying fish are important traditional fisheries resources in various Caribbean, Southeast Asian, and Southern Pacific regions and countries [21]. In the past, flying fish were economically important species for coastal fisheries with the amount of catch reaching the top twenty in fisheries production in Taiwan. Darkwinged flying fish (*Cypselurus poeicilopterus*), Limpidwing flying fish (*Cheilopogon unicolor*), Spotwing flying fish (*Cypselurus poeicilopterus*), and stained flying fish (*Cheilopogon spilonotopterus*) are the main edible species harvested in Taiwan [21]. In Taiwan, most flying fish were primarily consumed as dried flying fish, and the traditional process for dried flying fish involves back-cutting, degutting, salting, and sundrying for several days. Histidine at approximately 473 mg/100 g is the most prominent free amino acid (FAA) in the white muscle of flying fish, and accounts for 70% of the total FAAs in the fish [22]. However, no report exists on the presence of biogenic amines (e.g., histamine), histamine-forming bacteria, and related bacteria in dried flying fish products. Therefore, this study tested 30 dried flying fish products sold in fishing village stores in Taiwan to obtain a better understanding of the safety quality of these products.

## 2. Materials and methods

### 2.1. Samples

Thirty dried flying fish products (100–140 g each) were purchased from five fishing village stores in Taiwan from Lanyu Island (six samples), Ludao Island (nine samples), Liuqiu Island (five samples), Kaohsiung (five samples), and Hengchun (five samples). Drift gill net-caught flying fish (*Cypselurus poeicilopterus*) (120–200 g each) were commercially harvested off the Taiwan coast and delivered to fishing village stores from March 2011 to May 2011. The traditional process for drying flying fish involves back-cutting, degutting, salting, and sundrying for several days at fish village stores. In general, the dried flying fish products were not packed and were maintained at room temperature in the stores before purchase. All samples were wrapped in aseptic bags, placed in ice, and immediately transported to the laboratory for use within 8 hours.

### 2.2. Determination of pH value, salt content, water content, and water activity

Dried flying fish 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 measured using a Corning 145 pH meter (Corning Glass Works, Medfield, MA). The salt content in each sample was determined in accordance with AOAC International procedures [23]. Two grams of dried flying fish sample were homogenized with 18 mL of distilled water and then titrated with 0.1M silver nitrate ($AgNO_3$) using 10% w/v potassium chromate ($K_2CrO_4$) solution as the indicator. The water content was analyzed by the standard gravimetric method by drying 1–3 g of the test sample at 102.0°C ± 2.0°C under atmospheric pressure for 2 hours. The consistency of the mass was tested by additional 1-hour drying steps until the difference in the mass did not exceed 0.5 mg [23]. Water activity (Aw) was determined at 27°C using an electric hygrometer (Hygrodynamics, Inc., Silver Spring, MD) [23].

### 2.3. Microbiological analysis and isolation of histamine-forming bacteria

A 25-g portion of the dried flying fish sample was homogenized at high speed for 2 minutes in a sterile blender with 225 mL sterile potassium phosphate buffer (0.05M, pH 7.0). Before use, the blender was sterilized by autoclaving for 15 minutes at 121°C. The homogenates were serially diluted with a sterile phosphate buffer (1:9), and 1.0-mL aliquots of the dilutes were poured onto Petri dishes (9 cm diameter). Fifteen to 20 milliliters of aerobic plate count (APC) agar (Difco, Detroit, MI, USA), which contained 0.5% NaCl, was added and gently mixed at 45–50°C. The poured plates were allowed to solidify under a biological clean bench. Bacterial colonies were counted after the plates were incubated at 35°C for 48 hours. Bacterial numbers in the dried flying fish samples were expressed as $\log_{10}$ colony-forming units (CFU)/g [23].

To isolate histamine-forming bacteria, a 0.1-mL aliquot of the sample dilute was spread on histamine-forming bacterium isolation agar fortified with L-histidine [24]. After incubating the differential agar plates for 4 days at 35°C, blue or purple colonies on the plates were removed and streaked on trypticase soy agar (Difco) to obtain pure cultures. The ability of each isolate to produce biogenic amines was determined by inoculating the isolates in trypticase soy broth (TSB) (Difco) supplemented with 1% L-histidine (TSBH), and then incubating them without shaking at 35°C for 24 hours. One milliliter of the culture broth was withdrawn for the quantitation of biogenic amines.

Analyses of total coliforms (TCs) and *Escherichia coli* in these dried flying fish samples were conducted by using the three-tube most probable number method [25]. Lauryl sulfate

tryptose broth and brilliant green lactose bile (2%) broth incubated at 35°C for 48 hours were used for the presumptive test and the confirmation test for TCs, respectively. *E. coli* was determined by using lauryl sulfate tryptose broth and *Escherichia coli* (EC) broth incubated at 35°C and 44.5°C, respectively, for 48 hours. Cultures that showed positive production of gas in brilliant green lactose bile (2%) broth or EC broth were then confirmed by eosine methylene blue agar and by the indole, methyl red, Voges-Proskauer, and citrate tests.

### 2.4. Identification of histamine-forming isolates

The presumptive histamine-forming isolates were identified on the basis of morphology, Gram stain, endospore stain, and catalase and oxidase reaction. The identity of the histamine-forming isolates was further confirmed by amplifying and sequencing approximately 1400 bp of 16S ribosomal DNA (rDNA) of the bacteria [26,27]. Amplification of the 16S rDNA from histamine-forming bacteria was performed using the universal primers UNI-L (5′-AGAGTTTGATCATGGCTCAG-3′) and UNI-R (5′-GTGTGACGGGCGGTGTGTAC-3′) [26,27]. Bacterial cells were cultured overnight in 2 mL of TSB at 35°C and then centrifuged at 5000 × *g* for 10 minutes. The cell pellet was washed and resuspended in 0.5 mL of Tris-EDTA buffer (10mM Tris-HCl and 1mM EDTA at pH 8.0), and then lysed with 20% sodium dodecyl sulfate. The solution was boiled for 20 minutes and the cellular debris was discarded after centrifugation at 13,000 × *g* for 3 minutes. The total DNA in the supernatant was thereafter precipitated with 70% ethanol and used as the template DNA for polymerase chain reaction (PCR).

Polymerase chain reaction amplification was performed in 20 μL of a reaction mixture containing 14 μL of 10mM Tris-HCl (pH 8.3), 1.6 μL of 50mM potassium chloride (KCl), 1.6 μL of 1.5mM magnesium chloride ($MgCl_2$), 0.8 μL of 20 pmol each primer, 0.4 μL of a 0.2mM concentration for each of the four deoxynucleotide triphosphates (dNTPs), 0.4 μL of 0.5 U of *Taq* DNA polymerase (Applied Biosystems, Foster City, CA), and 1.2 μL of the template DNA (10 ng). Amplifications were performed for 35 cycles (94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds) in a GeneAmp PCR 2400 thermal cycler (Applied Biosystems) with an initial denaturation at 94°C for 4 minutes and a final extension at 72°C for 7 minutes [26,27]. Amplicons were detected by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) eluted in Tris-HCl (10 mM, pH 8.5) before sequencing. The amplified DNA was directly sequenced with the ABI TaqDye Deoxy Terminator cycle sequencing kit (Applied Biosystems) and the ABI Model 377 automated DNA sequencer (Applied Biosystems). The sequences were analyzed with the BLAST (Basic Local Alignment Search Tool) tool [National Center for Biotechnology Information (NCBI), Bethesda, MD] to identify histamine-forming bacteria.

### 2.5. Determination of the total volatile base nitrogen content

The total volatile base nitrogen (TVBN) content of the dried flying fish sample was measured by the Conway dish method for triplicate determinations [28]. The TVBN extract of the fish sample in 6% trichloroacetic acid (Sigma, St. Louis, Mo) was absorbed by boric acid and then titrated with 0.02N hydrogen chloride (HCl). The TVBN content was expressed in milligrams per 100 g of fish.

### 2.6. Biogenic amine analysis

In total, the content of eight biogenic amines (putrescine, cadaverine, tryptamine, 2-phenylethylamine, spermidine, spermine, histamine, and tyramine) in the dried flying fish samples was determined by triplicate assays. Each dried flying fish sample was ground in a Waring blender for 3 minutes. The ground samples (5 g) were transferred to 50-mL centrifuge tubes and then homogenized with 20 mL of 6% trichloroacetic acid for 3 minutes. The homogenate was centrifuged (10,000 × *g* for 10 minutes at 4°C) and filtered through Whatman no. 2 filter paper (Whatman, Maidstone, England). The filtrate was then placed in a volumetric flask, and trichloroacetic acid was added to bring the final volume to 50 mL. Samples of standard biogenic amine solutions and 1-mL aliquots of the dried flying fish extracts were derivatized with dansyl chloride in accordance with a previously described method [4]. One milliliter of each culture TSBH broth of a presumptive histamine-forming isolate was also dansylated using the same procedure used for the dried flying fish extracts. The dansyl derivatives were filtrated through a 0.45-μm filter, and 20-μL aliquots were used for high-performance liquid chromatography (HPLC) injection.

The content of biogenic amines in the dried flying fish samples was determined with an HPLC system (Hitachi) consisting of the model L-7100 pump, the Rheodyne model 7125 syringe loading sample injector, the model L-4000 ultraviolet-visible light detector (set at 254 nm), and the model D-2500 Chromato-integrator. A LiChrospher 100 RP-18 reversed-phase column (5 μm, 125 mm × 4.6 mm; E. Merck, Damstadt, Germany) was used for chromatographic separation. The gradient elution program began with 50:50 (vol/vol) acetonitrile:water at a flow rate of 1.0 mL/min for 19 minutes. This was followed by a linear increase to 90:10 acetonitrile:water (1.0 mL/min) during the next 1.0 minutes. The acetonitrile:water mix was decreased to 50:50 (1.0 mL/min) for 10 minutes.

### 2.7. Effect of NaCl content on histamine-forming bacteria

The effect of NaCl content on histamine production by histamine-forming bacteria was determined using 50 mL of TSBH medium in flasks containing 0.5%, 3%, 5%, 10%, 15%, or 20% of NaCl. One hundred microliters of the 20-hour-old bacterial cultures in 5 mL of TSBH medium (0.5% NaCl) at 35°C were inoculated into fresh TSBH to obtain an initial concentration of approximately 6.0 log CFU/mL. Bacterial growth and histamine production in TSBH were determined after incubation at 35°C for 12 hours, 24 hours, 36 hours, 48 hours, and 72 hours.

### 2.8. Statistical analysis

The data were subjected to one-way analysis of variance and expressed as the mean ± the standard deviation. Pearson

correlation was performed to determine the relationships between pH, salt content, water content, Aw, TVBN, APC, and histamine content in the 30 dried flying fish samples. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 9.0 for Windows software (SPSS Inc., Chicago, IL). A value of $p < 0.05$ indicated a significant deviation.

## 3. Results and discussion

### 3.1. *Chemical and microbiological quality of dried flying fish samples*

Table 1 presents the values of the pH, salt content, water content, Aw, TVBN, APC, TC, and *E. coli* in the 30 dried flying fish products. In the dried flying fish samples, the level of pH ranged 5.78–6.66; salt content, 1.38–7.60%; water content, 10.0–35.2%; Aw, 0.61–0.84; TVBN, 10.1–108.5 mg/100 g; and APC, 3.79–8.44 log CFU/g. None of these samples contained TC or *E. coli* (Table 1). The average salt content (6.03%) and water content (32.6%) were significantly higher in the Lanyu Island samples than in the other dried flying fish samples ($p < 0.05$). The average Aw (0.80), TVBN (70.8 mg/100 g), and APC (8.18 log CFU/g) were highest in the Ludao Island samples ($p < 0.05$). On the other hand, the average water content (11.6%), Aw (0.63), TVBN (11.0 mg/100 g), and APC (4.51 log CFU/g) were lowest in the Hengchun samples ($p < 0.05$) (Table 1).

The rate of unacceptable dried flying fish samples was 70.0% (21/30 samples) for TVBN, based on the decomposition limit level of 30 mg/100 g for fish quality determination, and 50.0% (15/30 samples) using the Taiwanese regulatory standard [29] of 6.47 log CFU/g for APC. The dried flying fish samples were sundried for several days, and then stored at room temperature, which allowed microorganisms to contaminate and multiply easily in the flying fish. Therefore, unhygienic handling or processing of dried samples resulted in poor chemical and microbiological quality in this study. With regard to hygienic quality, Hsu et al. [17] also reported that a tested dried milkfish product had the unacceptable rates of 68.8% for TVBN and 46.9% for APC.

### 3.2. *The content of biogenic amines in dried flying fish samples*

Table 2 summarizes the content of biogenic amines in the tested dried flying fish products. Except for histamine and cadaverine, the average content of the various biogenic amines in the tested samples was less than 3.5 mg/100 g. Among them, five samples collected from Kaohsiung had the highest average histamine content (79.88 mg/100 g) and cadaverine content (17.17 mg/100 g). The second highest average content of histamine (12.91 mg/100 g) and cadaverine (14.54 mg/100g) was obtained from the Lanyu Island samples (Table 2). Table 3 shows the distribution of the histamine content in the tested dried flying fish products. Eight (26.6%) of 30 samples contained more than 5.0 mg/100 g of histamine, which is the allowable limit of the U.S. Food and Drug Administration (U.S. FDA) for scombroid fish and/or products [30]. Four (13.3%) samples had more than 50 mg/100 g of

**Table 1 – The values of pH, salt content, water content, water activity, total volatile basic nitrogen, aerobic plate count, total coliform, and *Escherichia coli* in 30 dried flying fish products.**

| Sample source | No. of samples | pH | Salt content (%) | Water content (%) | Aw | TVBN (mg/100 g) | APC (log CFU/g) | TC (MPN/g) | *E. coli* (MPN/g) |
|---|---|---|---|---|---|---|---|---|---|
| Lanyu Island | 6 | 6.19–6.45 (6.33 ± 0.11) AB [a] | 5.01–7.60 (6.03 ± 0.95) A | 28.1–35.2 (32.6 ± 2.3) A | 0.74–0.80 (0.75 ± 0.02) B | 13.3–55.1 (32.0 ± 13.4) B | 4.05–6.55 (5.34 ± 0.99) C | <3 | <3 |
| Ludao Island | 9 | 5.78–6.13 (6.03 ± 0.10) C | 2.54–4.23 (3.27 ± 0.73) B | 18.9–27.6 (24.9 ± 2.8) B | 0.78–0.84 (0.80 ± 0.02) A | 49.1–108.5 (70.8 ± 18.2) A | 7.64–8.44 (8.18 ± 0.27) A | <3 | <3 |
| Liuqiu Island | 5 | 6.13–6.62 (6.28 ± 0.20) BC | 1.38–1.82 (1.60 ± 0.16) C | 15.0–17.1 (15.9 ± 0.8) D | 0.71–0.76 (0.74 ± 0.02) B | 29.6–38.0 (34.1 ± 3.3) B | 4.91–5.08 (5.01 ± 0.07) C | <3 | <3 |
| Kaohsiung | 5 | 6.52–6.64 (6.60 ± 0.05) A | 2.67–4.06 (3.19 ± 0.54) B | 19.2–21.0 (20.2 ± 0.8) C | 0.68–0.72 (0.70 ± 0.01) B | 37.8–70.9 (53.3 ± 15.8) AB | 7.60–7.77 (7.65 ± 0.07) B | <3 | <3 |
| Hengchun | 5 | 6.45–6.66 (6.52 ± 0.08) A | 1.40–1.89 (1.62 ± 0.17) C | 10.0–15.1 (11.6 ± 2.1) E | 0.61–0.64 (0.63 ± 0.01) C | 10.1–11.5 (11.0 ± 0.7) C | 3.79–4.81 (4.51 ± 0.41) D | <3 | <3 |

APC = aerobic plate count; Aw, water activity; CFU = colony-forming unit; *E. coli* = *Escherichia coli*; MPN = most probable number; TC = total coliform; TVBN = total volatile base nitrogen.

[a] The values in the parentheses indicate the mean ± the standard deviation. The different capital letters (A, B, C, and D) in the same column indicate a statistically significant difference ($p < 0.05$) between the means.

**Table 2 – The content of biogenic amines in 30 dried flying fish products.**

| Sample source | No. of samples | The content of biogenic amines (mg/100 g) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Try | 2-Phe | Put | Cad | His | Tyr | Spd | Spm |
| Lanyu Island | 6 | ND [a] | ND–2.98 (1.50 ± 0.50) | ND | ND–20.40 (14.54 ± 4.84) | 0.55–57.25 (12.91 ± 11.11) | ND | ND–0.52 (0.32 ± 0.19) | 0.26–0.50 (0.38 ± 0.08) |
| Ludao Island | 9 | ND | ND–2.27 (1.57 ± 0.80) | 0.37–10.05 (2.43 ± 1.98) | 0.46–35.27 (13.55 ± 11.98) | 0.19–9.59 (3.26 ± 2.56) | ND–5.31 (2.51 ± 1.39) | ND–0.28 (0.20 ± 0.08) | 0.25–0.41 (0.32 ± 0.07) |
| Liuqiu Island | 5 | ND | ND | ND–0.25 (0.20 ± 0.02) | ND–3.10 (1.64 ± 1.18) | ND–3.39 (1.79 ± 1.46) | ND | ND | ND–0.22 (0.19 ± 0.03) |
| Kaohsiung | 5 | ND | ND | ND–6.50 (3.50 ± 1.01) | 1.00–64.19 (17.17 ± 13.75) | ND–95.03 (79.88 ± 15.16) | ND–5.17 (2.28 ± 1.99) | ND–0.43 (0.23 ± 0.21) | ND–0.50 (0.42 ± 0.10) |
| Hengchun | 5 | ND | ND | ND–2.38 (1.27 ± 0.79) | 2.68–3.36 (3.00 ± 0.25) | ND–2.50 (1.41 ± 1.24) | 0.44–0.62 (0.50 ± 0.08) | 0.14–0.21 (0.17 ± 0.03) | 0.33–0.42 (0.37 ± 0.03) |

The data in parentheses indicate the mean ± the standard deviation.
Cad = cadaverine; His: histamine; ND = not detected; Phe = 2-phenylethylamine; Put = putrescine; Spd = spermidine; Spm = spermine; Try = tryptamine; Tyr = tyramine.
[a] For items denoted by ND, the amine level is less than 0.05 mg/100 g.

histamine, which is a level that can be hazardous to the health of consumers, based on data collected from numerous outbreak reports [31]. Among the four samples, one sample from Lanyu Island had a histamine content of 57.25 mg/100 g, whereas the remaining three samples from Kaohsiung had a histamine content of 56.51 mg/100 g, 80.90 mg/100 g, and 95.03 mg/100 g. We recently demonstrated that most (78.1%) tested dried milkfish products sold in Taiwan had histamine levels greater than the U.S. FDA guideline of 5 mg/100 g for scombroid fish and/or products, whereas 43.7% of the samples contained histamine levels greater than the 50 mg/100 g hazard action level [17]. Based on the finding that the higher levels of histamine, TVBN, and APC occurred in dried flying fish samples, we postulate that these samples had been seriously contaminated during food preparation and processing.

In most scombroid poisoning cases, the histamine levels in the fish that caused the illness have been above 20 mg/100 g, and often above 50 mg/100 g [30]. The Centers for Disease Control and Prevention also reported that histamine at 20 mg/100 g may be sufficient to cause the symptoms of scombroid poisoning [32]. High histamine content levels have been found in various types of fish implicated in scombroid poisoning. Hernandez-Herrero et al. [33] detected 68 mg/100 g of histamine in semipreserved Spanish anchovies that were implicated in an incident of scombroid poisoning. In one study, marlin implicated in a poisoning incident had a histamine content ranging 93.5–276 mg/100 g [1]. In a previous study, a high histamine content of 61.6 mg/100 g in the suspected dried milkfish product could have been the etiological factor for a fishborne poisoning in Taiwan [34]. Based on information on the toxicological levels of histamine in various seafood products in causing a health hazard, four (13.3%) samples could have caused disease symptoms when consumed, primarily because of their histamine content of greater than 50 mg/100 g (Table 3). Therefore, it is very important to be aware that dried flying fish could be a hazardous food item in causing histamine poisoning. Good and hygienic fish processing procedures such as cutting, salting, drying, and packing should eliminate the presence of histamine-forming bacteria and reduce the accumulation of histamine in dried flying fish products.

In this study, a high content of histamine was detected in dried flying fish products that were tested, although no case of foodborne histamine intoxication was reported because of consuming dried flying fish products. Symptoms of histamine poisoning are not particularly definitive. Therefore, histamine intoxication is frequently confused diagnostically with an allergic reaction. In addition, histamine poisoning is not a reportable illness, even in countries that maintain surveillance records [2]. High levels of putrescine and cadaverine were also present in dried flying fish products in this study (Table 2). Putrescine and cadaverine potentiate histamine toxicity when present in spoiled fish by inhibiting intestinal histamine-metabolizing enzymes [35].

Pearson correlation was conducted to determine if any relationship existed among the pH, salt content, water content, Aw, TVBN, APC, and histamine content of the tested 30 samples. In general, positive correlations existed between Aw and APC ($r = 0.85$, $p < 0.01$), pH and histamine ($r = 0.76$, $p < 0.05$), pH and TVBN ($r = 0.74$, $p < 0.05$), and histamine and

**Table 3 – Distribution of the histamine content in 30 dried flying fish products.**

| Content of histamine (mg/100 g) | No. of dried flying fish products | | | | | |
|---|---|---|---|---|---|---|
| | Lanyu Island | Ludao Island | Liuqiu Island | Kaohsiung | Hengchun | Total no. (% of samples) |
| <4.9 | 3 | 7 | 5 | 2 | 5 | 22 (73.3%) |
| 5.0–19.9 | 2 | 2 | 0 | 0 | 0 | 4 (13.3%) |
| 20.0–49.9 | 0 | 0 | 0 | 0 | 0 | 0 (0%) |
| >50.0 | 1 | 0 | 0 | 3 | 0 | 4 (13.3%) |
| Total | 6 | 9 | 5 | 5 | 5 | 30 (100%) |

TVBN ($r = 0.73$, $p < 0.05$). However, negative correlations were noted between pH and salt content ($r = -0.78$, $p < 0.05$), salt content and APC ($r = -0.73$, $p < 0.05$), and Aw and salt content ($r = -0.72$, $p < 0.05$).

### 3.3. *Isolation and identification of histamine-forming bacteria from dried flying fish samples*

Table 4 lists the identification of one histamine-forming bacterium isolated from the dried flying fish sample that had the highest histamine content (95.03 mg/100 g) and was collected from Kaohsiung. The identification was determined by 16S rDNA sequences, and they were then compared to reference strains using NCBI database analysis. This histamine-forming bacterial isolate was identified as *Staphylococcus xylosus*. It is capable of producing 507.8 ppm of histamine in trypticase soy broth supplemented with 1.0% L-histidine (TSBH) after incubation at 35°C for 24 hours. It also produced putrescine and cadaverine through the action of their respective decarboxylase enzymes on various amino acids that also existed in the culture medium (Table 4). Potential histamine-forming bacteria that have been isolated from various dried-salted fish products such as salted sardine and salted Spanish anchovies were *Staphylococcus* spp., *Vibrio* spp., and *Pseudomonas* III/IV-NH [36,37], *Staphylococcus epidermidis*, *S. xylosus*, *Klebsiella oxytoca*, *E. cloacae*, *Pseudomonas cepaciae*, and *Bacillus* spp. [33,38]. However, in this study, none of the histamine-forming bacteria (e.g., *S. xylosus*) isolated from the Kaohsiung sample was isolated from samples from Lanyu Island, Ludao Island, Liuqiu Island, and Hengchun. It is possible that the histamine-forming bacteria were killed or inhibited during the flying fish drying process (e.g., salting or drying).

*Staphylococcus* spp. were the most frequent reported histamine formers in fermented salted fish, and accounts for nearly 50% of histamine-forming microorganisms. They usually have powerful histamine-forming activity [36,37]. For example, *S. epidermidis* and *Staphylococcus capitis* isolated from salted Spanish anchovies produced more than 1000 ppm and 400 ppm of histamine, respectively, in TSBH broth [33]. In this study, *S. xylosus* was similarly a prolific histamine former and capable of producing 507.8 ppm of histamine in TSBH (Table 4). In addition, *S. capitis* isolated from mustard pickle and douchi products was a potent histamine former capable of producing more than 1000 ppm and 600 ppm of histamine, respectively, in TSBH broth [39,40]. However, the recently isolated *S. pasteuri* from miso products in Taiwan is a weak histamine former and produces only 28.1 ppm histamine in TSBH broth [41]. Because the staphylococci are a major microbial group that inhabits human skin, it is reasonable to expect that they would be transferred to food products through considerable human contact during food preparation and processing.

### 3.4. *Effect of NaCl content on histamine-forming bacterium*

Fig. 1 shows the growth and histamine production of *S. xylosus* strain Q2 in a TSBH medium containing 0.5%, 3%, 5%, 10%, 15%, or 20% NaCl. At 0.5% NaCl, bacterial growth was accelerated and reached approximately 9.9 log CFU/mL for 3 days; the histamine levels exceeded 400 ppm and were detectable after 36 hours of incubation. At 3% NaCl, the bacterial count increased gradually, until reaching approximately 9.7 log CFU/mL after 72 hours. The histamine level was below 50 ppm at 12 hours of incubation but increased to above 260 ppm and 320 ppm thereafter at 36 hours and 72 hours, respectively. At each incubation time, higher levels of histamine were detected in TSBH containing 0.5% NaCl, compared to TSBH containing 3% NaCl. When the medium's NaCl content was increased to 5% or 10%, bacterial growth was enhanced and eventually reached approximately 8.8 log CFU/mL in 3 days. During the incubation time, this bacterial strain produced low levels of histamine below 10 ppm. However, as the medium's NaCl content increased to 15%, the bacterial growth was gradually inhibited for 12 hours, and slowly increased thereafter. No histamine production was detected during 3 days of incubation. Bacterial growth was inhibited and no histamine production occurred when the NaCl content in TSBH was

**Table 4 – The identification of a histamine-forming bacterium isolated from dried flying fish samples collected from Kaohsiung, Taiwan by using 16S rDNA, based on the National Center for Biotechnology Information database, and its production of biogenic amines (ppm) in culture broth.**

| Strain | Organism identified | Histamine content in the original dried flying fish sample (mg/100 g) | Percentage identity (%) | GenBank accession number | His | Put | Cad |
|---|---|---|---|---|---|---|---|
| Q2 | *Staphylococcus xylosus* | 95.03 | 100% | HM352950.1 | 507.8 | 26.2 | 285.6 |

Cad = cadaverine; His = histamine; Put = putrescine.

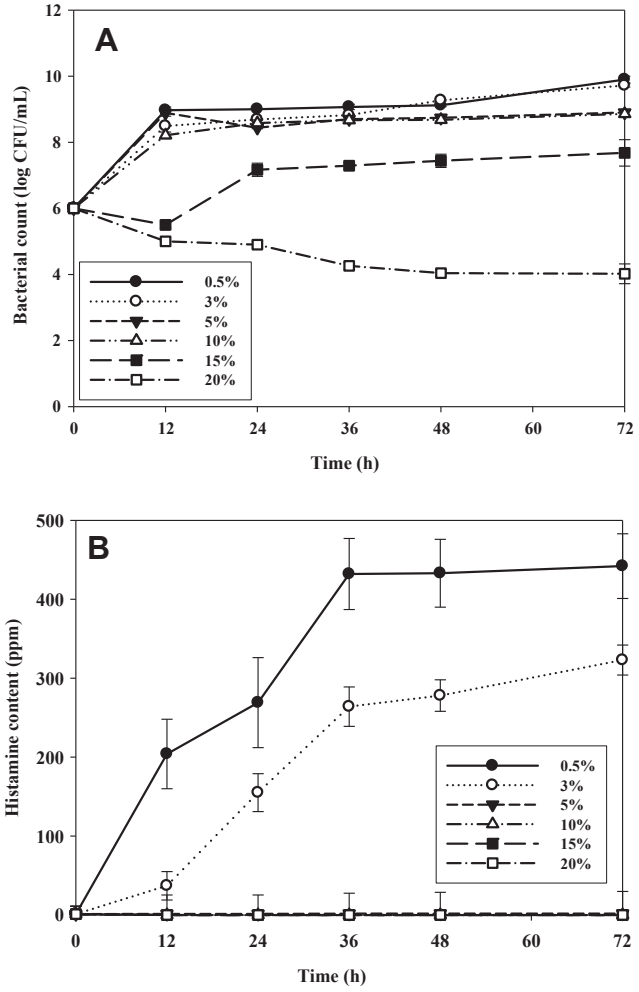


**Fig. 1 – (A) The growth and (B) the histamine production of *Staphylococcus xylosus* strain Q2 at 35°C in TSBH medium containing 0.5%, 3%, 5%, 10%, 15%, or 20% of sodium chloride (NaCl). Each value represents the mean ± the standard deviation in triplicate.**

increased to 20% (Fig. 1). Taylor and Speckard [12] report that 0.5–2.0% NaCl did not inhibit the growth of *M. morganii* and *K. pneumoniae* or inhibit their histamine production. *Enterobacter cloacae* from salted anchovies [33] and salted mackerel [14] 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% NaCl. In a previous study, we reported that NaCl concentrations of 1.5% and 3.5% had a stimulatory effect on histamine formation by *Raoultella ornithinolyticus*, whereas concentrations of NaCl in excess of 7.5% inhibited its growth and histamine formation [34]. However, Hernandez-Herrero et al [33] report that NaCl concentrations in the range of 0.5–10% have a stimulatory effect on histamine formation by *S. capitis* and *S. epidermidis*, whereas NaCl levels in excess of 20% inhibited their growth and histamine formation. *Staphylococcus capitis* isolated from mustard pickle and douchi products is capable of producing more than 1000 ppm of histamine in TSBH medium with an elevated NaCl content (10%) [39,40]. The differences in the effect observations of NaCl content could be because of the use of different histamine producers in these studies. It is interesting that the dried flying fish samples tested in this study had an average salt content of 1.60–6.03% (Table 1), whereas the optimal NaCl concentration for histamine formation in TSBH for *S. xylosus* strain Q2 was 0.5–5%. It is very possible that once the processed dried fish containing 0.5–5% NaCl content is contaminated with *S. xylosus* strain Q2 during manufacturing, this bacterium will grow under these optimal salt content conditions and produce hazardous levels of histamine. Furthermore, the halotolerant histamine-forming bacterium *S. xylosus* strain Q2 isolated from the dried flying fish sample that had the highest histamine content at 95.03 mg/100 g demonstrated a potential risk of contamination by this bacterium and risk of causing histamine poisoning under mild to high temperatures and moderate to long exposure times.

## 4. Conclusion

This study analyzed 30 dried flying fish samples sold in Taiwan and showed that the TVBN content in 21 samples exceeded the 30 mg/100 g decomposition limit. Eight (26.6%) of the 30 tested samples had histamine levels greater than the U.S. FDA guideline of 5 mg/100 g for scombroid fish and/or products. Four of the samples had the highest histamine content at greater than 50 mg/100 g. Consuming these products could lead to scombroid poisoning in consumers. *Staphylococcus xylosus* strain Q2 isolated from one dried fish sample that contained a high histamine content of 95.03 mg/100 g was a halotolerant histamine former capable of producing more than 300 ppm of histamine in TSBH medium containing 3% NaCl.

## Conflicts of interest

All authors declare no conflicts of interest.

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