Research article

Assessment of Antibacterial Activity of Lactic Acid Bacteria Isolated from Fermented Foods against *Escherichia coli* O157:H7 and *Proteus penneri* and Their Potential as Starter Cultures

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Abstract Fermented fish and vegetables produced by spontaneous fermentation are the most well-known traditional foods in Cambodia. Even though fermented foods provide health benefits related to nutrition, probiotics, and postbiotics, some of them are not safe due to the survival of bacteria harmful to humans. The purpose of this research is to examine *in vitro* the antibacterial activity of lactic acid bacteria (LAB) isolated from fermented fish and vegetables against *E. coli* O157:H7 and *Proteus penneri*, histamine forming LAB, and the survival of LAB in low pH levels and high salt concentration using pH and salt tolerance tests. LAB was isolated from 134 naturally fermented fish and vegetables from four different provinces and identified using the Biolog GEN III MicroStation semi-automated system. The antagonistic properties of these isolates against *E. coli* O157:H7, and *Proteus penneri* were examined using a co-culture method at 24, 48, and 72 hours of incubation times. As a result, 36 strains of LAB were identified from a total of 134 samples. Seven LAB species out of 36 strains can survive at pH 3 for 6 hours, while four of these seven LAB can tolerate pH 2. *Lactobacillus plantarum, Lactococcus lactis, Lactobacillus gasseri, Leuconostoc lactis,* and *Leuconostoc gilidum* have a significant capacity to inhibit *E. coli* O157:H7 and *Proteus penneri* growth when compared to control. These five LAB do not produce histamine and can tolerate up to a 10% salt concentration. All the results demonstrate that *Lactobacillus plantarum, Lactobacillus lactis, Lactobacillus gasseri, Leuconostoc lactis,* and *Leuconostoc gilidum* have great potential for use as starter cultures for suppressing pathogenic bacteria growth in fermented fish and vegetables.

Keywords fermented fish and vegetables, *E. coli* 0157:H7, lactic acid bacteria, antimicrobial activity, pH and NaCl tolerance

INTRODUCTION

The nutritional balance and food security of traditional fermented foods have made them popular throughout Asia. Across several Asian nations, methods for preserving crops, vegetables, meat, and fishery products seem to be well-developed. Moreover, Food preservation was the prime purpose of

fermentation, which was achieved by the synthesis of inhibitory metabolites including organic acid, ethanol, and antimicrobials combined with decreased water activity (Anal, 2019).

Cambodia is a kingdom in Southeast Asia that borders Vietnam, Laos, and Thailand. There are many varieties of fresh fish and vegetables caused by the Tonle Sap Lake in the north of Cambodia is recognized for its extensive river system and favorable climate for agriculture(LeGrand et al., 2020). Mostly in the traditional Cambodian diet, fermented foods play a significant role. There are different fermented foods ready to eat (Chrun et al., 2017). It is well known that five groups of fermented fish and vegetables including *Pra hok* (Fish paste), *Pha'ork* (Fermented fish), *Trey proheum* (Salt Fish), *Mam* (Fermented Fish), and *Chruk* (Fermented vegetable) (LeGrand et al., 2020) and have their benefit in term nutritional function such as vitamins, proteins, essential fatty acids, and amino acids (Nuraida, 2015). In addition, at the same time add value to agricultural products (Chrun et al., 2017).

Microbiological research on Asian fermented foods reveals that the identification of 68 samples of fermented vegetables from a local market in Cambodia contained *Enterobacter spp*. in 24% of the samples (Chrun et al., 2017). Another study that examined 13 samples of fermented fish in Cambodia discovered that the majority of the microbial communities were gram-positive cocci and rods such as Bacillus, Clostridium, Staphylococcus, and Tetragenococcus (Chuon et al., 2014). In the case of Vietnam was found that unprocessed fish and poultry are likely to be contaminated with *Salmonella* and in the absence of proper kitchen hygiene and may contaminate processed foods. Raw poultry samples were found high levels of contamination with *E. coli* (45%), *Campylobacter jejuni* (28.3%), and *Salmonella* (8.3%) and high-risk food classification. Additionally, raw fish, meat, and vegetables all contained *E. coli* at rates of 21.3%, 6.6%, and 18.5%, respectively. This article confirmed the importance of hygienic working practices when preparing food. (Thi et al., 2006). The purpose of this research is to examine in vitro the antibacterial activity of lactic acid bacteria (LAB) isolated from fermented fish and vegetables against *E. coli* O157:H7 and Proteus penneri, histamine forming LAB and and the survival of LAB in low pH level and high salt concentration using pH and salt tolerance tests.

OBJECTIVE

The objective of this research is to examine in vitro the antibacterial activity of lactic acid bacteria (LAB) isolated from fermented fish and vegetables against *E. coli* O157:H7 and *Proteus penneri*, histamine forming LAB and the survival of LAB in low pH levels and high salt concentration using pH and salt tolerance tests.

METHODOLOGY

Sample Collection Methods

Fermented fish and vegetable samples are divided into five groups such as *Pra hok* (Fish paste), *Pha'ork* (Fermented fish), *Mam* (Fermented fish), Salted fish (*trey proheum*) and *Chruk* (Fermented vegetable). All samples were collected from 4 provinces such as Kompong Thom, Siem Reap, Kandal, and Kompong Cham province. All samples are purchased from small-scale producers and local markets. There are 134 samples in total. For fish paste (*Pra hok*, n=17), Fermented fish (*Pha'ork,* n=30), Fermented fish (*Mam*, n=11), and Fermented vegetables (*Chruk*, n=76). All samples were sealed in plastic sample bags and stored in cool boxes during transportation to the laboratory which was less than 24 hours. After the arrival, all samples were immediately studied for their identification.

Sample Identification

Pure colonies were conducted with several tests before using Biolog System (Semi-Auto Microstation) for identification. These tests helped to avoid misidentification and save a lot of money since the Biolog System is costly. Most important tests such as Gram staining, Catalase, and Oxidase

are alternatives yet also help to give information on bacterial characteristics. The suspected pure LAB were sub-cultured two times to ensure the purification of the colony on MRS agar before culture on BUG Agar before identification with the Biolog system. By using GENIII-Microplate (BIOLOG) within protocol-C, inoculate pure colony from BUG Agar into Inoculating Fluid-C (IF-C) (provided by BIOLOG) within 92 to 95% Turbidity by using a Turbidity Meter (BIOLOG). Using an electric multi-dispense micropipette (SARTORIOUS), dispense 100 microliters in each microplate well then incubate the plate at 30^{o}c for 20 to 24 hours and using Microstation (BIOLOG) accompany with Microlog Software (BIOLOG) for interpreting the ID result of identification(Al-Dhabaan & Bakhali, 2017).

Antimicrobial Activities by Co-culture Method

10% of LAB suspension (9 Log CFU/ml) was inoculated with 6 Log CFU/ml of indicator *E. col*i 0157:H7 and *Proteus penneri* in each tube of MRS broth. This experiment was performed under aerobic conditions at 30 ^{0}C in the water bath for 72 hours. Every 24 hours, the presence of indicators was determined by streaking on selective Agar and incubating at 35^{0}C (Balouiri et al., 2016).

pH and Sodium Chloride Tolerance

LAB against *E. coli* 0157:H7 and *Proteus penneri* by co-culture method have been conducted with pH and sodium chloride tolerance. Selected LAB isolates were grown in MRS broth at 30°C overnight before being sub-cultured in new MRS broth by using HCl (0.1M) adjusted pH to 2 and 3 and used to adjust the salt content in MRS broth (5%, 6%, 7%, 8%, 9%, and 10%). Following an incubation period of 24 hours at 30°C (Dimic et al., 2015). After this, Streak Method on MRS Agar was used to confirm the tolerance of each LAB (Balouiri et al., 2016).

Determine the Concentration of Histamine Produced by Lactic Acid Bacteria

LAB was refreshed on MRS Agar at 30 ^{0}C for 24 hours. Inoculate one loop full colony of both Lactic acid bacteria in TSB broth with histidine and incubate at 30 °C for 24 hours centrifuge 6000 rpm/min for 15 min. Suspend 1 ml of supernatant in 9 ml of sterile saline for dilution 10-fold. After dilution that supernatant was used in the enzymatic histamine assay (Leszczyocha and Pytasz, 2018). Histamine concentration was calculated as follows.

$$\text{Histamine concentration (mg/L = ppm)} = (Es - Eb) \div (Estd - Ec) \times 4 \times 25 \times \text{dilution factor}$$

RESULTS AND DISCUSSION

The LAB species isolates were examined based on selective agar media called MRS and sub-cultured for few times until obtained a pure culture. The later suspected colony was Gram-positive, clustered cocci or bacilli, catalase-positive, and oxidase positive. LAB was later identified by using Biolog semi-auto system as shown in Fig. 1. Of 134 samples, 98 samples were rejected due to the absence of a suspected colony. group of *Mam* (n=11) isolated as *L. lactis* and *P. parvulus* (27%). There are 12% of group *Prahok* (n=17) isolated as *P. parvulus* and *P. pentosacues*. Other groups of *Pha'ork* (n=30) detected 10% were *L. garvieae, P. parvulus,* and *P. pentosaceus*. And 37% of group *Chruk* (n=76) isolated as *Lactobacillus, P. parvulus, P. pentosaceus, L. alimentarius, L. mali, L. gasseri, P. acidilactici, L. acidophilusBGA, L. lactis, P. sanguinis, Leu. gelidum, Tetra. solitarius, L. garvieae, L. reuteris, L. plantarum, Leu. citrem, L. bifermentans,* and *L. salivarius.* We found the relevance of the identification LAB by the API 50 CHL system from fermented food in Cambodia, thus three species of lactic acid bacteria were found, including *Lactobacillus acidophilus* and *Lactobacillus plantarum*. strains Y'11b,2, Y'11e,2, and Y'85,1. (Sophakphokea1 et al., 2021). According to (Ly et al., 2022) to study dentification, Classification and Screening for γ-Amino-butyric Acid Production in Lactic Acid Bacteria from Cambodian Fermented Foods by using Matrix-assisted laser

desorption/ionizing time-of-flight mass spectrometry (MALDI-TOF MS) and partial 16S rDNA sequencing were used to identify 68 LAB. And the result shows as one *Lactobacillus futsaii*, two *Lactobacillus namurensis*, and *three Lactobacillus plantarum* strains were found in fermented food in Cambodia.

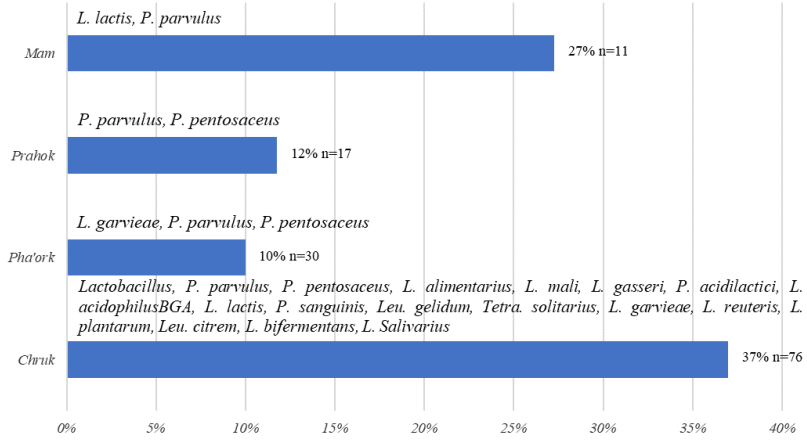

Fig. 1 Isolated LAB from fermented fish and vegetables in Cambodia

Table 1 Result of antimicrobial activities by co-culture method

Species	n	Time	*E. coli* 0157:H7	*Proteus penneri*
Leuconostoc lactis	3	24 h	+++	+++
		48 h	++	+
		72 h	+	-
Lactobacillus gasseri	3	24 h	++	+++
		48 h	-	+
		72 h	-	-
Lactobacillus plantarum	3	24 h	+++	++
		48 h	++	+
		72 h	+	-
Lactococcus lactis	3	24 h	++	+
		48 h	-	-
		72 h	-	-
Leuconostoc gelidum	3	24 h	+++	+++
		48 h	+	++
		72 h	+	-
Lactococcus garvieae	3	24 h	++	++
		48 h	+	+
		72 h	+	-
Lactobacillus mali	3	24 h	+++	+++
		48 h	++	+
		72 h	++	-

"+" represent to positive or resistance and "-" represent to negative or susceptible

According to the results shown in Table 1. Lactic acid bacteria are able to against *E. coli* 0157:H7 such as *Lactobacillus gasseri* and *Lactococcus lactis*. Moreover, Lactic acid bacteria able to against *Proteus penneri* were *Lactobacillus mali, Lactococcus garvieae, Leuconostoc gelidum, Lactococcus lactis, Lactobacillus plantarum,* and *Leuconostoc lactis*. Hassanzadazar et al. (2012) studied about antibacterial activity of Lactic Acid Bacteria against *Escherichia coli*, *Listeria monocytogenesis*, *Bacillus cereus*, and *Salmonella enteritidis*, and reported that lactic acid showed the strongest inhibitory activity against gram-positive bacteria than gram-negative bacteria. Similarly, Muzikowski (2009) also reported some species of *Lactobacilli* against different gram-positive and gram-negative bacteria were determined by agar-well diffusion assay. It also shows the antagonistic effect of the antibacterial agent on the growth of other gram-positive and gram-negative pathogenic

microorganisms which *E. coli, Listeria monocytogenes, Salmonella enterica, Staphylococcus aureus* and *Bacillus cereus*.

The pH tolerance is an important characteristic of species that hoped to affect the gastrointestinal tract. Moreover, tolerance to salt was an important cause of cell membrane structure can become damaged by salt. A study by Azam et al. (2017) focused on the isolation and characterization of *Lactobacillus spp.* from kefir samples in Malaysia mentions that *Lactobacillus spp.* isolated from kefir E was unable to survive in all pH conditions (pH 2, 3, and 4). All isolates tested did not survive pH 2.0, most of the isolated *Lactobacillus spp.* from kefir samples were able to tolerate the moderate pH levels of pH 3.0, pH 4.0, and all the isolated *Lactobacillus spp*. from kefir samples survived at 0.3-0.5% bile concentration after incubation. The *Lactobacillus spp*. from kefir H conferred the highest survival rate at 0.3% and 0.5% bile concentration, with a survival rate of 96.89 ± 0.02% and 96.84 ± 0.02%, respectively. Subsequently, the survival rate in bile salt condition was followed by isolated *Lactobacillus spp*. from kefir G and kefir C. (Hassanzadazar et al., 2012) research was done into the Investigation of antibacterial, acid, and bile tolerance properties of lactobacilli isolated from Koozeh cheese. The screening process involved 28 different *Lactobacillus* species that were obtained from Koozeh cheese, a typical cheese. A control pH of 7.5 was used while the acid tolerance test was investigated at pH 2.0 and 3.0 with 5 N HCl. Results showed that only one out of twenty-eight isolates have the ability to tolerate acid and bile salts. The ability to produce histamine of LAB was acceptable due to the concentration of histamine higher than 200 ppm will cause the disease in a human (Leszczyocha and Pytasz, 2018, Food Safety Authority of Ireland, 2005).

Table 2 Result of salt, pH tolerance test, and Histamine of identified bacteria from fermented samples

Bacteria species	pH and salt tolerance								Histamine concentration
	Salt (%)						pH		
	5	6	7	8	9	10	2	3	ppm
Lactococcus garvieae	+	+	+	+	+	+	0%	14%	2.35
Lactobacillus mali	+	+	+	+	+	+	21%	50%	4.70
Leuconostoc lactis	+	+	+	+	+	+	10%	13%	6.79
Lactobacillus gasseri	+	+	+	+	+	+	12%	18%	7.05
Lactobacillus plantarum	+	+	+	+	+	+	32%	52%	7.8
Lactococcus lactis	+	+	+	+	+	+	26%	44%	10.1
Leuconostoc gelidum	+	+	+	+	+	+	0%	20%	3.9

"+" represent to positive or resistance and "-" represent to negative or susceptible

CONCLUSION

Seven Lactic acid bacteria out of 36 species, such as *Lactobacillus mali, Lactococcus garvieae, Leuconostoc gelidum, Lactococcus lactis, Lactobacillus plantarum,* and *Leuconostoc lactis* were isolated and identification by using Biolog System (Semi-Auto Microstation) were found from fermented fish and vegetables. according to this study two species of LAB inhibition against *E. coli* 0157:H7 and all these seven species inhibitions against Proteus *penneri*. The identified bacteria isolated were subjected to different salt concentrations (5%, 6%, 7%, 8%, 9%, and 10%). Bacteria species *Lactococcus garvieae, Lactobacillus mali, Leuconostoc lactis, Lactobacillus plantarum, Lactococcus lactis, Leuconostoc gelidum* can tolerate with salt up to 10% and tolerate either pH3 in rate (14%, 50%, 13%, 18%, 52%, 44%, and 20%) nor pH2 in rate (21%, 10%, 12%, 32%, and 26%). In contrast, *Lactococcus garvieae* and *Leuconostoc gelidum* weren't tolerated with pH2. On the other hand, these seven species LAB were not histamine-producing bacteria due to the level of histamine concentration lower than 10 ppm. All the results demonstrate that *Lactobacillus plantarum, Lactococus lactis, Lactobacillus gasseri, Leuconostoc lactis,* and *Leuconostoc gelidum* have a great potential for use as starter cultures for suppressing pathogenic bacteria growth in fermented food.

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