

Histamine contents and histamine-forming bacteria in sufu products in Taiwan

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Abstract

Twelve white and ten brown sufu products sold in the supermarkets in southern Taiwan were purchased and tested to determine the occurrence of histamine and histamine-forming bacteria. The levels of pH, salt content, and aerobic plate count (APC) in all samples ranged from 4.6 to 6.6, 6.2% to 12.0%, and 3.0 to 7.9 log CFU/g, respectively. None of these samples contained total coliform and *Escherichia coli*. Although the average content for each of the nine biogenic amines in all samples was less than 5 mg/100 g, only one brown sufu sample had histamine content (15.8 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 1.33 mg/100 ml and 1.34 mg/100 ml of histamine in trypticase soy broth (TSB) supplemented with 1.0% L-histidine (TSBH) were identified as *Bacillus subtilis* by 16S rDNA sequencing with PCR amplification.

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1. Introduction

Sufu or furu (Chinese cheese) is a traditional Chinese fermented soybean curd which can be used in a similar way as cheese (Steinkraus, 1996). Sufu is used directly as a condiment or is cooked with vegetables or meats in China and Taiwan. Production of sufu consists of four steps: (1) preparation of tofu by salt precipitation from boiled soymilk, (2) preparation of pehtze (pizi) by spray-inoculation of diced tofu with a pure fermentation starter culture, (3) salting, and (4) ripening in a dressing mixture. The pure starter cultures consist mainly of moulds – Mucoraceae (*Actinomucor*, *Mucor*, and *Rhizopus*), or bacteria – *Micro-*

coccus and *Bacillus* spp. (Han, Beumer, Rombouts, & Nout, 2001).

Biogenic amines are basic nitrogenous compounds occurring in many foods, especially fermented foods such as cheese, sauerkraut, wine and fermented meat due to amino acid decarboxylation activities of certain microbes during fermentation (Arnold & Brown, 1978). High levels of histamine in foods can have important vasoactive effects in humans (Lehane & Olley, 2000; Taylor, 1985). Although no sufu has been incriminated in incident of histamine poisoning, the other biogenic amines present in sufu products might have contributed to enhanced histamine toxicity. Cadaverine and putrescine are known to enhance histamine toxicity by inhibiting histamine metabolizing enzymes such as diamine oxidase and histamine methyl transferase (Arnold & Brown, 1978; Bjeldanes, Schutz, & Morris, 1978; Lehane & Olley, 2000), and sufu was reported to contain these two biogenic amines (Yen, 1986).

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Histamine is formed mainly through the decarboxylation of histidine by exogenous decarboxylase released by many bacterial species known to possess histidine decarboxylase. These bacteria have been isolated not only from fish and other seafood products, but also other types of foods such as cheese, fermented sausage, and wine (Taylor, 1986). In these fermented foods, several species of histamine-producing lactic acid bacteria belonging to the *Lactobacillus*, *Leuconostoc*, and *Pediococcus* genera have been isolated (Kung et al., 2005; Stratton, Hutkins, Summer, & Taylor, 1992; Stratton, Hutkins, & Taylor, 1991; Tsai et al., 2005a). Recently, our research group isolated histamine-formers *Staphylococcus* spp., *Enterobacter cloacae*, and *Candida* spp. from mustard pickle products in Taiwan (Kung, Lee, et al., in press).

Although a pure culture is used for pehtze fermentation, the process of sufu manufacture itself is carried out under non-sterile conditions. Consequently, microbial contamination could occur during sufu production. Yen (1986) studied the occurrence of biogenic amines in sufu products in Taiwan. However, no information was available concerning the hygienic quality and histamine-forming bacteria in the product. This research was undertaken by testing 22 sufu products sold in the supermarkets in Taiwan to better understand their safety, including the contents of total coliform, *E. coli*, and histamine, for the purpose to better protect the consumers.

2. Materials and methods

2.1. Materials

Twelve white and ten brown sufu products sold in the supermarkets in southern Taiwanese were purchased in August and September of 2005. Based on the color and flavor, the sufu products are classified into two types – the white and the brown sufu, depending on the ingredients used in the dressing mixture during ripening. The dressing mixture for the white sufu consists of salt, sesame oil, alcoholic beverage, sugar, flour (or soybean) paste and some spices; while the brown sufu has similar ingredients as the white sufu for the dressing mixture but without sesame oil. Rice is also included in the dressing mixture of the brown sufu. The sufu products sold in the supermarkets were kept at room temperature. Once purchased, they were immediately transported to the laboratory for analysis.

2.2. pH value and salt content

Samples of sufu product (10 g) were homogenized in sterile blenders with 10 ml of distilled water to make 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 sufu sample with 18 ml of distilled water. The homogenate was titrated with 0.1 M AgNO₃ using 10% w/v K₂CrO₄ solution as an indicator.

2.3. Microbiological analysis and isolation of histamine-forming bacteria

A 25-g portion of the sufu 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 sufu samples were expressed as log₁₀ colony forming units (CFU)/g.

To isolate histamine-forming bacteria, a 0.1 ml aliquot of the sample dilute 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 color 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 milliliters of the culture broth were taken for quantitation of biogenic amines.

Analyses of total coliform and *E. coli* in these sufu 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. 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 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 sulfate (SDS). After the solution was boiled for 20 min and the cellular

Table 1

Values of the pH, salt content, aerobic plate count (APC), total coliform (TC), and *E. coli* in tested sufu products

Samples	No. of samples	pH	Salt content (%)	APC (log CFU/g)	TC (MPN/g)	<i>E. coli</i> (MPN/g)
White sufu	12	4.6–6.6 (5.9 ± 0.6) ^a A	6.5–12.0 (8.8 ± 1.9)A	3.0–6.8 (4.5 ± 1.3)B	<3	<3
Brown sufu	10	4.8–5.9 (5.3 ± 0.3)B	6.2–11.3 (8.7 ± 1.6)A	3.0–7.9 (6.2 ± 1.4)A	<3	<3

The white and brown sufu differ in their ingredients of dressing mixtures. The dressing mixture of the white sufu contains salt, sesame oil, alcoholic beverage, sugar, flour paste, and some spices; while that of the brown sufu contains salt, rice, alcoholic beverage, sugar, flour paste, and some spices.

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

debris was discarded following centrifugation at 13,000g 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 MgCl₂, 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 cycle (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. Biogenic amine analysis

Each sufu 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 (10,000g, 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 sufu 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 using the same procedures for sufu 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 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 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.

2.6. Statistical analysis

Pearson correlation was carried out to determine relationships between pH, salt content, APC and histamine contents in the 22 sufu samples. All statistical analyses were performed using the Statistical Package for Social Sciences, SPSS Version 9.0 for windows (SPSS Inc., Chicago, IL, USA). Value of $P < 0.05$ was used to indicate significant deviation.

3. Results and discussion

Values of the pH, salt content, aerobic plate count (APC), total coliform, and *E. coli* in the sufu products are presented in Table 1. The levels of pH, salt content, and APC in all samples ranged from 4.6 to 6.6, 6.2% to 12.0%, and 3.0 to 7.9 log CFU/g, respectively. These results are in agreement with those previously reported by Han, Beumer, et al. (2001) for commercial sufu products in China and The Netherlands. The average pH value of the white sufu (5.9) was significantly ($P < 0.05$) higher than that of the brown sufu (5.3). In contrast, the average APC level in the white sufu products (4.5 log CFU/g) was significantly ($P < 0.05$) lower than that of the brown sufu products (6.2 log CFU/g). None of these samples contained total coliform and *E. coli* (Table 1). The higher salt contents (>6%) in these sufu samples apparently had some inhibitory effect on coliform bacterial growth. Similar results were observed in an earlier study (Han, Rombouts, & Nout, 2001) in which inactivation of Enterobacteriaceae was demonstrated to occur steadily during the salting and aging steps of sufu production and sufu products contained no such bacteria. Pearson correlation was conducted to determine if there existed any relationship among the pH values, salt contents, APC, and histamine contents of the tested samples. In general, no

Table 2
Contents of biogenic amines in tested sufu products

Samples	No. of samples	Content of biogenic amine (mg/100 g)								
		Put	Cad	Try	Phe	Spd	Spm	His	Tyr	Agm
White sufu	12	ND–4.5 (1.51 ± 1.96)	ND–4.1 (0.70 ± 0.88)	ND–8.1 (1.62 ± 1.81)	ND	ND	ND–8.2 (1.64 ± 1.83)	ND–2.0 (0.46 ± 0.75)	ND–4.0 (0.80 ± 0.85)	ND–5.6 (1.12 ± 1.30)
Brown sufu	10	ND–1.0 (0.24 ± 0.23)	ND–37.1 (5.00 ± 12.05)	ND	ND	ND	ND	ND–15.8 (2.72 ± 3.20)	ND–6.5 (1.08 ± 1.33)	ND–5.6 (1.36 ± 1.18)

Put: putrescine; Cad: cadaverine; Try: tryptamine; Phe: 2-phenylethylamine; Spd: spermidine; Spm: spermine; His: histamine; Tyr: tyramine; and Agm: agmatine.

ND: Not detected (amine level less than 0.1 mg/100 g).

The values in the parentheses represent Mean ± SD.

Table 3
Identification of histamine-forming bacteria isolated from sufu products that contains 15.8 mg/100 g of histamine by 16S rDNA, basing on the output results from NCBI database analysis, and their production of histamine and other biogenic amines (mg/100 ml) in culture broth

Strain	Organism identified	Percentage identity (%)	Gene bank accession number	His ^a	2-Phe	Spm
M49-1	<i>Bacillus subtilis</i>	100	AY462217.1	1.34	0.28	ND
M49-2	<i>B. subtilis</i>	100	AY462217.1	1.33	ND	1.28

ND: Not detected (amine level less than 0.1 mg/100 ml).

^a His: histamine, 2-Phe: 2-phenylethylamine, Spm: spermine.

correlation existed among the pH values, salt contents, APC, and histamine contents in the 22 tested samples.

None of the 22 tested sufu samples contained 2-phenylethylamine and spermidine (Table 2). Although the average content for each of the remaining seven biogenic amines in all samples was lower than 5.0 mg/100 g, only one of the brown sufu samples had a histamine content of 15.8 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: Scombrotoxin (histamine) formation]). Therefore, based on the content of histamine in the test products, a 4.5% (1/22) unacceptable rate was obtained with these sufu samples. Yen (1986) reported that the average amine contents in 15 samples of commercial sufu from Taiwan were: cadaverine (3.9 mg/100 g), histamine (8.8 mg/100 g), 2-phenylethylamine (6.3 mg/100 g), putrescine (47.3 mg/100 g), tryptamine (15.3 mg/100 g), and tyramine (48.5 mg/100 g). Unlike these reported results by Yen (1986), the sufu products tested in this study did not contain high levels of biogenic amines. The amounts of dressing mixtures, the microbiological composition, and the conditions and duration of fermentation could contribute to variations in the contents of biogenic amines in soybean fermented products (Chin & Koehler, 1983; Nout, Ruiker, & Bouwmeester, 1993).

The tested sufu samples produced 12 purple colonies on the differential HBI agar plates. Only two of them (16.7%) produced histamine in TSBH medium. The remaining 10 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 presumptive histamine-producers that they isolated from Niven's medium were actually false-positives when grown in a histidine-supplemented culture broth. Thus, our

results confirm the previous observations that Niven's medium may yield false-positive isolates of histamine-producers, because other alkaline products of bacterial origin can also cause color changes of the colonies on the agar plates (Ababouch, Afilal, Rhafiri, & Busta, 1991; Tsai, Kung, Lee, Lin, & Hwang, 2004; Tsai et al., 2005b).

Table 3 listed the identity 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 M49-1 and M49-2 had a 100% homology with *Bacillus subtilis*. In addition to the substantial amounts of histamine (1.34 and 1.33 mg/100 ml) in TSBH medium, these two histamine-forming isolates also produced different amounts of 2-phenylethylamine and spermine 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 *B. subtilis* strains M49-1 and M49-2, were only isolated from the sufu sample that contained the highest amount of histamine at 15.8 mg/100 g. Han, Beumer, et al. (2001), Han, Cao, Rombouts, and Nout (2004) reported that *B. subtilis* was the main spore-forming bacterial species found in a number of randomly picked isolates from sufu samples.

Although these two *B. subtilis* isolates from the test sufu 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 15.8 mg/100 g. The *Bacillus* spp. isolates from salted anchovies produced low levels of histamine at 10.5 ppm and 12.4 ppm, respectively (Hernandez-Herrero, Roig-Sagues, Rodriguez-Jerez, & Mora-Ventura, 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, Lin, et al., 2006). *B. amyloliquefaciens*, *B. subtilis* and *B. megaterium* were also weak histamine-formers isolated from miso products in Taiwan (Kung, Tsai, & Wei, in press).

4. Conclusion

This study to determine the safety of 22 sufu products sold in Taiwan showed that white sufu products had lower levels of APC than brown sufu products. The average content for each of the nine tested biogenic amines in these samples was less than 5 mg/100 g, although one of them had a histamine content of 15.8 mg/100 g. The two *B. subtilis* strains isolated from this test sample were found to produce low level of histamine in culture broth. To our knowledge, this is first report to demonstrate the occurrence of histamine-forming bacteria in sufu products.

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