Research article

Determination of Histamine Level and Its Correlation with Viable Bacterial Count in Cambodian Fermented Fish

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Abstract Nem trey (NT), Sangvak (SV) (minced fish), and Nem sbek chrouk (NC) (minced fish mixed with pork skin) are fermented fish products popular in Cambodia; NC and NT are ready for direct consumption after two or three days of fermentation, while SV must be additionally grilled before consuming. However, food safety standards for these products including control of biogenic amines such as histamine are neglected, which could pose a health problem. Biogenic amines are nitrogenous organic chemical compounds mainly produced from the decarboxylation of amino acid, histidine, by the activity of certain bacteria associated with fish products processed under poor hygiene and unstable temperature control. This study aimed to determine the histamine level in NT, SV, and NC products and studied its correlation with the viable bacterial count (BVC). Twenty-six samples, NT (n=12), SV (n=5), and NC (n=9), were collected from different producers from Battambang (BB) and Kratie (KT) provinces. A histamine test kit utilizing a colorimetric enzymatic assay, was used to determine histamine level. The BVC was done using the spread plate technique on Luria Bertani agar media incubated at 37°C for 24h. For KT province, NT and NC contained BVC (log_{10} CFU/g) in the range of 5.74±0.02 to 6.57±0.01 and 5.52±0.01 to 5.98±0.01, while histamine (mg/kg) ranged from 0 to 156.43±0.13 and 28.76±0.71 to 73.87±0.13, respectively. SV, NT, and NC from BB province contained the BVC (log_{10} CFU/g) ranging from 6.02±0.16 to 6.56±0.15, 5.97±0.15 to 6.04±0.15, and 5.69±1.18 to 6.12±0.11, while histamine (mg/kg) ranging from 6.10±0.44 to 54.52±0.00, 0 to 10.90±0.14, and 0 to 38.54±0.00, respectively. The BVC and histamine showed a significant correlation, with a value of 0.448 (p-value < 0.05). Therefore, raw materials, processing, and storage conditions along the production chain should be evaluated in further studies to ensure safety of the fermented fish products.

Keywords biogenic amine, viable bacterial count, fermented fish, food safety

INTRODUCTION

Fish and fishery products have long been associated with people's socioeconomic lives. To preserve fish, fermentation is one of the oldest and most cost-effective methods (Uochoi, 2019). Fish fermentation is practiced in many parts of the world, but the most popular practice is found in Southeast Asia, including Cambodia. Generally, the common fermented fish product in Cambodia includes prahok (fish paste), teuk trey (fish sauce), trey praherm; trey proma (salted fish), and mam trey, paork, and nem (other fermented fish) that have played an essential part of the typical diet in Cambodia involved to fermented fish (Ly et al., 2018, 2020). Among these types of Cambodian fermented fish products, Nem such as Nem trey (NT), Sangvak (SV) (minced fish), and Nem sbek chrouk (NC) (minced fish mixed with pork skin) are the fermented fish products that usually take two to three days for fermentation. These ready-to-eat appetizers, except for Sangvak, which needs to be grilled before consumption, are popular among Cambodians.

Potentially pathogenic bacteria such as *Bacillus*, *Clostridium*, and *Staphylococcus* were found in traditional Cambodian fermented fish products (Chuon et al., 2014; Ly et al., 2018). So, fermented fish products can contain microorganisms that affect human health. Chemical compounds and microbiological contamination can cause acute poisoning or long-term disorders such as cancer (Ruiz-Capillas and Herrero, 2019). Another study reported that many bacterial isolates from Cambodian fermented fish products were from the non-lactic acid bacteria (LAB) genera, including *Staphylococcus, Bacillus*, *Clostridium, Virgibacillus, Kocuria*, *Clostridium, Lisinibacillus, Psychrobacter, Lantibacillus,* and *Micrococcus* (Chuon et al., 2014).

Next to microorganisms, a toxic chemical substance such as biogenic amines (BAs) is the most common compound associated with fermented fish (Visciano et al., 2012). Biogenic amines are basic nitrogenous chemicals with various structures that may be found in various foods, including fish and fishery products, meat and animal products, cheeses, wine, beer, and other fermented meals and beverages (Durak-Dados et al., 2020). Histamine, tyramine, putrescine, cadaverine, and b-phenylethylamine are the most significant BAs in foods and drinks, both qualitatively and quantitatively, as a result of the decarboxylation of histidine, tyrosine, ornithine, lysine, and phenylalanine, respectively (Spano et al., 2010). Fish and fish products show the highest levels of BAs in previous studies (Linares et al., 2011; Durak-Dados et al., 2020). Among all types of biogenic amines, histamine (HIS) is the most concern BAs related to food safety (EFSA, 2011) as histamine has been shown to cause severe symptomatology such as skin rashes, headache, nausea, diarrhea, and variations in blood pressure (Botello-Morte et al., 2022). The synthesis of histamine in mackerel and other marine fish carrying high levels of endogenous histidine is linked to the action of microflora rather than the activity of a decarboxylase found natively in fish (Durak-Dados et al., 2020). Histamine consumption of 8-40 mg, 40-100 mg, and more than 100 mg may generate mild, moderate, and severe poisoning, respectively (Sahu et al., 2016). European Union limited the histamine level in food products by 100 mg/kg on average, no one sample exceeds 200 mg/kg, and no more than two samples may each have a value of more than 100 ppm, but less than 200 ppm for nine samples tested (EFSA, 2007).

Microorganisms, known as factors, that affect the formation of biogenic amines and histamine formation, have been reported. The genus *Photobacterium*, *Aeromonas hydrophila*, and Enterobacteria such as *Morganella morganii*, *Enterobacter aerogenes*, *Raoultella planticola*, and *Klebsiella oxytoca* are involved in the accumulation of histamine in fish and seafood products (Fernández-No et al., 2010; Küley et al., 2013). Some fungi (yeast and molds) are implicated in BAs formation, although their significance is debatable in several ways (Tristezza et al., 2013). Poor raw material quality and improper handling are two reasons for including BAs in fermented fish (Ruiz-Capillas and Herrero, 2019).

OBJECTIVE

According to the relation between histamine level and microorganisms, this study aimed to determine the histamine level and identify the correlation between histamine level and viable bacterial count in Cambodian fermented fish, Nem, collected from two provinces, including Battambang and Kratie. This study provides insight into predicting the safety of the fermented fish product and can be a supporting document for standard quality development for the fermented fish product in Cambodia.

METHODOLOGY

Samples Collection

Twenty-six samples were collected from local producers in two provinces in Cambodia, Kratie (KT) and Battambang (BB) provinces. From KT, there were 11 samples collected, and separated into three types of samples such as fermented fish packaged by banana leaves, Nem Trey (KTNTB) three samples; fermented fish packaged by plastic, Nem Trey (KTNTP) four samples; and fermented fish mixed with pork skin, Nem Sbek Chrouk (KTNCB) four samples were collected. Whereas for BB province, 15 Nem samples were collected including Songvak (BTBSV) five samples, Nem Trey (BTBNT) five samples and fermented fish with pork skin, Nem Sbek Chrouk (BTBNC) five samples.

Table 1 Description of collected samples

Province	Type and number of samples	Sample code	Main ingredient
Battambang	Nem trey (fermented fish) (n=5)	BTBNT	Fish
	Nem sbek chrouk (fermented fish mixed with pork skin) (n=5)	BTBNC	Fish and pork skin
	Songvak (fermented fish) (n=5)	BTBSV	Fish
Kratie	Nem trey (packaged by banana leave) (n=3)	KTNTB	Fish
	Nem trey (packaged in plastic) (n=4)	KTNTP	Fish
	Nem sbek chrouk (packaged by banana leave) (n=4)	KTNCB	Fish and pork skin

Viable Bacterial Count Analysis

The enumeration of viable bacterial count (BVC) was done using the spread plate technique. For each Nem product, a homogenized sample of 10g was diluted with 90 mL of phosphate-buffered saline (PBS). Appropriate dilutions of the samples were prepared using the same diluent. Then 0.1mL aliquots of each dilution were applied to Luria Bertani agar media and spread on its surface until completely absorbed. Plates were then incubated at 37℃ for 24 hours. The colonies counting of BVC were presented as logarithms colony-forming units per gram ($\log_{10}$ CFU/g).

Histamine Concentration Analysis

The sample preparation and extraction followed the manufacturer's instructions for the extraction kit (Kikkoman Biochemifa, Tokyo, Japan). 10 g of each Nem sample was first homogenized, and 1 g of the homogenized sample was diluted with 24 ml of sample treatment buffer (EDTA) in a centrifuged tube. The tube was vortexed for 30 mins and then boiled for 20 mins. The tube was cold by placing it on an ice box (until < 20℃). The tube was vortexed again and put in an ice box to separate the phase. The tube was centrifuged 10 000 x g for 5 min; then the supernatant was collected. 11 ml of deionized water was added to the colorimetric reagent vial. 6 ml of buffer vial was added to the enzyme reagent vial. The concentration of histamine (mg/kg or ppm) (HIS) was calculated by Eq. (1).

$$HIS = (Es - Eb) \div (Estd - Ec) \times 100 \quad (1)$$

The absorbance of the sample, Es, was measured by mixing extracted sample solution, colorimetric reagent, and enzyme solution. Eb stands for absorbance of sample blank obtained by mixing extracted sample solution, colorimetric reagent, and buffer. The absorbance of standard solution, Estd, by mixing standard histamine solution, colorimetric reagent, and enzyme solution. The last absorbance of reagent blank, Ec, got by mixing Distilled water, colorimetric reagent, and buffer. Absorbance was measured using a spectrophotometer (UV-1280, Shimadzu, Japan) with a 1 cm optical wavelength of 470 nm.

Statistical Analysis

Results units of the quantitative bacterial viable count were $\log_{10}$ CFU/g. The histamine content and viable bacterial count were analyzed with statistical analyses using the Statistical Package for the Social Sciences (SPSS, Version 20.0.0 for Windows, 2018; IBM Co., Somers, NY, USA). Data were analyzed for the degree of variation by calculating the result's mean and standard deviations (SDs). The significance of differences was evaluated using analysis of variance (ANOVA). A *p*-value of less than 0.05 was considered statistically significant. The correlation value was determined using the Pearson Correlation coefficient.

RESULT AND DISCUSSION

Evaluation of Viable Bacterial Count and Histamine Level

Figures 1 and 2 described the result of HIS and BVC, respectively. For KT province, Nem trey packaged by banana leaves and plastic (KTNTB and KTNTP) contained HIS (mg/kg) in the range from 6.35$\pm$0.48 to 156.43$\pm$0.13, while BVC ($\log_{10}$ CFU/g) showed a range from 5.74$\pm$0.02 to 6.57$\pm$0.01. However, Nem sbak chrouk described the HIS (mg/kg) in the range 228.76$\pm$0.71 to 73.87$\pm$0.13. At the same time, BVC ($\log_{10}$ CFU/g) presented from 5.52$\pm$0.01 to 5.98$\pm$0.10. On the other hand, from BB province, NT showed the range of HIS (mg/kg) from 0 to 10.90$\pm$0.14, while BVC (log10 CFU/g) represented from 5.97$\pm$0.15 to 6.04$\pm$0.15. NC showed a range of HIS (mg/kg) and BVC ($\log_{10}$ CFU/g) from 0 to 38.54$\pm$0.00 and 5.69$\pm$1.18 to 6.12$\pm$0.11, respectively. For Songvak, the data represented 6.10$\pm$0.44 to 54.52$\pm$0.00 and 6.02$\pm$0.16 to 6.56$\pm$0.15 for HIS (mg/kg) and BVC ($\log_{10}$ CFU/g), separately.

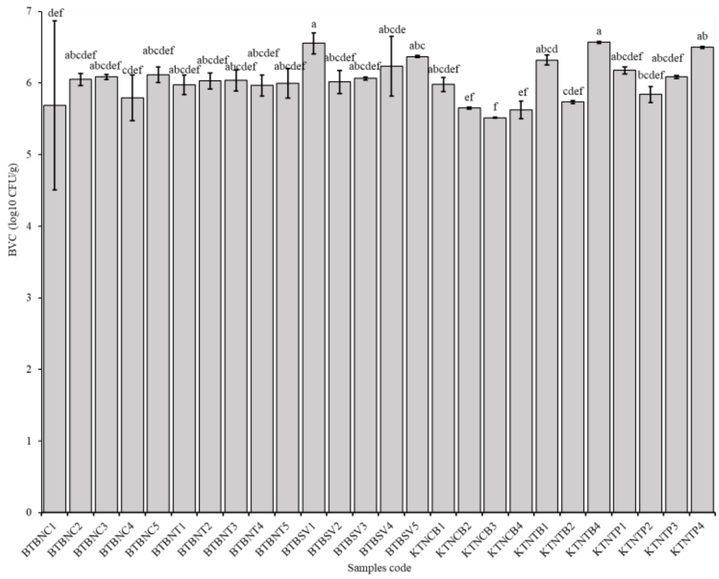

Fig. 1 Viable bacterial count in all samples

The result of all samples was compared with maximum limits. The US Food and Drug Administration (FDA) set the maximum of histamine in fish and fish products at 50 mg/kg (Ly et al., 2020), while European Food Safety Authority set the limit of histamine in fishery products at 200 mg/kg (EFSA, 2007). Seven in twenty-six samples exceed 50 ppm, and no one above 200 mg/kg. The safety of these fermented products should be slightly concerning. Previous studies on Cambodian fermented fish showed that the histamine concentration loaded in products ranged from 32 to 840 mg/kg (Ly et al., 2020). This result showed higher histamine levels, but it might be comparable with a concentration of histamine studied in this paper as the Nem products required only a few days to consume, while other fermented fish mentioned in that previous study were other types of fermented products that require a longer time to consume (Paork Chav, Paork Chau, and Mam Trey). The Malaysian fermented fish, *Budu*, detected 187 mg/kg of histamine concentration (Saaid et al., 2009). Histamine was found at a high concentration in *Feseekh*, Egyptian fermented fish (521 mg/kg) (Rabie et al., 2011).

The microbial ranged from 5.52 to 6.32 $\log_{10}$ CFU/g. The total cell counts identified on other Cambodian fish products were 5 to 7 $\log_{10}$ CFU/g for Prahok (fermented fish paste), 5 to 8 $\log_{10}$ CFU/g for Kapi (fermented fish), and 2 to 6 for Toeuk Trey (fermented fish sauce) (Chuon et al., 2014). The viable plate count found 6 $\log_{10}$ CFU/g in the Indonesian salt-fermented fish product (Kusmarwati et al., 2020). This information may be compared with each other for the same type of products (fermented fish products).

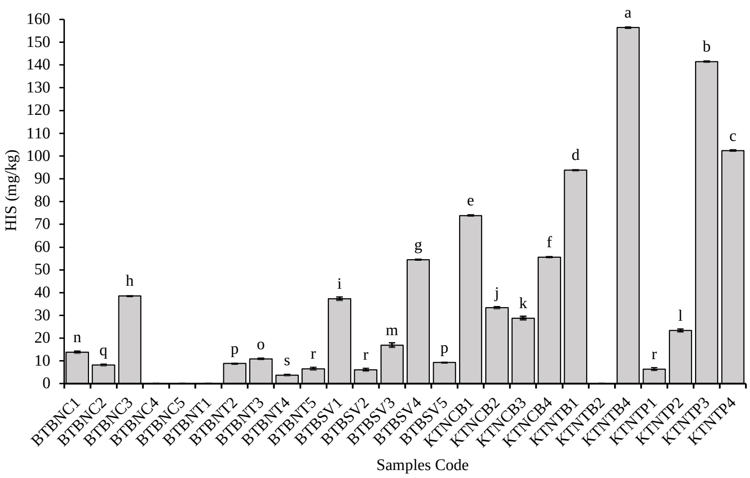

Fig. 2 Histamine content in all samples

As determined by One-way ANOVA and LSD tests in SPSS 2018. The significant differences of all samples are shown in script on the bar chart in Fig 1 and 2 for BVC and HIS, respectively. Statistically significant differences (p<0.05) were found among the defined concentration of histamine, except BTBSV2, KTNTP1, and BTBNT5 showed non-significant differences (p>0.05) and pair of BTBNT2 and BTBSV5 found that non-significant differences also (p>0.05). However, for the viable bacterial count, statistically significant differences (p<0.05) were found, but some of the group samples were described that they are non-significant differences (p>0.05). Moreover, comparing histamine levels with 50 ppm showed non-significant differences (p>0.05). In contrast, the significance differences (p<0.05) were found in analyzing 200 mg/kg histamine levels using the One Sample T-test. Table 2 showed the significant moderate correlation between histamine concentration and bacterial viable count value r=0.448, p<0.05, n=26 was found using Pearson Correlation. Sample form Kratie showed a non-significant different (p>0.05) between banana leave packaging and plastic packaging through Paired Samples T-test.

Table 2 Correlation between BVC and HIS by Pearson Correlation

		Viable bacterial count
Histamine content	Pearson Correlation	0.448
	Significant	0.022
	Number of samples	26

The histamine concentration and microbial loaded in these Nem samples were correlated. Therefore, to prevent the increase of histamine levels in Nem products, the elimination of microbial through applied raw material quality, good hygiene production, and cold processing line play an important factor in reducing histamine in food.

CONCLUSION

The histamine level, a formation chemical concerning food safety, in Cambodian fermented fish products, was studied on 26 samples collected from 2 different provinces in Cambodia. The range of histamine concentration was detected from 0 to 156.43±0.13 mg/kg, with seven samples exceeding the maximum limit of 50 mg/kg set by the FDA. Microbial loads of each product were also determined, and the results ranged from 5.52±0.01 to 6.32±0.07 $\log_{10}$ CFU/g. The correlation between histamine and bacterial concentration was significantly correlated with a moderate correlation on the positive side (r=0.48). This result is important for setting limits of food safety criteria for local fermented products. It is important to note that to reduce the risk of the formation of histamine concentration of the Nem product, reduction or elimination of certain bacteria species through the hygienic practice through food supply chains such as raw material, operating, and temperature controlling shall be applied.

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