Research article

Evaluation of the Effect of Lactic Acid Bacteria on Histamine-producing Bacteria Isolated from Cambodian Prahok

SAMOEUN DANA*
Faculty of Agro-Industry, Royal University of Agriculture, Phnom Penh, Cambodia
Email: samoeundana99@gmail.com/sdana@rua.edu.kh

TOP SOTHALONG
Faculty of Agro-Industry, Royal University of Agriculture, Phnom Penh, Cambodia

BUNTONG BORARIN
Division of Research and Extension, Royal University of Agriculture, Phnom Penh, Cambodia

CHHE CHINDA
National Institute of Science, Technology, and Innovation, Phnom Penh, Cambodia

CHRUN RITHY
Faculty of Agro-Industry, Royal University of Agriculture, Phnom Penh, Cambodia

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Abstract Prahok (fermented fish paste) is commonly used as a side dish or condiment in Cambodian cuisine. Because of the condition of the raw material and inadequate hygiene during processing, this type of fermented fish is frequently loaded with histamine toxicity. The purpose of this study was to examine the potential histamine-producing bacteria (HPB) isolated from prahok collected from three provinces in Cambodia and to evaluate the efficacy of local lactic acid bacteria (LAB) in controlling those HPB. For HPB isolation, a modified Niven's agar was used and the bacteria species were identified using the Biolog GEN III Semi-automatic system, while histamine concentration was quantified using a colorimetric enzyme assay. Ten LAB strains from the stock collection of Laboratory Food Biotechnology, Faculty of Agro-Industry, were used to evaluate their antibacterial activities against HPB using the well diffusion method and the co-culture method, and compared to the control which involved growing HPB without LAB. As a result, five identified HPBs isolated from prahok, including *Enterobacter aerogenes*, *Klebsiella oxytoca*, *Morganella morganii*, *Proteus penneri*, and *Staphylococcus xylosus*, were found to produce histamine at levels of more than 200 ppm in vitro. A well diffusion and co-culture examination demonstrated that the growth of all potential HPB cultured with three LABs (*Lactobacillus plantarum, Lactobacillus gasseri,* and *Lactococcus lactis*) of a total of 10 LAB strains was inhibited compared to the control. In conclusion, because the three LABs listed above cannot create histamine, they are promising candidate LABs to apply in Prahok processing to control HPB as well as histamine levels in the final product.

Keywords Prahok, histamine producing bacteria, histamine, lactic acid bacteria, antimicrobial activity

INTRODUCTION

Fish is a vital source of food for millions of Cambodians, who live in a country where bodies of water abound. The Mekong River, Sap River, Bassac River, and Tonle Sap Lake, Southeast Asia's largest freshwater lake, are home to hundreds of thousands of fish species, which locals harvest and process into a variety of fermented dishes such as Prahok, Pa ork, and many others, moreover, a technique that has been around since ancient times and continues to this day (Chuon et al., 2014). However,

the majority of the production of these fermented fish is still traditionally produced by local families, where knowledge of hygiene is limited (Ly et al., 2018). Otherwise, the outbreak of foodborne disease caused by bacteria in Cambodia is not publicly reported or taken seriously by the authority, which ultimately becomes normal in people's livelihoods, obviously, this can cause serious harm to human health (Chrun et al., 2017).

One of the key chemicals that contributes to food-borne intoxication is histamine ($C_5H_9N_3$), a biogenic amine that will cause significant health effects such as diarrhea, rash, nausea, vomiting, fever, abdominal pain, and a variety of other symptoms similar to allergic reactions when consumed at high levels by humans (Shimoji et al., 2019; Surya et al., 2019). Foods with high histamine content can lead to histamine intoxication, also known as scombroid fish poisoning. Histidine is decarboxylated into this biogenic amine by bacterial decarboxylases (Madejska et al., 2022). The maximum level of histamine content should be 100 mg/L and not exceed 200 mg/L, according to trustworthy food regulators like the European Commission (regulation No. 2073/2005/EC), Australia-New Zealand Food Standards (ANZFS), the Food Safety and Standards Authority (FSSAI), and CODEX (Surya et al., 2019; CODEX.,2015). Histamine has been found in raw fish, salted or fermented fish and smoked fish, and other fish products (Kuda et.al., 2007; Madejska et.al., 2022; Moon et.al., 2013; Pawul-Gruba and Osek, 2021; Sokvibol et.al., Tao et.al., 2022).

A popular strategy to combat these bacteria recently is known as biocontrol, in which lactic acid bacteria (LAB) are used to produce amine oxidase enzymes, which are used to de-structure the biogenic amine. LAB has been widely accepted as a safe (GARS) biocontrol in food processing by many food producers due to its potency as an anti-pathogen (Lim, 2016). The main purpose of this study was to examine the potential histamine-producing bacteria (HPB) isolated from Prahok collected from three provinces in Cambodia and to evaluate the efficacy of local lactic acid bacteria (LAB) in controlling those HPB.

OBJECTIVE

The objectives of this study are to examine the potential histamine-producing bacteria isolated from Prahoks collected from three provinces of Cambodia and to evaluate the efficacy of local lactic acid bacteria (LAB) in controlling those histamine-producing bacteria (HPB).

METHODOLOGY

Sample Selection

The sampling for the analysis is collected randomly from three separate provinces' local markets such as Kampong Thom, Kampong Cham, and Siem Reap. A total of 15 samples were gathered, which were then sealed in polyethylene bags in an ice box and delivered right away to the microbiology Laboratory, Faculty of Agro-Industry, Royal University of Agriculture, Phnom Penh.

Isolation and Identification of HPB

The samples were accurately weighed out at 1.0 g, added to 10 mL of sterile TSB, supplemented with TSB (3.0%), histidine monohydrochloride (0.1%), and pyridoxal hydrochloride (0.5%), cultured for 24 h at 30 °C (Lim, 2016). The presence of histamine was demonstrated by streaking on Niven's agar (Niven et al., 1981), which was incubated at 35 °C for 24 hrs. By monitoring how well the isolates grew in Niven's medium, supplemented with Tryptone (0.5%), Yeast extract (0.5%), L-Histidine 2HCl (2.0%), NaCl (0.5%), $CaCO_3$ (0.1%), Agar (3.0%), and Bromecresol purple (0.006%), it was determined that the isolates produced histamine. A doubt colony with a purple halo surrounding it and a yellowish backdrop were found (Joosten and Northolt, 1989; Mavromatis and Quantick, 2002; Refai et al., 2020). The HPB species have been identified by using the Biolog Semi-automatic MicroStation with Microlog Software System (Model: MicroStation Brand: BIOLOG) (Al-Dhabaan and Bakhali, 2017).

Colorimetric Enzyme Assay

To measure the level of PHB, a single colony was chosen and grown in 5 ml of TSB-Histidine broth for 24h at 35°C and then the result was read by Spectrophotometer (Model: DNM-9602G, Brand: Nanjing Perlove Medical) using the formula of Histamine (Karami et al., 2021) (Eq. 1).

$$Concentration\ (ppm) = [(Es - Eb) \div (Ed - Ec) \times 40 \quad \text{Eq. 1}$$

Antimicrobial Activity Testing by Applying Well Diffusion and Co-Culture Method

Well diffusion method: Ten LAB kept in the microbiology laboratory at the Faculty of Agro-Industry, Royal University of Agriculture were cultured in 2 ml of De Man, Rogosa, and Sharpe broth (MRS broth) for 24 hrs. at 30 °C, and then separated using a centrifuge engine (Model: Z 326, Brand: HERMLE - Germany) running at 6,000 rpm for 15min. HPB is disseminated on Mueller-Hinton agar (MHA), measured at 90% equal to 10^{-8} (% transmittance) using a Biolog Turbidimeter (model: MicroStation, brand: BIOLOG), and then holes with a 6 mm diameter are made. The perforated hole was filled with 100 microliters of LAB, which was incubated for 24 h at 35±2 °C (Cotton et al., 2019; Jahangirian et al., 2013).
Co-culture method: 10% of LABs suspension 9 Log CFU/ml were inoculated with 6 Log CFU/ml of HPB in mixed broth (MRS broth + TSB) in each tube. This experiment was performed under aerobic conditions at 30 °C in the water bath for 72 hrs. Every 24 h, the mixed broths have been streaked on selective agar to observe the presence of HPB (Mellefont et al., 2008).

RESULTS AND DISCUSSION

Isolation and Identification of HPB

HPBs were investigated in Prahok from 3 provinces, Kampong Thom, Kampong Cham, and Siem Reap. After incubating at 30°C for 24 hrs., 15 homogenate Prahok samples were determined to be histamine positive. In the overall 15 Prahok samples, 8 (53%) had histamine levels from 0<100 ppm which is the level allowed by USFDA (2001), and 6 (40%) were detected with histamine 100<200 ppm had histamine levels beyond Cambodian regulation standards (CS, 2015), and 1 (7%) was the highest amount of histamine found in Prahok Trey Sandaiy purchased from Kampong Cham province which was contained 228 ppm of histamine concentration over Cambodian regulation standards >200 ppm (Table 1). Similar to Sokvibol (2022) research on the assessment of biogenic amine levels from Cambodia fermented fish products showed in the three samples of Prahok products brought from Kampot, Phnom Penh, and Kampong Chhnang had histamine levels were over the Cambodian guideline of 200 ppm, while those in 11 samples (Prahok and Teuk Trey) met the FDA's recommended level of 50 ppm (Sokvibol et al., 2022).

The identification of HPB by the Biolog GEN III Semi-automatic system analysis detected 7 species. 5 of 7 species that can produce histamine over 1000ppm were chosen to evaluate the effect of LAB on HPB (Table 2) including *Enterobacter aerogenes* (1610 ppm), *Proteus penneri* (1576 ppm), *Staphylococcus xylosus* (1420 ppm), *Morganella moganii* (1507 ppm), and Klebsiella oxytoca (1520 ppm). In contrast, 2 more species, *Enterococcus faecalis* (67 ppm) and *Citrobacter youngae* (153ppm) also generate histamine, although at levels that were beyond 200 ppm (Table 1). As research of Moon (2013) investigated the Isolation and Characterization of Histamine- Producing Bacteria in Fermented Products and found bacteria that could produce histamine such as *Morganella moganii* and *Enterobacter aerogenes* by rRNA testing. (Moon et al., 2013). According to researcher Tao (2022) studied on Prevalence of Histamine-Forming Bacteria in Two Kinds of Salted Fish at Town Markets of Guangdong Province of South China was identified six bacteria species in the samples of salted fish pickled overnight, there are *Vibrio alginolyticus, Vibrio rumoiensis* (360 to 363 mg/kg), *Staphylococcus saprophyticus* (95 to 113 mg/kg), *Staphylococcus xylosus* (65 to 96 mg/kg), *Lactococcus lactis* (3 mg/kg), and *Morganella morganii* (2,000 mg/kg) and 1 specie from

dry salted fish, *Enterobacter aerogenes* (351 to 352 mg/kg). (Tao et al., 2022).

The presence of histamine found in Prahok products may be caused by several variables, including the type of fish used, the species of fish used, the location of the fish, and producer processing techniques. However, histamine is heat stable, and if it was there before automated processing began, it can be detected in the final products (Madejska et al., 2022). As a result, it is not surprising that foodborne outbreaks are common in Cambodia because in Cambodian food, people use Prahok as an ingredient in many dishes, and it can be eaten raw by mixing it with lemon and chili. (Ly et al., 2020; Norng et al., 2011).

Table 1 Histamine level and HPB identification from prahok products in the three different provinces

Province	Type of Prahok Trey	Scientific name	Histamine level* (ppm)	Identification bacteria species	Histamine level** (ppm)
Kampong Thom	Prahok Trey Chomrus	-	144	ND	-
	Prahok Trey Chomrus	-	87	*Klebsiella oxytoca*	1520
	Prahok Trey Kampleanh	*Osphronemidae*	157	ND	-
	Prahok Trey Chdor	*Channa micropeltes*	1	*Enterococcus faecalis* *Citrobacter youngae*	- 67 153
	Prahok Trey Sanday	*Wallagonia attu*	228	*Enterobacter aerogenes*	- 1610
	Prahok Trey Riel	*Cyprinidae*	13	ND	-
Kampong Cham	Prahok Trey Chongva	*Rasbora tornieri*	157	ND	-
	Prahok Trey Lait	-	52	ND	-
	Prahok Trey Riel	*Cyprinidae*	157	ND	-
Siem Reap	Prahok Trey Kampleanh	*Osphronemidae*	47	ND	-
	Prahok Trey Kampleanh	*Osphronemidae*	124	*Staphylococcus xylosus* *Proteus penneri* *Morganella moganii*	1422 1576 1507
	Prahok Trey Kampleanh	*Osphronemidae*	18	ND	-
	Prahok Trey Ros	*Channa striata*	118	ND	-
	Prahok Trey Ros	*Channa striata*	53	ND	-
	Prahok Trey Pra	*Pangasiidae*	36	ND	-

Histamine level= Histamine level in Prahok samples Histamine,*
*Histamine level**= Histamine level produced by HPB*

Antimicrobial Activity Testing by Applying Well Diffusion and Co-culture Method

Table 2 displays the results of the inhibition zone measurements for 10 LAB against *Enterobacter aerogenes*, *Proteus penneri*, *Staphylococcus xylosus*, *Morganella moganii*, and *Klebsiella oxytoca*. These substances have significant antibacterial activity against both Gram-positive (*Staphylococcus xylosus*) and Gram-negative (*Enterobacter aerogenes*, *Proteus penneri*, *Morganella moganii*, and *Klebsiella oxytoca*) bacteria. According to Table 2, all 10 LAB showed increased antibacterial activity when compared to the five HPB, however, *Lactobacillus plantarum*, *Lactococcus lactis*, and *Lactobacillus gasseri* had greater zone values. According to the five LAB strains found in Myeolchi-jeot, the research of the Inhibitory Effect of bacteriocin-producing lactic acid bacteria against histamine-forming bacteria isolated from Myeolchi-jeot shared 98.3–100% sequence identity with the following bacteria: *Pediococcus acidilactici*, *Leuconostoc mesenteroides*, *Enterococcus faecium*, *Lactobacillus sakei*, and *Lactobacillus acidophilus* produced an antibiotic substance that inhibited the growth of HPB like *B. licheniformis*, *S. marcescens*, *S. xylosus*, *A. hydrophila*, or *M. morganii* (Lim, 2016).

Table 2 Growth inhibition of HPB by 10 LABs using well diffusion method

LAB	Time (hrs.)	HPB [Zone value (mm)]				
		Staphylococcus xylosus	*Enterobacter aerogenes*	*Proteus penneri*	*Morganella morganii*	*Klebsiella oxytoca*
Lactobacillus mali	24	0	0.0	0.0	0.0	0.0
	48	0	0.0	0.0	0.0	0.0
	72	0	0.0	10.0	10.5	0.0
Lactococcus garvieae	24	0	0.0	0.0	0.0	0.0
	48	0	0.0	0.0	12.0	0.0
	72	0	12.4	10.5	14.0	8.6
Lactobacillus plantarum	24	0	0.0	0.0	0.0	0.0
	48	0	10.5	8.6	12.0	0.0
	72	0	10.5	10.5	13.6	11.0
Lactococcus lactis	24	0	0.0	0.0	0.0	0.0
	48	0	10.6	0.0	12.4	0.0
	72	0	13.9	11.0	15.8	0.0
Lactobacillus gasseri	24	0	0.0	0.0	0.0	0.0
	48	8	0.0	0.0	12.0	0.0
	72	0	13.9	11.0	17.0	0.0
Leuconostoc gelidum	24	0	0.0	0.0	0.0	0.0
	48	0	0.0	0.0	11.5	0.0
	72	0	0.0	0.0	12.4	0.0
Tetragenococcus solitarius	24	0	0.0	0.0	0.0	0.0
	48	0	12.4	0.0	12.0	0.0
	72	0	0.0	0.0	13.0	11.0
Pediococcus parvulus	24	0	0.0	0.0	0.0	0.0
	48	0	0.0	0.0	9.6	0.0
	72	0	00.0	0.0	10.5	0.0
Lactobacillus curvatus	24	0	00.0	0.0	0.0	0.0
	48	0	00.0	0.0	10.0	0.0
	72	0	00.0	9.0	10.5	0.0
Leuconotoc citrerum	24	0	00.0	0.0	0.0	0.0
	48	0	00.0	0.0	10.0	0.0
	72	0	00.0	10.5	10.5	0.0

Table 3 Three effective LABs performed co-culture analysis to find the presence of HPB

LAB	N	Time (hrs.)	HPB				
			Staphylococcus xylosus	*Enterobacter aerogenes*	*Proteus penneri*	*Morganella morganii*	*Klebsiella oxytoca*
Lactobacillus plantarum	3	24	(-)	(-)	(-)	(-)	(-)
		48	(-)	(-)	(-)	(-)	(-)
		72	(-)	(-)	(-)	(-)	(-)
Lactococcus lactis	3	24	(-)	(-)	(-)	(-)	(-)
		48	(-)	(-)	(-)	(-)	(-)
		72	(-)	(-)	(-)	(-)	(-)
Lactobacillus gasseri	3	24	(+)	(+)	(+)	(+)	(+)
		48	(+)	(-)	(-)	(-)	(+)
		72	(-)	(-)	(-)	(-)	(-)
Mixed LAB	3	24	(-)	(+)	(+)	(+)	(+)
		48	(-)	(+)	(-)	(-)	(-)
		72	(-)	(-)	(-)	(-)	(-)

Since they produced better results in good diffusion, *Lactobacillus plantarum*, *Lactococcus lactis*, and *Lactobacillus gasseri* were chosen to examine the co-culture strategy to find HPB. According to Table 3, 2 LAB, which included *Lactobacillus plantarum* and *Lactococcus lactis*, could not be detected when co-cultured for 24 hours at 30 °C with the five HPBs. In contrast, *Lactobacillus gasseri* proved effective against *Enterobacter aerogenes*, *Proteus penneri*, and *Morganella moganii* for 48 hours at 30 °C, whereas *Proteus penneri*, *Morganella moganii*, and *Klebsiella oxytoca* were

examined for after 48 hrs and *Staphylococcus xylosus* after 24 hrs in mixed LABs.

In research of the control of tyramine and histamine accumulation by lactic acid bacteria using bacteriocin-forming lactococci showed that *L. lactis subsp. lactis* produced nisin Z induced the death of the histamine-producing strain *S. thermophilus*. However, *L. lactis subsp. lactis* EG46- produced lacticin 481 was able to reduce its growth extent and histamine accumulation (Tabanelli et al., 2014)

CONCLUSION

This study showed that the seven different bacterial species, including *Enterobacter aerogenes*, *Proteus penneri*, *Staphylococcus xylosus*, *Morganella moganii*, *Klebsiella oxytoca*, *Citrobacter freundii*, and *Citrobacter youngae*, were found in Prahok products, according to this study. Five of these seven are known histamine makers since samples show levels of histamine that are higher than the 200ppm threshold set by Cambodian regulations. Therefore, Prahok producers must follow the regulatory limitations on histamine that have been defined by international and national standards to maintain adequate hygiene procedures to prevent cross-contamination and proper handling methods to control the bacterial development dependent on the fermented fish products. Antibacterial activity of *Lactobacillus plantarum*, *Lactococcus lactis*, and *Lactobacillus gasseri* was stronger than other 7 LAB by using well diffusion method to evaluate the effeteness of grate zone values, whereas in co-culture the antibacterial activity of *Lactobacillus plantarum* and *Lactococcus lactis* were stronger than *Lactobacillus gasseri* in inhibited *Enterobacter aerogenes*, *Proteus penneri*, *Staphylococcus xylosus*, *Morganella moganii*, *Klebsiella oxytoca*, *Citrobacter freundii*, and *Citrobacter youngae*. Therefore, *Lactobacillus plantarum* and *Lactococcus lactis* can be used as potential antibacterial agents in Prahok products. In a study performed in Sweden, bacteria on samples were reduced by cleaning with water (Uhlig et al., 2017). Therefore, to rid the Prahok product of bacteria and histamine, the prahok manufacturer should clean the fish multiple times with water.

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