Antimicrobial and Probiotic Properties of Lactic Acid Bacteria Isolated From *Pha-Ork*, A Traditional Fermented Pangasius Fish of Cambodia

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ABSTRACT

Cambodia's traditional fermented Pangasius fish paste, locally known as Pha-ork, is a central component of aquatic food systems. Fermentation processing preserves fish, enhances flavor and texture, and introduces beneficial microorganisms with high potential for food and health applications. This study isolated 15 lactic acid bacteria (LAB) from 153 samples of the fermented fish product from Kampong Thom, Siem Reap and Phnom Penh markets, comprising 5 species - *Lactobacillus pentosus* (4 isolates), *Enterococcus faecalis* (5 isolates), *Enterococcus hirae* (2 isolates), *Lactococcus garviae* (2 isolates), and *Pediococcus pentosaceus* (2 isolates). Most of these isolates exhibited moderate antibacterial activity against *Aeromonas* and *Vibrio* species isolated from fresh Pangasius fish from the same markets. Among the LAB isolates, only one isolate of *Pediococcus pentosaceus* stood out as a strong candidate for probiotic use due to its tolerance to low pH (2.3), bile salts, and phenol. These findings have important implications in developing LAB-based antimicrobial strategies to reduce reliance on antibiotics use and promote traditional fermentation as a sustainable and ecological alternative to achieve food, nutrition and livelihood security.

Keyword: Aquatic Food System, Fermentation, Natural Antimicrobial, Food Preservation, Food Safety

INTRODUCTION

Cambodia's traditional, fermented fish is a pillar of food, nutrition, and livelihood security. It is the most important staple food after rice; serves as a key source of protein and essential nutrients; and sustains rural processing economies (Shern, 2023). It is a high-value product priced at 9-16 times higher than fresh fish; absorbs surplus harvests; and reduces food loss by repurposing fish that would otherwise be unsold and wasted. As an embedded staple in Khmer cuisine, fermented fish is a central component of Cambodia's aquatic food systems, recognized as vital for national food security and heavily prioritized in the National Strategy for Food Security and Nutrition (NSFSN 2024–2028).

Fermentation processing of fish offers significant economic and nutritional benefits, including improved value-added for agriculture, diversification of socioeconomic growth, and enhanced nutritional, organoleptic and probiotic functions (Rastogi et al., 2022). Fermentation preserves fish, enhances flavor and texture, and introduces beneficial microorganisms and bioactive compounds with potential health benefits (Chrun et al.,

2020; Khongthaw et al., 2026). Fermented foods could contain beneficial Lactic Acid Bacteria (LAB), which are generally recognized as safe (GRAS), with some strains considered probiotic (Nuraida, 2015). Probiotics are live microorganisms that, when consumed in adequate amounts, provide a health benefit to the host. LAB promotes human health by improving gastrointestinal health, boosting the immune system, aiding digestion and nutrient absorption, and reducing the risk of certain diseases (Ayivi et al., 2020). LAB are known for their ability to inhibit the growth of various pathogens (LeGrand et al., 2020). However, traditional fish fermentation relies on spontaneous microflora, which can lead to unpredictable quality, spoilage, or health hazards. Some species of LAB (e.g. *Streptococcus*, *Lactococcus*, and *Enterococcus*) are pathogenic to humans and animals. By isolating and characterizing LAB, a traditional preservation method can be transformed into a controlled, safe, and highly nutritious process. Characterizing LAB in fermented fish products ensures food safety and preservation as it identifies strains that produce compounds (e.g. lactic acid, hydrogen peroxide, and bacteriocins) that quickly decrease pH to inhibit deadly foodborne pathogens like *Vibrio* spp. and *Aeromonas* spp. which are usually associated with aquatic environments (Duk et al., 2024). It also unlocks LAB strains with probiotic traits, such as resistance to harsh stomach acids and bile salts for survival during human digestion. A thorough understanding of LAB is crucial to maximize their technological, nutritional, and health applications while avoiding the potential risks (Lars et al., 2024).

MATERIALS AND METHODS

Sample collection

Samples of Khmer traditional fermented Pangasius fish (Figure 1) were collected from three markets in each of the three target areas: (1) Kampong Thom Province (KPT) - Phsar Kampong Thom (PKPT), Phsar Stoung (PST) and Phsar Romlong (PRL) markets; Siem Reap Province (SRP) - Phsar Sot Nikum (PSN), Phsar Prasat Bakong (PPB) and Phsar Leu Thom Tmey (PLTT) markets; and (3) Phnom Penh (PP) - Phsar Prek Pnov (PPP), Chamkar Doung (PCD) and Phsar Deum Kor (PDK) markets. From each market, 17 samples were collected, giving a total of 153 samples. The samples were taken from different retailers in each market and immediately brought to the Microbiology Lab of the Faculty of Agro-Industry, Royal University of Agriculture, Phnom Penh.

Figure 1. Khmer fermented Pangasius fish (taken by Mr. Duk Seyha)

As an indication of the presence and activity of LAB, pH of the samples was measured using a digital pH meter. Samples from Kampong Thom markets had a pH of 3.4-4.9; Siem Reap markets, 3.8-5.1; and Phnom Penh markets, 3.7-4.7.

Isolation and identification of LAB

LAB were isolated and identified following the method of Haitham et al (2017) (Figure 2). From each sample, 10 g was added to 90 ml of 1% peptone and sterile saline (0.9% NaCl) and then homogenized for 1 min. Serial dilutions (10^{-1}– 10^{-5}) were prepared. Afterward, 1 ml of each dilution was inoculated to de Man, Rogosa, and Sharpe (MRS) broth (Merck-Darmstadt, Germany) while 0.1 ml of each dilution was spread on MRS agar, both incubated anaerobically at 30°C for 24-48 hr. Gram staining and catalase test were performed to confirm the isolates were Gram positive and catalase negative, the typical characteristics of LAB. After this, bacterial cells

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were extracted by the ethanol formic acid extraction method and a matrix solution was prepared for deposit in the MALDI target plate for MALDI-TOF (Matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry analysis (Neelja, et al., 2015). The mass spectrum was compared with the reference database (Bruker Daltonics MALDI BioTyper database) for identification of LAB. The results were confirmed by API 50CH and API 50 CHL. API 50 CHL is a test kit for the identification of Lactobacillus and related genera and is used together with API 50 CH strip to identify bacteria based on carbohydrate fermentation profiles.

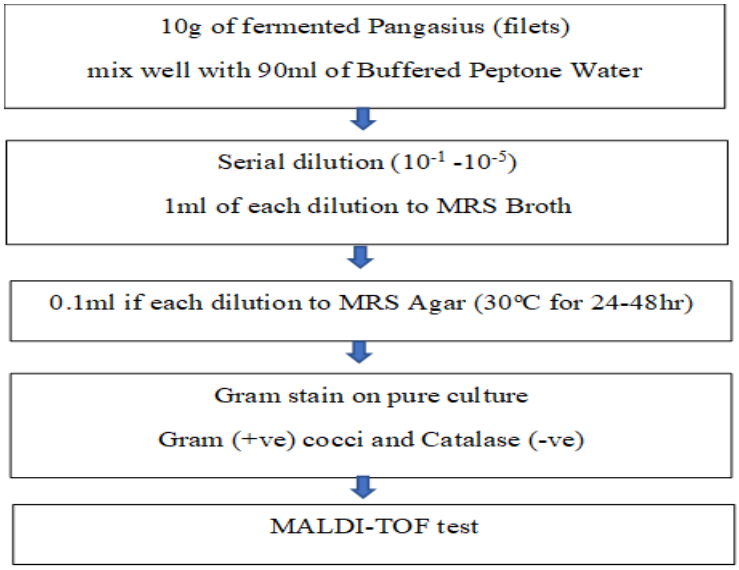

Figure 2. Isolation and identification of LAB (Haitham et al., 2017).

Antibacterial activity of LAB isolates

The LAB isolates were evaluated for antibacterial activity against *Aeromonas* and *Vibrio* species as indicator microorganisms which were isolated from fresh Pangsius fish in a preceding study (Duk et al., 2024). The agar well diffusion method was followed (Figures 3) (Pratiksha, 2023). The indicator microorganisms were inoculated onto 16 ml of the Mueller Hinton Broth (MHB) medium at a final concentration of 10^5 CFU ml/L. After homogenizing, the agar was poured into petri dishes (90mm diameter); and after 30 min, 6mm-diameter wells were prepared with sterile metal cylinder. 100 µl of the cell-free supernatant of LAB isolate was loaded in the well at 4°C for 1 h. After which, the plates were incubated at 37°C for 24 h. The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the wells. Based on the zone of inhibition diameter, antimicrobial activity of LAB was classified as weak = <5 mm, moderate = 5-10 mm, strong = 11-20 mm, and very strong = >20 mm (Sanam et al., 2022).

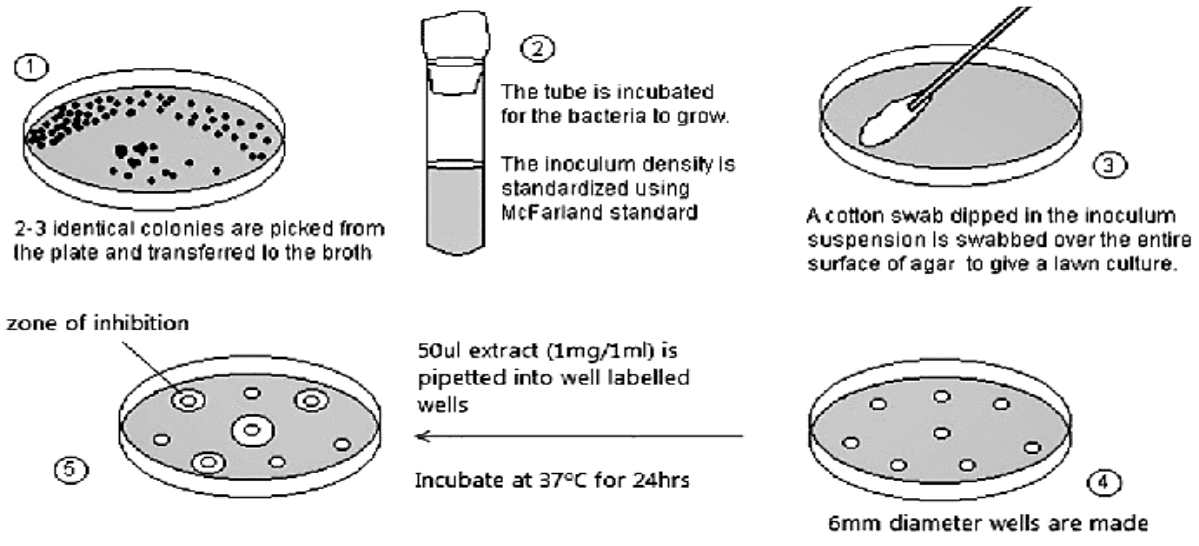

Figure 3. Antimicrobial screening by the agar well diffusion method (Pratiksha, 2023).

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Probiotic properties of LAB isolates

Probiotic potential of the LAB isolates from Khmer traditional fermented Pangasius fish was evaluated based on bile salt, acid and phenol tolerance tests. LAB isolates were grown in MRS broth, a specialized culture medium designed for the growth and isolation of Lactobacillus and other LAB. Acid tolerance test was performed following the method of Charteris et al (1998); bile tolerance test by the method of Matijasic and Rogelj (2000); and phenol test by the method of Aswathy et al (2008).

Statistical analysis

Descriptive statistics was employed by taking the mean as a measure of central tendency. In addition, boxplots for antibacterial activity of LAB were created to highlight the differences in central tendency (mean) and variability (standard deviation of the mean) (Field, 2018).

RESULTS AND DISCUSSION

LAB isolates

LAB were isolated from 15 of the 153 samples (9.80%), comprising 5 species, namely; *Lactobacillus pentosus* (4 isolates, 2.61%), *Enterococcus faecalis* (5 isolates, 3.26%), *Enterococcus hirae* (2 isolates, 1.30%), *Lactococcus garviae* (2 isolates, 1.30%), and *Pediococcus pentosaceus (2 isolates,* 1.30%) (Table 1). The Bruker database confirmed the identify of these LAB isolates.

Table 1. LAB species in Khmer traditional fermented Pangasius fish from different locations as identified by MALDI-TOF mass spectrometry and Bruker database.

No.	Sample code	Lab species	Location[1]
1	204018-2	*Lactobacillus pentosus*	PKPT- KPT
2	204018-3	*Lactobacillus pentosus*	PKPT-KPT
3	204018-4	*Lactobacillus pentosus*	PKPT-KPT
4	204018-5	*Enterococcus hirae*	PST- KPT
5	204018-6	*Enterococcus hirae*	PST- KPT
6	204018-7	*Enterococcus faecalis*	PRL- KPT
7	204018-8	*Lactobacillus pentosus*	PRL- KPT
8	204011-9	*Enterococcus faecalis*	PLTT-SRP
9	204020-1	*Lactococcus garviae*	PPP-PP
10	204020-2	*Lactococcus garviae*	PPP-PP
11	204020-5	*Enterococcus faecalis*	PCD-PP
12	204020-7	*Enterococcus faecalis*	PCD-PP
13	204020-8	*Enterococcus faecalis*	PCD-PP
14	204020-9	*Pediococcus pentosaceus*	PDK-PP
15	204020-10	*Pediococcus pentosaceus*	PDK-PP

[1]*see target areas (province and market) enumerated under 'Sample collection'.*

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Fermentation of traditional fish products is done using spontaneous fermentation under anaerobic condition in which microorganisms, specifically LAB, already present in the raw materials become the normal flora at the time of fermentation. Studies have demonstrated that LAB are part of the normal microbiota in fresh fish. Nair and Surendran (2005) found LAB in fresh fish, including *Streptococcus spp, Leuconostoc spp, Pediococcus spp.* and *Lactobacillus spp.*, with *L. plantarum* as the most predominant species. Quach et al (2024) isolated LAB (*Lactobacillus* and P*ediococcus*) from the intestine of fresh Pangasius fish. Earlier, the same LAB species were isolated from catfish, red tilapia and tilapia (Quach et al., 2023). During fermentation, the production of lactic acid from LAB decreases the pH of the product thereby decreasing the number of other microorganisms. The typical acid-forming LAB rapidly increase in number, becoming the predominant microorganisms after fermentation has started and reach maximum density at the end of fermentation.

The type of LAB in fermented fish may vary due to differences in products and production processes, duration and stages of fermentation, and environmental conditions, which in turn differ with geographic location. This is evidenced by the results of the present study in which the type of LAB differed with location and market. In earlier studies, common LAB species isolated from fermented Pangasius fish included *Lactobacillus plantarum, L. pentosus, Pediococcus spp., Lactococcus spp., Enterococcus spp., Streptococcus spp.* and *Leuconostoc spp.* (Ohshima and Giri, 2014). In the Indonesian fermented freshwater fish (including Pangasius) called Bekasam, 62 isolates out of the total 74 isolates belong to LAB (Desniar et al., 2013). LAB included *Lactobacillus spp., Aerococcus spp., Streptococcus spp., Pediococcus spp., Staphylococcus spp., Erysipelothrix,* and *Gemella* (Setiarto et al., 2024). In another Indonesian fermented fish product called Rusip, Yuliana et al (2018) found *Streptococcus* as more common in early fermentation, *Lactococcus* as more common in mid-fermentation, and *Leuconostoc* at the end of fermentation. In Thailand's fermented fish product called Plaa-som, the predominant LAB species included *Lactococcus garvieae, Weissellacibaria, Pediococcus pentosaceus, Streptococcus bovis, L. plantarum*, and *L. fermentum* (Kopermsub and Yunchalard, 2010). In Malaysian fermented freshwater fish product called Pekasam, LAB isolates were mainly *L. plantarum* and *L. pentosus* (Muryany et al., 2017). Some of these LAB species were obtained in the present study.

Antimicrobial activity of LAB isolates

The 15 isolates of 5 LAB species (4 *Lactobacillus pentosus*; 5 *Enterococcus faecalis*; 2 each for *Enterococcus hirae, Lactococcus garviae* and *Pediococcus pentosaceus*) were tested against *Vibrio* and *Aeromonas* species isolated from fresh Pangasius fish from markets in Kampong Thom (3 *Vibrio* and 4 *Aeromonas* species), Siem Reap (3 *Vibrio* and 2 *Aeromonas* species), and Phnom Penh (2 *Vibrio* and 1 *Aeromonas* species); in contrast, fermented Pangasius fish had no *Aeromonas* and *Vibrio* species detected (Duk et al., 2024).

Against the *Vibrio* and *Aeromonas* species isolated from Kampong Thom markets, all LAB isolates had moderate antibacterial activity (5-10 mm diameter of zone of inhibition), except for the following: (a) one *Lactobacillus pentosus* isolate (code 204018-8) from PRL-KPT was ineffective against *V. parahaemolyticus* and the 4 *Aeromonas* species; (b) one *Enterococcus faecalis* isolate (code 204011-9) from PLTT-SRP was antagonistic only to *V. cholerae*; (c) 2 *Enterococcus faecalis* isolates (code 204020-7 and 204020-8) from PCD-PP were not effective against *V. cholerae*, and had weak antibacterial activity (<5 mm diameter of zone of inhibition) against *A. hydrophila* similar to one *Pediococcus pentosaceus* isolate (code 204020-10) from PDK-PP; (d) the 2 isolates of *Lactococcus garviae* (204020-1 and 204020-2) from PCD-PP) were effective only against *V. cholerae*; and (e) one isolate of *Pediococcus pentosaceus* (code 204020-10) from PDK-PP had no antibacterial activity against *V. parahaemolyticus* (Table 2). Boxplot shows the moderate antibacterial activity within the range of 7-10 mm diameter of the zone of inhibition of the 5 LAB species against the *Vibrio* and *Aeromonas* species (Figure 4).

Table 2. Antibacterial activity of 15 LAB isolates against *Aeromonas* and *Vibrio* species isolated from Kampong Thom markets.

Sample code	LAB isolate	Average diameter of zone of inhibition (mm)						
		V. cholerae	*V. fluviales*	*V. parahaemolyticus*	*A. hydrophila/ caviae*	*A. caviae*	*A. sobria*	*A. hydrophila*
204018-2	*Lactobacillus pentosus*	9.5	9.0	9.0	8.0	8/0	8.0	8.0

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204018-3	*Lactobacillus pentosus*	9.5	8.0	8.5	9.0	8.0	8.0	8.0
204018-4	*Lactobacillus pentosus*	9.0	8.0	8.0	8.0	8.0	8.0	8.0
204018-8	*Lactobacillus pentosus*	8.0	7.0	-	-	-	-	-
204018-7	*Enterococcus faecalis*	8.0	8.0	8.0	8.0	8.0	8.0	7.0
204011-9	*Enterococcus faecalis*	7.0	-	-	-	-	-	-
204020-5	*Enterococcus faecalis*	8.0	8.0	8.0	8.0	8.0	8.0	7.0
204020-7	*Enterococcus faecalis*	-	7.0	7.0	7.0	7.0	7.0	3.5
204020-8	*Enterococcus faecalis*	-	7.0	7.0	7.0	7.0	7.0	3.5
204018-5	*Enterococcus hirae*	9.5	7.0	7.0	8.0	8.0	8.0	8.0
204018-6	*Enterococcus hirae*	8.5	8.5	8.5	7.5	7.5	7.0	7.0
204020-1	*Lactococcus garviae*	7.5	-	-	-	-	-	-
204020-2	*Lactococcus garviae*	7.0	-	-	-	-	-	-
204020-9	*Pediococcus pentosaceus*	7.0	7.0	7.0	7.0	7.0	8.0	7.0
204020-10	*Pediococcus pentosaceus*	7.0	7.0	-	7.0	7.0	7.0	**3.5**

Antimicrobial activity based on inhibition zone diameter: weak = <5 mm; moderate = 5-10 mm; strong = 11-20 mm; very strong = >20 mm

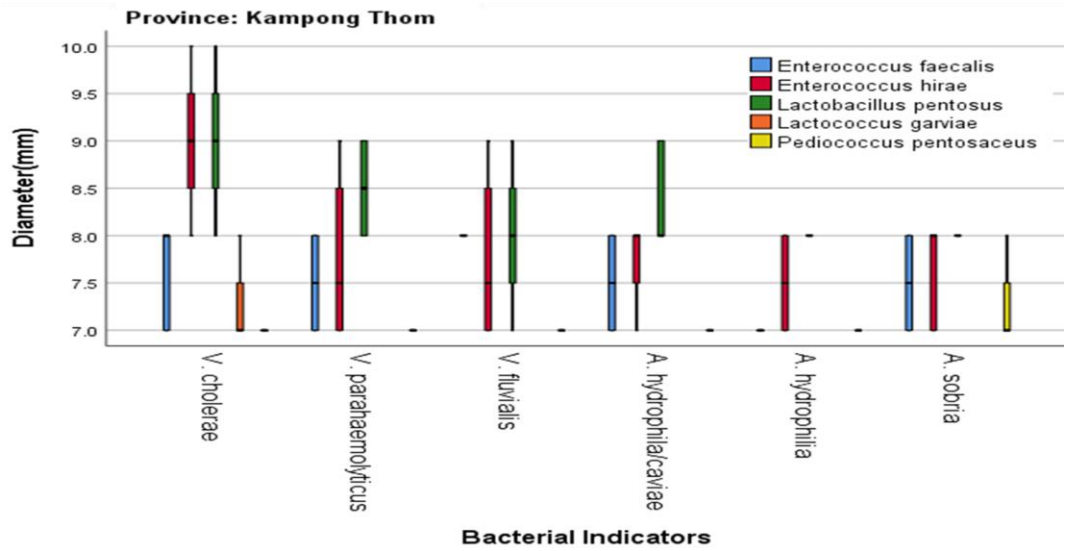

Figure 4. Boxplot of antibacterial activity of 5 LAB species against *Aeromonas* and *Vibrio* species isolated from Kampong Thom markets.

Similarly, all LAB isolates had moderate antibacterial activity against the *Vibrio* and *Aeromonas* species isolated from Siem Reap markets, except for the strong antibacterial activity (11-20 mm diameter of zone of inhibition) of one isolate of *Lactobacillus pentosus* (code 204018-2) from PKPT- KPT against *A. hydrophila/caviae* and *A. hydrophila*, and one isolate of *Enterococcus faecalis* (code 204018-7) from PRL- KPT and *Lactococcus garviae* (code 204020-1) from PPP-PP against *V. aglinoluticus* (Table 3). In addition, one isolate of *Lactococcus garviae* (code204020-2) from PPP-PP had no antibacterial activity against *V. cholerae* and *V. parahaemolyticus.* Boxplot clearly shows the strong antibacterial activity (ranging from 12-17 mm diameter) of 2 LAB species - *Lactobacillus pentosus* and *Lactococcus garviae* while that of *Enterococcus faecalis* is not visible (Figure 5).

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The rest of LAB species had antibacterial activity ranging from about 7 mm diameter (moderate) to 10.5 mm (almost strong).

Table 3. Antibacterial activity of LAB isolates against *Aeromonas* and *Vibrio* species isolated from Siem Reap markets.

Sample code	LAB isolate	Average diameter of zone of inhibition (mm)				
		V. cholerae	*V. parahaemolyticus*	*V. aglinoluticus*	*A. hydrophila/caviae*	*A. hydrophila*
204018-2	*Lactobacillus pentosus*	9.0	9.0	9.0	**14.5**	**17.0**
204018-3	*Lactobacillus pentosus*	9.0	7.5	9.0	10.0	9.5
204018-4	*Lactobacillus pentosus*	9.5	8.0	8.0	8.0	8.0
204018-8	*Lactobacillus pentosus*	8.0	7.0	7.0	7.0	7.0
204018-7	*Enterococcus faecalis*	8.0	8.0	**15.0**	8.0	8.0
204011-9	*Enterococcus faecalis*	8.0	8.0	8.0	8.0	8.0
204020-5	*Enterococcus faecalis*	7.0	7.5	7.0	7.5	7.0
204020-7	*Enterococcus faecalis*	8.0	7.5	7.5	8.0	7.5
204020-8	*Enterococcus faecalis*	8.5	8.0	7.0	9.5	9.0
204018-5	*Enterococcus hirae*	9.5	8.0	8.0	8.0	8.0
204018-6	*Enterococcus hirae*	8.5	8.5	8.5	7.5	7.0
204020-1	*Lactococcus garviae*	9.0	8.5	8.5	9.0	8.5
204020-2	*Lactococcus garviae*	-	-	**16.0**	10.5	10.5
204020-9	*Pediococcus pentosaceus*	10.5	9.0	8.5	8.0	8.5
204020-10	*Pediococcus pentosaceus*	10.5	9.5	10.5	9.0	9.5

Antimicrobial activity based on inhibition zone diameter: weak = <5 mm; moderate = 5-10 mm; strong = 11-20 mm; very strong = >20 mm

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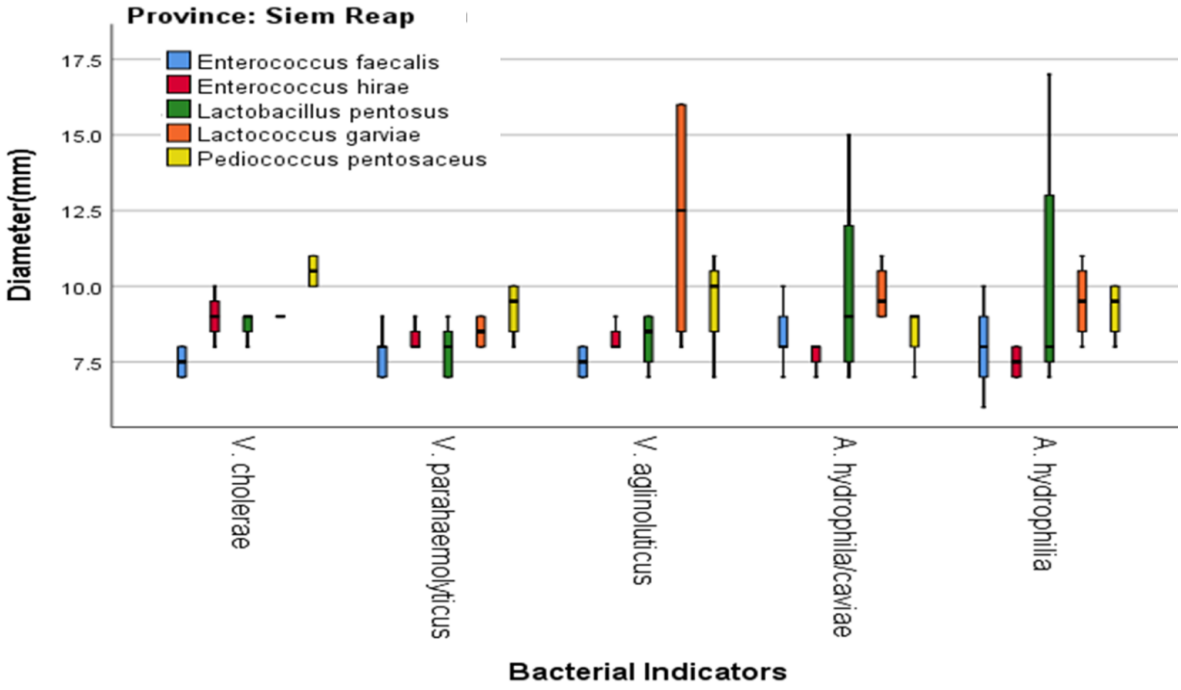

Figure 5. Boxplot of antibacterial activity of 5 LAB species against *Aeromonas* and *Vibrio* species isolated from Siem Reap markets.

Against the *Vibrio* and *Aeromonas* species isolated from Phnom Penh markets, all LAB isolates had also moderate antibacterial activity, except that one *Enterococcus faecalis* isolate (code 204018-7) had no antibacterial activity (Table 4). However, one *Pediococcus pentosaceus* isolate (code 204020-10) had strong antibacterial activity, which can be clearly seen from the boxplot of antibacterial activity of the 5 LAB species (Figure 6).

Table 4. Antibacterial activity of LAB isolates against *Aeromonas* and *Vibrio* species isolated from Phnom Penh markets.

Sample code	LAB isolate	Average diameter of zone of inhibition (mm)		
		V. cholerae	*V. parahaemolyticus*	*A. hydrophila/caviae*
204018-2	*Lactobacillus pentosus*	8.0	8.5	8.5
204018-3	*Lactobacillus pentosus*	8.0	8.5	8.0
204018-4	*Lactobacillus pentosus*	7.5	8.0	8.0
204018-8	*Lactobacillus pentosus*	8.0	7.0	7.0
204018-7	*Enterococcus faecalis*	-	-	-
204011-9	*Enterococcus faecalis*	9.0	8.0	9.0
204020-5	*Enterococcus faecalis*	9.5	10.5	10.0
204020-7	*Enterococcus faecalis*	8.0	8.0	8.5
204020-8	*Enterococcus faecalis*	9.0	9.5	10.0
204018-5	*Enterococcus hirae*	9.5	8.5	9.0

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204018-6	*Enterococcus hirae*	8.5	10.0	8.0
204020-1	*Lactococcus garviae*	9.0	9.0	8.0
204020-2	*Lactococcus garviae*	8.5	9.0	8.0
204020-9	*Pediococcus pentosaceus*	8.0	9.5	9.0
204020-10	*Pediococcus pentosaceus*	**11.5**	**11.0**	**11.0**

Antimicrobial activity based on inhibition zone diameter: weak = <5 mm; moderate = 5-10 mm; strong = 11-20 mm; very strong = >20 mm

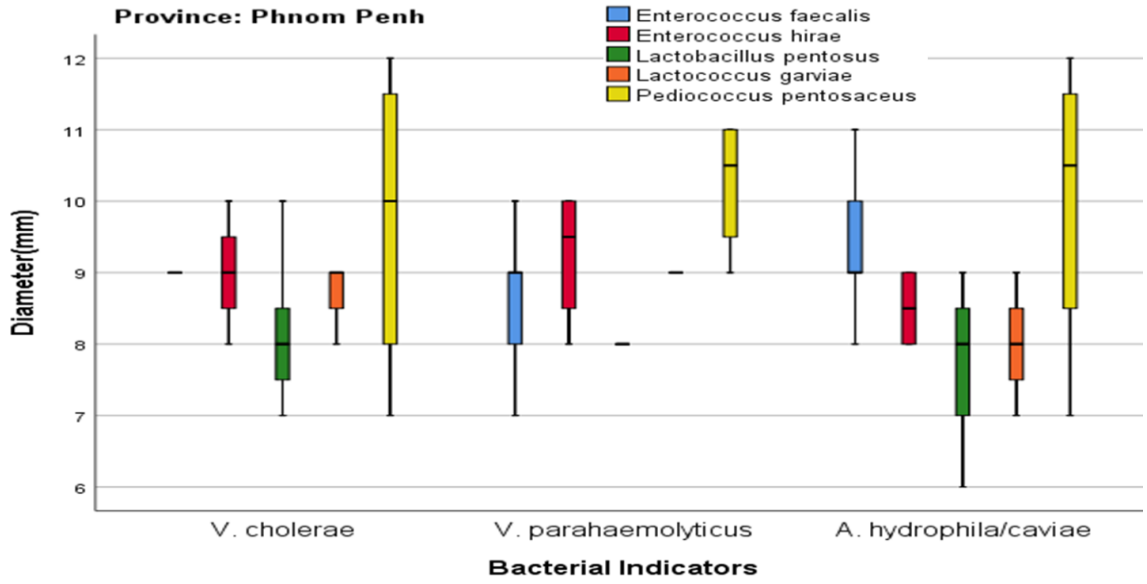

Figure 6. Boxplot of antibacterial activity of 5 LAB species against *Aeromonas* and *Vibrio* species isolated from Phnom Penh markets.

The results show that most of the LAB species have moderate antibacterial activity against the *Vibrio* and *Aeromonas* species isolated from fresh Pangasius fish from markets in Kampong Thom, Siem Reap and Phnom Penh. Previous studies highlight the antimicrobial potential of LAB as reviewed by several researchers (Gao et al., 2019; Ibrahim et al., 2021). The antimicrobial action of LAB has been attributed to several modes of action, including production of antimicrobial compounds (e.g. lactic acid, hydrogen peroxide, CO_2, diacetyl, acetaldehyde, D-isomers of amino acids, reuterin, bacteriocins and defensins); acidification and reduction in luminal pH; inhibition of pathogen adhesion; competitive exclusion of pathogenic microorganisms; and enhancement of barrier function. Further, the results show variations in the effects of different isolates of the same LAB species as some isolates have strong antimicrobial activity while others have weak or no antimicrobial activity. Several factors contribute to this. The effectiveness of LAB isolates against *Aeromonas* and *Vibrio* species can vary depending on the location of the isolates due to differences in the specific strains, virulence factors, and environmental conditions (Pessoa et al., 2019; Loo et al., 2022). While the LAB isolate may be effective against some strains from a particular location, it might not be effective against strains from another location due to these variations. *Aeromonas* and *Vibrio* species have a diverse range of virulence factors, including toxins, enzymes, and adhesion molecules. The presence and expression of these virulence factors can vary between strains and locations, influencing their susceptibility to the LAB isolate.

Probiotic properties of LAB isolates

Acid tolerance test showed that among 15 LAB isolates, only 204020-10 (*Pediococcus pentosaceus*) survived and grew at pH 2.3 (Table 5). This was indicated by higher absorbance value than that before incubation. The other 14 LAB isolates had zero or lower absorbance values than that before incubation. At neutral pH (pH 7),

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all LAB isolates survived, except isolates 204020-5, 204020-7 and 204020-8 (all *Enterococcus faecalis*) and 204020-9 (*Pediococcus pentosaceus*) which did not show any growth with zero absorbance value.

Table 5. Acid tolerance test for 15 LAB isolates (absorbance at 620 nm before and after incubation)

No.	Sample code	Supernatant	Before incubation	After incubation at		Bacterial growth*	
				pH 2.3	pH 7	pH 2.3	pH 7
1	204018-2	*Lactobacillus pentosus*	0.899	0.000	1.990	-	+
2	204018-3	*Lactobacillus pentosus*	0.944	0.132	1.845	-	+
3	204018-4	*Lactobacillus pentosus*	0.824	0.096	2.617	-	+
4	204018-8	*Lactobacillus pentosus*	0.967	0.000	1.221	-	+
5	204018-5	*Enterococcus hirae*	0.992	0.127	2.444	-	+
6	204018-6	*Enterococcus hirae*	0.912	0.000	1.991	-	+
7	204018-7	*Enterococcus faecalis*	0.972	0.000	1.889	-	+
8	204011-9	*Enterococcus faecalis*	0.899	0.000	1.138	-	+
9	204020-1	*Lactococcus garviae*	0.900	0.000	1.232	-	+
10	204020-2	*Lactococcus garviae*	0.902	0.000	1.199	-	+
11	204020-5	*Enterococcus faecalis*	0.899	0.000	1.110	-	+
12	204020-7	*Enterococcus faecalis*	0.879	0.000	1.200	-	+
13	204020-8	*Enterococcus faecalis*	0.992	0.000	1.181	-	+
14	204020-9	*Pediococcus pentosaceus*	0.952	0.000	1.160	-	+
15	204020-10	*Pediococcus pentosaceus*	0.886	1.198	2.090	+	+

Note: A higher absorbance value before incubation than that after incubation indicates negative result.

**Bacterial growth: + (positive), - (negative)*

In the bile salt tolerance test, all LAB isolates, except 204018-6 (*Enterococcus hirae*) from PST- KPT, survived at 0.1% bile salt indicated by higher absorbance values than that before incubation (Table 6). At higher bile salt concentration of 0.3%, only LAB isolates 204011-9 (*Enterococcus faecalis*) from PLTT-SRP and 204020-5 (*Enterococcus faecalis*) from PCD-PP and 204020-9 and 204020-10 (both *Pediococcus pentosaceus*) from PDK-PP survived, the latter two LAB isolates were the only ones that survived at the highest bile salt concentration of 0.6%.

Table 3.6. Bile salt tolerance test for 15 LAB isolates (absorbance at 620 nm before and after incubation)

No.	Sample code	Supernatant	Before incubation	After incubation with bile salt at		
				0.1%	0.3%	0.6%
1	204018-2	*Lactobacillus pentosus*	0.886	1.318	0.811	0.664

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2	204018-3	*Lactobacillus pentosus*	0.944	1.455	0.712	0.551
3	204018-4	*Lactobacillus pentosus*	0.824	1.551	0.772	0.442
4	204018-8	*Lactobacillus pentosus*	0.992	1.122	0.789	0.456
5	204018-5	*Enterococcus hirae*	0.991	0.887	0.881	0.511
6	204018-6	*Enterococcus hirae*	0.999	1.221	0.812	0.565
7	204018-7	*Enterococcus faecalis*	0.899	1.323	0.891	0.575
8	204011-9	*Enterococcus faecalis*	0.889	1.112	0.981	0.757
9	204020-1	*Lactococcus garviae*	0.992	1.331	0.891	0.551
10	204020-2	*Lactococcus garviae*	0.991	0.999	0.981	0.887
11	204020-5	*Enterococcus faecalis*	0.899	0.992	0.901	0.665
12	204020-7	*Enterococcus faecalis*	0.871	0.877	0.811	0.771
13	204020-8	*Enterococcus faecalis*	0.881	0.999	0.881	0.773
14	204020-9	*Pediococcus pentosaceus*	0.897	1.331	1.122	1.221
15	204020-10	*Pediococcus pentosaceus*	0.879	1.318	1.208	1.199

A different response was obtained in the phenol tolerance test, except that LAB isolate 204020-10 (*Pediococcus pentosaceus*) from PDK-PP also showed resistance to both phenol concentrations of 0.2g and 0.5g (Table 7). In addition to this, LAB isolates 204018-2, 204018-3 and 204018-4 (all *Lactobacillus pentosus*) and 204018-5 (*Enterococcus hirae*) had the same response as LAB isolate 204020-10. All other LAB isolates failed to survive in the presence of phenol, except 204020-2 (*Lactococcus garviae*) at 0.2g phenol.

Table 7. Phenol tolerance test for 15 LAB isolates (absorbance at 620 nm before and after incubation)

No.	Sample code	Supernatant	Before incubation	After incubation with phenol at	
				0.2g	0.5g
1	204018-2	*Lactobacillus pentosus*	0.119	0.787	0.222
2	204018-3	*Lactobacillus pentosus*	0.111	0.481	0.229
3	204018-4	*Lactobacillus pentosus*	0.214	2.010	0.245
4	204018-8	*Lactobacillus pentosus*	0.114	2.213	0.365
5	204018-5	*Enterococcus hirae*	0.121	0.100	0.067
6	204018-6	*Enterococcus hirae*	0.133	0.100	0.058
7	204018-7	*Enterococcus faecalis*	0.131	0.099	0.077
8	204011-9	*Enterococcus faecalis*	0.129	0.089	0.069

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9	204020-1	*Lactococcus garviae*	0.122	0.099	0.000
10	204020-2	*Lactococcus garviae*	0.123	0.980	0.000
11	204020-5	*Enterococcus faecalis*	0.118	0.079	0.000
12	204020-7	*Enterococcus faecalis*	0.115	0.099	0.000
13	204020-8	*Enterococcus faecalis*	0.101	0.077	0.050
14	204020-9	*Pediococcus pentosaceus*	0.113	0.076	0.000
15	204020-10	*Pediococcus pentosaceus*	0.114	2.213	0.365

The results show that only LAB isolate 204020-10 (*Pediococcus pentosaceus*) has a strong potential as a probiotic as it is the only one that was tolerant to all three conditions of low pH, bile salt and phenol, in addition to their antibacterial activity against *Aeromonas* and *Vibrio spp.* LAB are evaluated for their tolerance to acid, bile salts, and phenol to assess their ability to survive and function in the human gastrointestinal tract (Ahire et al., 2021). These tests are key indicators of a LAB strain's potential as a probiotic. Tolerance to acids, bile salts and phenol ensures that the LAB can maintain its beneficial functions, such as producing beneficial metabolites or inhibiting pathogens, within the host. The acid tolerance test determines the ability of LAB to survive the acidic conditions of the stomach (Ahire et al., 2021; Nath et al., 2021). The bile salt tolerance test assesses the LAB's ability to withstand bile salts present in the small intestine, which can be inhibitory to microbial growth. Humans have a digestive fluid known as bile which enables the emulsification and solubilization of lipids and lipid-soluble vitamins in the body for proper digestibility (Nath et al., 2021). A higher bile concentration is injurious to the microflora and cell membranes of the host. A 0.3% w/v bile concentration is critical and sufficient for checking the bile tolerance of LAB strains. The phenol tolerance test determines the LAB's ability to tolerate phenol. Phenols are toxic byproducts of digestion released through bacterial deamination of amino acids sourced from dietary proteins. It is essential for bacteria to possess the capability to tolerate the minute amounts of phenols present within the gastrointestinal tract to be considered candidate probiotic strains (Huligere et al., 2023). Recently, a study reported the isolation of LAB *Lactiplantibacillus plantarum* which significantly increase in growth after 24 h of incubation with 0.4% phenol (Meena et al., 2022).

The results further show that some LAB isolates were tolerant to either bile salt or phenol. This is not sufficient to declare them as probiotics (Divyashree et al., 2024; Guney et al., 2025). While bile salt and phenol tolerance are important characteristics for survival in the gastrointestinal tract, they are not the only criteria for probiotic status. Probiotics should be able to withstand the harsh conditions of the stomach (low pH) and small intestine (bile salts and digestive enzymes); adhere to the intestinal lining and potentially colonize it, which is crucial for long-term beneficial effects; exhibit antimicrobial activity; and safe or non-pathogenic or not producing harmful substances. Therefore, while bile salt and phenol tolerance are positive attributes for a potential probiotic, they are not sufficient on their own to declare a LAB isolate as a probiotic. A more comprehensive evaluation of the isolate's probiotic potential is needed.

CONCLUSION

LAB were isolated in 15 out of 153 (9.8%) samples of fermented Pangasius fish, comprising five species: *Lactobacillus pentosus, Enterococcus faecalis, Enterococcus hirae, Lactococcus garviae,* and *Pediococcus pentosaceus.* They showed moderate antibacterial activity against most *Aeromonas* spp. and *Vibrio* spp., with some isolates, particularly *Lactobacillus pentosus* (code 204018-2) and *Pediococcus pentosaceus* (code 204020-10) showing stronger antibacterial activity. Only one LAB isolate (*Pediococcus pentosaceus*, code 204020-10) showed strong probiotic potential with high tolerance to low pH, bile salts, and phenol, in addition to its antibacterial activity. Future work may delve into the molecular characterization of probiotic genes, screening for antibiotic resistance and virulence factors, and whole-genome sequencing to confirm biosafety before recommending strains for commercial applications. Also, additional *in vivo* experiments using animal models could be carried out to provide stronger evidence for probiotic efficacy beyond laboratory assays.

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Conflict Of Interest

The authors declare no potential conflict of interest.

Compliance With Ethical Standards

This article does not cover any studies with human contributors or animals performed by any of the author.

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