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DETERMINATION OF HISTAMINE-FORMING BACTERIA, TOTAL COLIFORM AND ESCHERICHIA COLI IN FERMENTED FOODS

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ABSTRACT: Fermented fishery food products have been generally sold in the local market, Thailand. These products were frequently contaminated with various microorganisms due to raw material and unhygienic production process. Determination of microbial contamination of fermented food products helps to assure safety for consumers. Therefore, the aims of the study were to determine total bacteria, total coliform, *Escherichia coli* and histamine producing bacteria from fermented fishery food products commercially distributed in several regions of Chumphon and Surat Thani provinces. Twenty-two samples of fermented fishery food products were randomly collected for bacterial contamination assay. All samples revealed the levels of pH and salt content in the range of 3.67-7.46 and 8.46%-29.69%, respectively. The bacterial contamination of the samples was tested by total plate count method. Determination of coliform bacteria and *E. coli* was examined by multiple-tube fermentation technique. Total bacteria, total coliform and *E. coli* of all samples were in the range of 2.15-7.54 log CFU/g, < 3-460 MPN/g and < 3-93 MPN/g, respectively. In addition, thirteen histamine-forming bacteria were found on histamine-forming bacteria isolation agar after incubation at 37°C for 4 days. Five isolates of histamine-forming bacteria named M1, M3, M9, M11, and M13 were selected to identify by molecular techniques. M1 and M3 were obtained from shrimp paste (Kapi) and M11 was obtained from fermented fish (Pla Pang Dang). They were identified as *Enterobacter* sp. M9 and M13 isolated from fermented fish (Pla Som and Pla Ra) were identified as *Citrobacter farmeri* and *Staphylococcus kloosii*, respectively.

Keywords: Fermented food, Histamine, Enterobacter sp., *Citrobacter farmeri, Staphylococcus kloosii*

1. INTRODUCTION

Fermented fishery food products such as fermented fish products (Pla Som, Pla Pang Dang, and Pla Ra), fermented fish entrails, fermented mollusks, and shrimp pastes are the Thai traditional fermented products. Fermented fish entrails and shrimp pastes (Kapi) are mainly consumed in southern parts of Thailand. However, Pla Ra is normally consumed in the northeastern regions of Thailand.

Biogenic amines are nitrogenous compounds formed by microbial decarboxylation of specific amino acids or fermentation. They are present in foods, especially fermented foods such as kimchi [1], mustard pickle products [2], fermented soybean products (miso, douchi, natto and soybean paste) [3]-[6], fermented meat [7], and fermented seafood products [8]-[11]. Normal microflora present in raw materials, the food composition, and other factors critically influence the quantity and type of biogenic amine during processing and storage of fermented food products [12].

Histamine, a biogenic amine is a causative toxin of scombroid (a food-borne chemical agent). Scombroid poisoning causes a variety of symptoms such as flushing, urticaria, itching of the skin, nausea, vomiting, and diarrhea [13]. Free amino acid, histidine was catalyzed to histamine through exogenous decarboxylases produced by many bacterial strains. Histamine-forming bacteria were found in fermented food products such as *Bacillus coagulans* [9], *B. megaterium* [9], [11], *B. subtilis, Staphylococcus pasteuri* [4,5], *S. capitis* [4,5], *Enterobacter* sp. [14]. A large number of biogenic amines (particularly histamine) and food-borne pathogens in fermented foods are considered to evaluate food safety. However, a few reports have been presented in histamine-forming bacteria and pathogenic bacteria in fermented fishery food products in Thailand. The objective of the research was, therefore, to determine the contamination of pathogenic bacteria in traditional fermented fishery food products sold in the local market of the southern areas (Chumphon and Surat Thani provinces) of Thailand.

2. MATERIALS AND METHODS

2.1 Sample Collection

Twenty-two samples of fermented fishery food products, including 8 shrimp pastes (Kapi),

1 fermented shrimp, 8 fermented fish (1 Pla Som, 2 Pla Pang Dang, and 5 Pla Ra), 2 fermented green mussel, and 2 fermented fish entrails (Tai Pla) were purchased from the local market in Chumphon and Surat Thani provinces, Thailand. After purchase, they were transported to the laboratory for chemical and microbiological analysis.

2.2 Determination of pH Measurement and Salt Content

Each fermented food product (10 g) was homogenized in 10 ml of distilled water with the sterile blender to make the slurry product. The pH of the slurry product was measured using a pH meter. Salt content of each sample was investigated according to the AOAC method [15]. Two grams of samples were homogenized in 18 ml of distilled water. The homogenate was then titrated with 0.1 M silver nitrate ($AgNO_3$) solution using 10% w/v potassium chromate (K_2CrO_4) solution as an indicator.

2.3 Microbial Analysis and Isolation of Histamine-forming bacteria

Twenty-five grams of fermented food products were added into 225 ml of 0.05 M potassium phosphate buffer (pH 7.0) and homogenized with sterile blender for 2 min. The homogenates were serially diluted (1:10) with a sterile potassium phosphate buffer (pH 7.0) in triplicate. The suspension (1 ml) was poured into Petri dishes (9 cm diameter). Then, plate count agar (PCA) containing 0.5% NaCl was added and gently mixed. After solidification of the agar plate at room temperature, total bacterial colonies were determined after the plates were incubated at 37°C for 48 h. The number of bacteria in fermented fishery food products was then expressed as logarithm of colony forming unit per gram (log CFU/g). Total coliform and *E. coli* in fermented food samples were performed using the three-tube most probable number (MPN) method [16].

For histamine-forming bacteria isolation, the diluted sample (0.1 ml) was spread on histamine-forming bacterium isolation agar (HBI agar) supplemented with L-histidine according to the method of [17]. After 4 days of incubation at 37°C, blue or purple colonies on the agar plates were selected and subsequently streaked on trypticase soy agar (TSA) to obtain pure colonies.

2.4 Molecular Identification of Histamine-forming isolates

The identity of the presumptive histamine-forming isolates was carried out by amplifying and sequencing approximately 1,400 bp of 16S ribosomal DNA (rDNA). The 16S rDNA gene was amplified with the universal primers: F27 (5'AGAGTTTGATC(A/C)TGGCTCAG3') and R1492 (5'TACGG(C/T)TACCTTGTTACGACTT 3') [18].

The selected presumptive histamine-forming isolates were inoculated into 2 ml of trypticase soy broth (TSB) and incubated at 37°C for 16-24 h. After incubation, the culture was centrifuged at 11,000 rpm for 10 min. The cell pellet was mixed with 1 ml of TE buffer (10 mM Tris-HCl; 1 M EDTA; pH 8.0) and lysozyme. The mixture was incubated at 37°C for 90 min. Subsequently, 200 µl of 10% (w/v) sodium dodecyl sulfate (SDS) was added into the mixture and the reaction was incubated at 60°C for 30 min. Proteinase K (20 µl) was further added into the mixture. After incubation at 37°C for 30 min, the equal volume of phenol: chloroform: isoamyl alcohol was mixed with the mixture and centrifuged at 11,000 rpm for 10 min. The supernatant was collected and mixed with the 2 volumes of isopropanol to precipitate the genomic DNA at -20°C for 30 min. The mixture was centrifuged at 11,000 rpm for 10 min. The DNA pellet was washed with 200 µl of 70% ethanol. The air-dried DNA pellet was resuspended in 100 µl of the sterile water. The quality and quantity of extracted DNA were analyzed by measuring the absorbance at 260 and 280 nm and using agarose gel electrophoresis.

The PCR reaction was performed by using 10 ng of extracted DNA in a reaction volume of 25 µl, containing a final concentration of 0.2 µM of each primer, 1X TE buffer, 10 µM of each dNTP, and one unit of Taq DNA polymerase. Amplification conditions were 98°C for 3 min followed by 30 cycles at 98°C for 10 s, 55°C for 10 s, 72°C for 30 s, and then subjected to a final concentration of 72°C for 5 min. The PCR products were then analyzed with an automatic sequencing system (Apical Scientific Sdn Bhd, Malaysia). Sequence homology was aligned using the BLAST tool (NCBI). Phylogenetic tree was conducted by MEGA 7.0 using the neighbor-joining method.

3. RESULTS AND DISCUSSION

Twenty-two fermented fishery food products were categorized into 2 groups (salty taste and salty and sour taste) based on the taste of fermented food products. The fermented food products with salty taste were Kapi, Pla Ra, and Tai Pla. The products with salty and sour taste were Pla Som, Pla Pang Dang, fermented shrimp, and fermented green mussel. The levels of pH, salt content, total bacteria, total coliform and *E. coli* in all products were in the range of 3.67-7.46, 8.46%-29.69%, 2.15-7.54 log CFU/g, < 3-460 MPN/g, and < 3-93 MPN/g, respectively as shown in Table 1.

The average pH value (6.03) and the average salt content (24.40%) of the fermented food products with salty taste was higher than the average pH value (4.27) and the average salt content (15.39%) of the products with salty and sour taste. However, fermented food products according to Thai Community Product Standards reveals that the pH value of Pla Som and fermented shrimp must not exceed 4.6. In addition, the salt content of Kapi, Tai Pla and Pla Ra are not lower than 12%. The results

Table 1 Values of pH and salt content of fermented fishery food products, total bacteria, total coliform and *E. coli*

Category of fermented food sample	No. of Sample	pH	Salt content (%)	Total bacteria (log CFU/g)	Total coliform (MPN/g)	E. coli (MPN/g)
Salty taste - Shrimp paste (Kapi) - Fermented fish (Pla Ra) - Fermented fish entrails	15	4.49-7.46 (6.03)	18.02-29.69 (24.40)	2.15-7.54	< 3-460	< 3-43
Salty and sour taste - Fermented fish (Pla Som) - Fermented shrimp - Fermented fish (Pla Pang Dang) - Fermented green mussel	7	3.67-4.76 (4.27)	8.46-27.14 (15.39)	2.79-7.52	< 3-53	< 3-93

Note: the value in the parenthesis is the average value of each data.

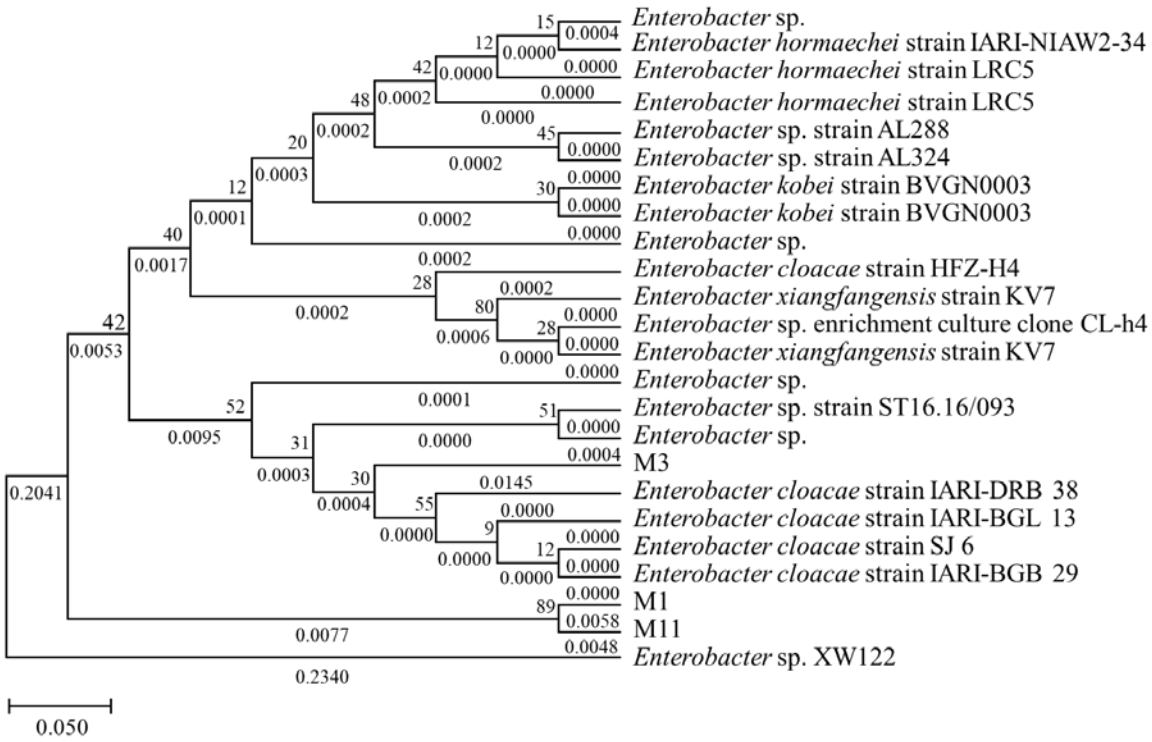

Fig. 1 Phylogenetic analysis of M1, M3 and M11 was constructed based on 16S rDNA sequences using the neighbor-joining method in the MEGA 7.0. The numbers in the phylogramnodes indicated the bootstrap value (%).

the pH value and salt content of fermented food products did not relate to the number of total bacteria, total coliform, and *E. coli.* Thai Community Product Standards are a requirement of quality that makes reliable and acceptable community products and increases confidence to consumers. The standard regulation of some of pH value and salt content in this study were similarity with the several previous reports. Values of pH and salt content in fish sauce, fish paste, and shrimp paste were 4.8-6.5 and 16.2%-45.3%, respectively [9]. The traditional fermented products (soybean and black bean douchi products) had the pH values and salt contents of 4.7-5.9 and 4.4%-

14.0%, respectively [4]. The level of pH and salt content in fish sauces were in the range of 4.8-5.7 and 15.6%-25.7%, respectively [19]. The traditional soybean pastes of Doenjang had pH values in the range of 4.8-6.0 [6]. The traditional Rihaakuru (fish paste) from the Maldives had the pH values of 5.62-6.18, whereas salt content of 1.4%-1.6% was lower than this study [10]. The pH values and salt contents in salted seafood products were in the range of 3.58-7.47 and 2.34%-21.51%, respectively [11]. Amino acid decarboxylase activity is stronger in an acidic condition, thus the pH is an important factor for the formation of biogenic amines during the fermentation process [20].

The quantity of total bacteria was 2.15-7.54 log CFU/g obtained from fermented food products in this study. However, the standard regulation of total bacteria in fermented food is not reported in Thai Community Product Standards. Several previous studies determined aerobic plate count (APC) in fermented food products. Fermented fish products were obtained 1.0-4.2 log CFU/g of APC [9]. Soybean douchi and black bean douchi showed 6.6 log CFU/g of APC and 7.5 log CFU/g of APC, respectively [4]. Moreover, salted seafood products had 1.08-7.2 log CFU/g of APC [11].

Total coliform and *E. coli* in all tested samples were < 3-460 MPN/g and < 3-93 MPN/g, respectively as shown in Table 1. In addition, twelve fermented

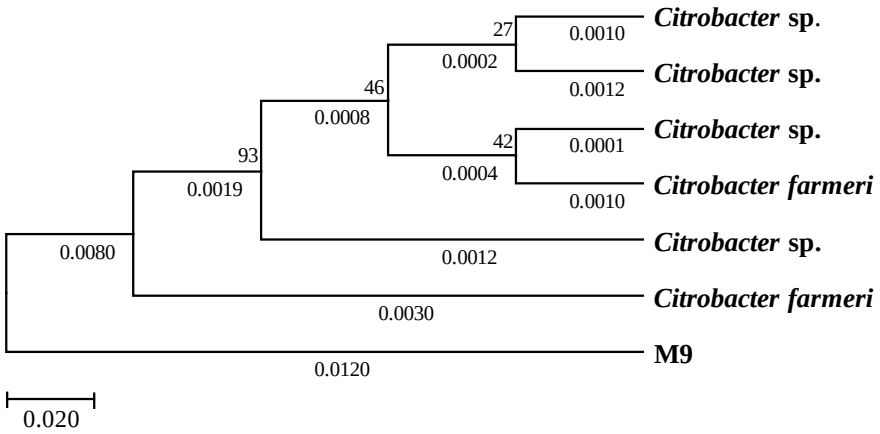

Fig. 2 Phylogenetic analysis of M9 was constructed based on 16S rDNA sequences using the neighbor-joining method in the MEGA 7.0. The numbers in the phylogramnodes indicated the bootstrap value (%).

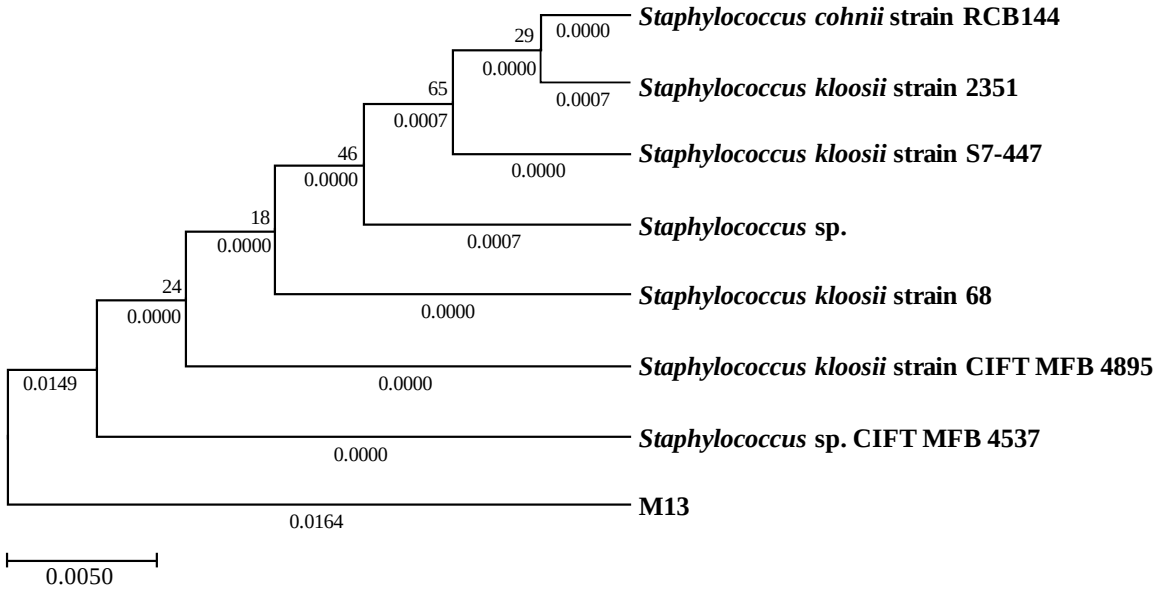

Fig. 3 Phylogenetic analysis of M13 was constructed based on 16S rDNA sequences using the neighbor-joining method in the MEGA 7.0. The numbers in the phylogramnodes indicated the bootstrap value (%).

food samples were found < 3 MPN/g of total coliform and *E. coli*. The quantities of *E. coli* in the fermented foods according to Thai regulatory level must be < 3 MPN/g whereas the quantity of total coliform in these products is not reported. Comparison with fermented food products such as mustard pickle products, soybean douchi products, and salted seafood products in previous studies, none of these products contained total coliform and *E. coli* [2], [4], [11]. The results of the study indicated that the bacterial contamination of fermented fishery food products may be caused by bacterial flora in the raw materials and bacterial contamination during the fermentation process.

From twenty-two fermented fishery food samples, thirteen histamine-forming bacteria were obtained after incubation at 37°C for 4 days. Five bacterial isolates (M1, M3, M9, M11, and M13) were selected to identify by the universal primers targeted to the 16S rDNA gene. The 1,400-bp amplicons obtained from each isolate were analyzed and compared with other reference strains from GenBank using the BLAST tool. Bacterial isolates (M1 and M3) were isolated from Kapi. The other isolates (M9, M11, and M13) were isolated from Pla Som, Pla Pang Dang, and Pla Ra, respectively. The M1, M3 and M11 isolate were identified as *Enterobacter* sp. (Fig. 1). The M9 isolate was highly homologous to *Citrobacter farmeri* (Fig. 2). The M13 isolate had a homology with *Staphylococcus kloosii* (Fig. 3). The *Enterobacter* sp. was identified as histamine former isolated from tuna dumpling [14]. *E. cloacae* isolated from salted mackerel were found to be weak histamine former [8]. *E. cloacae* were also identified as weak histamine former isolated from mustard pickle products [2]. *Staphylococcus* sp. was mostly found as histamine formers in fermented foods. *S. capitis* and *S. pasteuri* were found as a histamine former in the mustard pickle products [2] and douchi [4].

4. CONCLUSIONS

Total bacteria, total coliform, *E. coli*, and histamine-forming bacteria were determined in fermented fishery food products. The presence of *E. coli* found in some products were out of Thai regulatory level (< 3 MPN/g). Moreover, the study of the research demonstrated the incidence of histamine-forming bacteria (*Enterobacter* sp., *C. farmer*, and *S. kloosii*) in fermented fishery food products. Therefore, further studies are to determine the quantity of histamine contents in fermented fishery food products for food safety assessment.

5. ACKNOWLEDGMENTS

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