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Hygienic Quality and Incidence of Histamine-forming *Lactobacillus* Species in Natural and Processed Cheese in Taiwan

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ABSTRACT

Thirty-one natural cheese and 39 processed cheese products were collected from food markets in Taiwan and analyzed for bacterial content and histamine-related quality. The natural cheese samples had <1 to 6.84 log colony forming unit (CFU)/g of aerobic plate count (APC) and <3 to 60 most probable number (MPN)/g of total coliform (TC). On the other hand, the processed cheese products had <1 to 4.57 log CFU/g of APC and <3 to 30 MPN/g of TC. None of the tested samples contained *Escherichia coli*. Only 2 of the natural cheese and 2 of the processed cheese products contained more than 10 MPN/g of TC, which is beyond the regulatory limit of hygienic quality. The tested natural cheese products had an average histamine content of 7.9 mg/100 g, while 17 of them (54.8%) had histamine content greater than the 5 mg/100 g limit set by the U.S. Food and Drug Administration for scombroid fish and/or product. In contrast, only 6 processed cheese products (15.4%) had histamine levels greater than this limit. The average content of the other 8 biogenic amines in all tested samples was lower than 2 mg/100 g. Among the 37 presumptive histamine-forming bacterial colonies isolated from the natural cheese products, 15 produced histamine, ranging from 6.4 to 16.4 ppm, in MRS broth supplemented with 0.25% L-histidine. These histamine-producing bacteria were identified as *Lactobacillus* spp. Of the 7 *L. brevis* identified, one produced 71.2 ppm of tyramine in histidine-supplemented MRS broth.

Key words: cheese, histamine, biogenic amine, hygienic quality, histamine-forming bacteria

INTRODUCTION

Biogenic amines are basic nitrogenous compounds present in meat, fish, cheese, and wine products mainly due to amino acid decarboxylation of certain microbes⁽¹⁾. High levels of histamine in foods have important vasoactive effects in humans^(2,3). In fact, outbreaks of histamine poisoning have occurred following the consumption of cheese containing high levels of histamine^(4,5,6). In the United States, all cheese-related histamine poisoning outbreaks had involved Swiss cheese that contained greater than 100 mg/100 g of histamine⁽⁶⁾. Cases of cheese-related histamine poisoning involving individuals on drug therapy have also been reported. In Canada, an isoniazid-treated patient became ill after consuming extremely aged Cheddar cheese that was later found to contain 40 mg/100 g of histamine⁽⁷⁾. In France, Gruyere cheese was implicated in a single incident and was detected to contain 30 mg/100 g of histamine⁽²⁾.

Histamine is formed mainly through the decarboxyla-

tion of histidine by exogenous histidine decarboxylase produced by many bacterial species. These bacterial species have been isolated from fish and other seafood products, as well as other types of foods such as cheese, fermented sausage, and wine⁽⁸⁾. In 1985, a histamine-forming strain of *Lactobacillus buchneri*, which was capable of producing 420 ppm of histamine, was isolated from Swiss cheese that had been implicated in an outbreak of histamine poisoning⁽⁹⁾. Stratton *et al.*⁽¹⁰⁾ isolated 5 species of histamine-producing bacteria and identified them as *L. fermentum*, *L. helveticus*, *Enterococcus faecium*, and *L. lactis* subsp. *lactis*. Other lactobacilli capable of producing histamine included *L. casei*, *L. acidophilus*, and *L. arabinose*⁽⁶⁾.

Both natural and processed cheese products are popular in Taiwan. Although the microbiological quality of cheese products in Taiwan has been studied⁽¹¹⁾, no data is available on the occurrence of biogenic amines and histamine-forming bacteria in cheese sold in Taiwan. This research was undertaken to study the histamine-related hygienic quality of natural and processed cheese sold in supermarkets in Taiwan. The occurrence, species identity,

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and histamine-producing capability of the isolated histamine-forming bacteria were also determined.

MATERIALS AND METHODS

I. Cheese Samples

Seventy samples of imported cheese (31 natural cheese products and 39 processed products) were purchased from supermarkets in Taiwan from July to October, 2002. The processed cheese is made from a blend of natural cheese with smaller amounts of dairy products such as cream, milk, or whey, and subjected to heat treatment to give the desirable texture and flavor⁽¹²⁾. All samples were wrapped in aseptic bags, kept at 4°C, and immediately transported to the laboratory.

II. pH Value

To determine pH of the cheese, 10-g portions of the test samples were homogenized in an Osterizer blender (Oster, Milwaukee, WI, USA) at high speed 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).

III. Microbiological Analysis

A 25-g portion was cut from each cheese sample and homogenized at high speed for 2 min in a sterile blender with 225 mL of sterile potassium phosphate buffer (0.01M, pH 7.0). The homogenates were serially diluted with sterile phosphate buffer, and 1.0 mL aliquots of the dilutes were poured with aerobic plate count (APC) agar (Difco, Detroit, MI, USA) containing 0.5% NaCl in duplicate. Bacterial colonies were counted after the plates were incubated at 35°C for 48 hr, and the numbers expressed as log₁₀ colony forming units (CFU)/g of cheese.

Histidine decarboxylating *Lactobacillus* is generally responsible for histamine buildup in cheese^(6,9,10). To isolate histamine-forming bacteria, a 1.0-mL aliquot of the dilute was mixed with differential plating medium fortified with histidine⁽¹³⁾. The medium used to detect histidine decarboxylating *Lactobacillus* in cheese contained 0.5% tryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl, 0.1% glucose, 0.05% Tween 80, 0.02% MgSO₄·7H₂O, 0.01% CaCO₃, 0.006% bromocresol purple, 0.005% MnSO₄·4H₂O, 0.004% FeSO₄·7H₂O, 2% agar, and 2% L-histidine, with final pH at 5.0 ± 0.1. Following incubation of the plates at 35°C for 4 days, colonies with blue or purple color on the differential agar were picked and further streaked on MRS agar (Difco) to obtain pure cultures. These bacteria were identified on the basis of Gram stain, spore stain, catalase and oxidase reactions. From these preliminary tests, the bacteria were classified as *Lactobacillus*. Further identification of the pure isolates to the species

level was accomplished by a variety of biochemical tests using API 50CHL (BioMerieux, France). To determine the histamine-forming capabilities, these bacterial isolates were incubated (without shaking) in MRS broth supplemented with 0.25% L-histidine 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 cheese samples were conducted using the methods described by Food and Drug Administration (FDA) (1992)⁽¹⁴⁾.

IV. Biogenic Amine Analysis

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

Each cheese sample was ground in a Waring Blender for 3 min. 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 a final volume of 50 mL. Samples of standard amine solutions and 2-mL aliquots of the cheese extracts were derivatized with benzoyl chloride according to the previously described method⁽¹⁵⁾. Two milliliters of each bacterial culture in histidine-supplemented MRS broth were also benzoylated following the same procedures as the cheese extracts. The benzoyl derivatives were dissolved in 1 mL of methanol, and 20-μL aliquots were used for HPLC analysis.

The biogenic amines contents in the test samples were determined with a Hitachi liquid chromatograph (Hitachi, Tokyo, Japan), consisting of a Model L-6200 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 separation. The gradient elution program began with methanol/water (50/50, v/v) at a flow rate of 0.8 mL/min for the first 0.5 min, followed by a linear increase to methanol/water (85/15, 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. The detection limit of the nine biogenic amines was 1 ppm.

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

V. Statistical Analysis

Pearson correlation was carried out to determine relationships between pH, APC and histamine contents in samples collected from Taiwan. All statistical analyses were performed using the Statistical Package for Social Sciences, SPSS Version 9.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

I. Analysis of pH and Microbiological Quality

Values of pH, APC, total coliform, and *E. coli* in natural and processed cheese are presented in Table 1. The pH of natural and processed cheese products ranged from 4.58 to 6.24 and 5.00 to 7.00, respectively. These results are in agreement with those previously reported by Huang *et al.*⁽¹¹⁾ for commercial cheese products in Taiwan. No close relationship was observed to exist among these quality-related parameters for pH, APC and histamine ($r = 0.02-0.15$). The natural cheese products had < 1 to 6.84 log

CFU/g of APC and < 3 to 60 MPN/g of total coliform, while the processed cheese products had < 1 to 4.57 log CFU/g of APC and < 3 to 30 MPN/g of total coliform. Two out of the 31 natural cheese products contained 20 and 30 MPN/g of total coliform, and 2 out of the 39 processed cheese products contained 10 and 60 MPN/g of total coliform. None of the tested cheese samples contained *E. coli*. The rates of unacceptable natural and processed cheese products were 6.5% (2/31) and 5.1% (2/39), respectively, based on the Taiwanese regulatory limit for total coliform (Table 1). The processed cheese is made from a blend of natural cheese with smaller amounts of dairy products such as cream, milk, or whey, and subjected to heat treatment to give the desirable texture and flavor⁽¹²⁾. This heat treatment might cause destruction of the microbes to result in the lower levels of APC for the processed cheese as shown in this study ($p < 0.05$, Table 1). Huang *et al.*⁽¹¹⁾ reported similar findings with the hygienic quality of the marketed cheese in Taiwan.

II. Analysis of Biogenic Amines in Cheese

The levels of biogenic amines in natural and processed cheese are summarized in Table 2. Except for histamine, the average content of the various biogenic amines in tested samples was less than 2 mg/100 g. The average histamine content in natural cheese was 7.90 mg/100 g, and 2.37 mg/100 g in processed cheese. Table 3 shows the distribution of histamine contents in tested samples, with 17 natural (54.8%) and 6 processed cheese (15.4%) products containing more than 5 mg/100 g of histamine, which was the allowable limit of the U.S. Food and Drug Administration (FDA) for scombroid fish and/or product. Only 2 out of the 31 natural cheese products had histamine content of 34.1

Table 1. pH value, aerobic plate count (APC), total coliform (TC) and *E. coli*, and the percentage (%) of unacceptable cheese products

Type of cheese	No. of sample	pH	APC (log CFU/g)	TC (MPN/g)	<i>E. coli</i> (MPN/g)	Percentage (%) of unacceptability ^b		
						APC	TC	<i>E. coli</i>
Natural cheese	31	5.00 - 7.00 (5.90 ± 0.53) ^a A	< 1 - 6.84 (5.65 ± 2.81) A	< 3 - 60	< 3.6	— ^c	6.5 (2/31) ^d	0 (0/31)
Processed cheese	39	4.58 - 6.24 (5.80 ± 0.30) A	< 1 - 4.57 (2.84 ± 1.47) B	< 3 - 30	< 3.6	—	5.1 (2/39)	0 (0/39)

^aMean ± S.D. Values for the same column with different letters are statistically different ($p < 0.05$).

^bThe regulatory limits of total coliform and *E. coli* for cheese products in Taiwan are <10 MPN/g and 3.6 MPN/g (negative), respectively.

^cNo regulatory level.

^dNumber of unacceptable samples/number of total samples tested.

Table 2. Biogenic amine levels in tested natural and processed cheese products

Type of cheese	No. of samples	Range of amine level (mg/100 g)								
		Put ^a	Cad	Try	Phe	Spd	Spm	His	Tyr	Agm
Natural cheese	31	ND ^b ~21.5 (1.80 ± 3.10) ^c	ND~8.1 (1.34 ± 2.07)	ND~9.1 (0.30 ± 1.24)	ND~10.7 (0.65 ± 2.27)	ND	ND	ND~39.6 (7.90 ± 6.20)	ND~18.4 (1.21 ± 2.41)	ND~5.4 (0.17 ± 0.69)
Processed cheese	39	ND~2.0 (0.13 ± 0.27)	ND~7.3 (0.83 ± 1.44)	ND~3.0 (0.08 ± 0.38)	ND	ND	ND~9.8 (0.41 ± 1.34)	ND~23.6 (2.37 ± 4.57)	ND~7.6 (0.40 ± 1.20)	ND~5.7 (0.31 ± 1.08)

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

^bND: not detected (amine level below 0.1 mg/100 g).

^cMean ± S.D.

and 39.6 mg /100 g. These two natural cheese samples with greater than 30 mg/100 g of histamine might pose health hazard to some individuals since Gruyere cheese implicated in a histamine poisoning incident in France contained 30 mg/100 g of histamine⁽²⁾, and the extremely aged Cheddar cheese that caused illness in a Canadian tuberculosis patient being treated with isoiazid had 40 mg/100 g of histamine⁽⁷⁾.

Voigt *et al.*⁽¹⁶⁾ consistently detected tyramine in Cheddar cheese, which was found in 81 of 85 samples tested and tyramine concentration ranged from nondetectable to 70 mg/100g cheese. Histamine was found in 24 of 85 samples; concentrations varied from nondetectable to 130 mg/100g cheese. Moreover, histamine in Swiss cheese ranged from 1 to 250 mg/100g cheese, and putrescine and cadaverine values ranged from nondetectable to 7 mg/100g cheese⁽⁶⁾. Stratton *et al.*⁽⁶⁾ reported that the Swiss cheese which was responsible for the histamine poisoning outbreaks in the United States had greater than 100 mg/100 g of histamine. The Swiss cheese suspected to cause another outbreak in 1980 involving six individuals on a military vessel had an average of 187 mg/100 g of histamine⁽¹⁷⁾. Although the tested natural cheese in this study did not contain as high histamine level as the above reported Swiss or Cheddar cheese, the presence of putrescine, cadaverine, tyramine, tryptamine, and agmatine, and/or 2-phenylethylamine and spermine may synergistical-

ly enhance histamine toxicity. Putrescine and cadaverine have been shown to potentiate histamine toxicity when present in spoiled fish by inhibiting the intestinal histamine metabolizing enzyme^(3,18,19,20,21).

III. Isolation of Histamine-forming Bacteria and Productivity of Biogenic Amines

The tested natural cheese produced 37 purple colonies on the differential plating medium. Following incubation in MRS broth supplemented with 0.25% histidine, 15 out of the 37 isolates (40.5%) showed the ability to produce histamine, as determined by HPLC analysis of the culture broth. However, no histamine-forming bacteria was isolated from processed cheese products. These 15 histamine-forming bacterial isolates were identified as *Lactobacillus brevis* (7 isolates), *L. curvatus* (2 isolates), *L. plantarum* (1 isolate), *L. para. paracasei* (3 isolates), *L. pentosus* (1 isolate), and *Lactobacillus* sp. (1 isolate); and they produced 6.4 - 16.4 ppm of histamine in the culture broth (Table 4). *L. brevis* strain 29MT produced 71.2 ppm of tyramine, but low levels of putrescine and 2-phenylethylamine; while *L. para. paracasei* strain 50MT2 produced 20.6 ppm of putrescine and 13.9 ppm of 2-phenylethylamine, but 1.6 ppm of tyramine and 1.0 ppm of cadaverine (Table 4). *L. curvatus* strain 41MH2 produced low levels of putrescine and cadaverine, while *L. curvatus* strain 41MH3 produced 1.6 ppm of cadaverine, and *L. para. paracasei* strain 30MH3 produced 6.2 ppm of tyramine in the culture broth (Table 4). In this study, the levels of histamine and tyramine formed in the tested cheese products by lactobacilli isolates are relatively low as compared to those previously reported by Roig-Sagues *et al.*⁽²²⁾, Stratton *et al.*⁽¹⁰⁾, and Bover-Cid and Holzapfel⁽²³⁾. Our results seem to show that histamine-forming bacteria

Table 3. Histamine content in natural and processed cheese products

Histamine content (mg/100 g)	No. of natural cheese products	No. of processed cheese products
< 4.9	14	33
5.0—19.9	14	5
20.0—100.0	3	1
Total	31	39

Table 4. Production of histamine and other biogenic amines in histidine-supplemented MRS broth by histamine-forming bacterial isolates from natural cheese products

Strain	Histamine former	Histamine content in original natural cheese sample (mg/100g)	His ^a	Tyr	Put	Cad	Phe
41MT2	<i>L. brevis</i>	39.6	15.9	ND ^b	ND	ND	ND
41MH2	<i>L. curvatus</i>	39.6	16.4	ND	1.2	1.4	ND
41MH3	<i>L. curvatus</i>	39.6	11.6	ND	ND	1.6	ND
34MT	<i>L. plantarum</i>	34.1	7.1	ND	ND	ND	ND
47MT	<i>L. brevis</i>	23.6	7.9	ND	ND	ND	ND
29MT	<i>L. brevis</i>	0	12.9	71.2	2.3	ND	1.1
50MT2	<i>L. para.paracasei</i>	5.8	7.8	1.6	20.6	1.0	13.9
50MH3	<i>L. brevis</i>	15.8	11.4	ND	ND	ND	ND
25MT	<i>L. para.paracasei</i>	15.0	7.6	ND	ND	ND	ND
24MH	<i>L. brevis</i>	13.6	8.6	ND	ND	ND	ND
39M2	<i>L. brevis</i>	12.1	9.2	ND	ND	ND	ND
28MT	<i>L. sp.</i>	12.0	10.2	ND	ND	ND	ND
30MH	<i>L. brevis</i>	10.5	16.2	ND	ND	ND	ND
30MH3	<i>L. para.paracasei</i>	10.5	14.5	6.2	ND	ND	ND
67MH	<i>L. pentosus</i>	10.1	6.4	ND	ND	ND	ND

Values are in ppm.

^aHis: histamine; Tyr: tyramine; Put: putrescine; Cad: cadaverine; and Phe: 2-phenylethylamine

^bND: not detected (amine level less than 1 ppm).

could only be isolated from natural cheese products that contained higher content of histamine. Further study is still needed to determine the role of these histamine-forming bacteria in contribution to the histamine contents in natural cheese products. Furthermore, there existed no correlation between the higher contents of histamine in the cheese samples and the presence of histamine-forming bacteria that produced higher levels of histamine in the culture broths. It was possible that the major histamine-forming bacteria that contributed to the higher levels of histamine in the cheese samples were killed or inhibited during cheese-making process and storage condition (such as pasteurization, addition of preservatives, and low temperature storage, etc.)

Potent histamine-forming bacteria have been isolated from cheese samples. For example, *L. buchneri* capable of producing 420 ppm of histamine was isolated from the Swiss cheese implicated in an outbreak of histamine poisoning⁽⁹⁾. Joosten and Northolt⁽¹³⁾ isolated five strains of *L. buchneri*-like histamine producers from Gouda cheese. Recsei & Snell⁽²⁴⁾ identified a strain of *L. delbreukii* that was capable of producing high level of histamine, and Stratton *et al.*⁽¹⁰⁾ isolated *L. fermentum*, *L. helveticus*, *E. faecium* and *L. lactis* subsp. *lactis* from Swiss cheese. However, none of these species matched with the histamine-formers we isolated from the natural cheese. *L. brevis* accounted for 47% (7/15) of the total histamine-forming isolates collected in this study. In addition, *L. brevis* 29MT also produced higher level of tyramine at 71.2 ppm. Previous reports by Joosten and Northolt⁽¹²⁾, Maijala⁽²⁵⁾, and Bover-Cid and Holzapfel⁽²³⁾ indicated that *L. brevis* isolated from meat, meat products, and cheese was the most intensive biogenic amine former particularly for tyramine formation. Roig-Sagues *et al.*⁽²²⁾ isolated *L. curvatus* from ripened sausages as a potent histamine-former. Bover-Cid and Holzapfel⁽²³⁾ also reported *L. curvatus* as an intensive tyramine producer along with *L. para. paracasei* and *L. plantarum*. Our results support the observation that most of the histamine-forming bacteria isolated from cheese products belonged to the genus of *Lactobacillus*. The differences in the pH of the cheese products, the decarboxylase activity of the bacterial isolates, and the incubation condition (such as time, temperature, and aerobic/anaerobic condition, etc.) might affect the formation of these biogenic amines. The optimal growth conditions of these lactobacilli isolates and their histamine-producing ability are being studied.

CONCLUSIONS

This research studying the bacterial and histamine-related hygienic quality of the natural and processed cheese products sold in Taiwan showed that processed cheese products had lower levels of APC than natural cheese products. The natural cheese products had an average histamine content of 7.9 mg/ 100 g in comparison to 2.37 mg/ 100 g in the processed cheese. Only 2 of the 31 tested

natural cheese samples had histamine levels greater than 30 mg/ 100 g which might induce health hazard to some individuals if consumed. All 15 histamine-producing isolates were identified as *Lactobacillus* spp. and were found to be weak histamine formers.

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