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Probiotic potential of lactic acid bacteria isolated from Vietnamese sour-fermented fish product

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ABSTRACT

Aims: To isolate and characterize the lactic acid bacteria (LAB) strains from the “mam chua ca ro” (sour fermented fish) in the South of Vietnam and investigate their potential anti-bacterial properties.
Methodology and results: Four LAB strains (MCR1, MCR2, MCR3 and MCR4) were isolated from the "mam chua ca ro" product and their anti-bacterial activity was determined using the spot assay and the paper disc diffusion method. The isolated LABs can inhibit *Escherichia coli* ATCC 25922, *Staphyloccocus aureus* ATCC 25923 and *Vibrio parahaemolyticus* BV016 and produce bacteriocin to control the growth of *E. coli* ATCC 25922 and *S. aureus* ATCC 25923, except *V. parahaemolyticus*. MCR2 was chosen to sequence 16S rRNA of *Pediococcus acidilactic*.
Conclusion, significance and impact of study: On the basis of their prominent anti-pathogenic bacteria activity, LAB strains isolated from Vietnamese sour-fermented fish products were verified as prospective probiotics.

Keywords: Pediococcus acidilactic, lactic acid bacteria, sour-fermented fish

INTRODUCTION

Lactic acid bacteria (LAB) are prevalent in fermented foods, contributing to their flavour, texture and preservation from spoilage. Lactic acid bacteria are beneficial microbes that account for 0.01-1.8% of the major colonic bacterial groups in the human large intestine (Louis *et al.*, 2007). Certain LAB strains have been used in the food industry as probiotics in fermented products and dietary supplements, conferring health advantages by balancing the microbial community.

“Mam chua ca ro” is a Vietnamese traditional sour fermented fish product. Vietnam has a dense network of streams, lakes, rivers and a coastline of 3400 km from North to South. Therefore, Vietnam has enormous potential for the fishery industry. Fish is an essential daily food for people. However, due to the tropical monsoon climate, the food gets spoiled easily (Ly *et al.*, 2018). The aim of making sour fermented fish is to prolong the storage life of perishable freshwater and seawater fish. The activity of LAB produces lactic acid and reduces the pH of the product, which inhibits the pathogenic food microorganisms and contributes to the flavour and texture of the sour fermented fish (Chelule *et al.*, 2010; Ngasotter *et al.*, 2020). Moreover, Tran *et al.* (2013) reported that before fermentation, the natural LAB cell density in the mixture was 10^6-10^7 CFU/g. After fermentation, it increased to 2.61×10^8-1.14×10^9 CFU/g. To keep the fish sauce fresh, lactic acid bacteria produce substances that block harmful bacteria as they proliferate, thus preventing the food from spoiling. These LABs can (1) live in environments with high salinity, (2) be capable of lactic fermentation and (3) produce secondary products such as bacteriocin and organic compounds.

Bacteriocins-natural peptides are a type of proteinaceous molecule produced during fermentation. These peptides vary in size, structure, activity and receptors (De Filippis *et al.*, 2020). Previous *in vitro* and *in vivo* studies reported the noticeable bactericidal action against other bacteria species or archaea, even with antibiotic-resistant bacteria. Particularly, mersacidin is the product of the *Bacillus* sp. (strain HIL-Y85/54728) to control methicillin-resistant *Staphylococcus aureus* strains in mice (Kruszewska *et al.*, 2004). Galvin *et al.* (1999) reported that lacticin 3147, produced by *L. lactis* subsp. *lactis*, was capable of inhibiting *S. aureus*, methicillin-resistant *S. aureus* and vancomycin-resistant *E. faecalis* (VRE). In addition, Dicks *et al.* (2011) indicated that bacteriocins are the potential candidates in the fight against various systemic infections (urogenital, gastrointestinal, respiratory and skin). Additionally, several LAB species have been certified as "generally

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recognized as safe" (GRAS) by the United States Food and Drug Administration (US FDA), indicating that the bacteriocins produced by the LAB are also generally acknowledged as safe (Abanoz and Kunduhoglu, 2018; De Filippis *et al.*, 2020). Therefore, some types of bacteriocins derived from LAB have been used in food to control infectious pathogens (Cleveland *et al.*, 2001). This study aimed to isolate LAB strains from local sour-fermented fish, a traditional Vietnamese product with an abundance of LABs and evaluate the pathogenic bacteria inhibition of the isolated strains.

MATERIALS AND METHODS

Materials

Sour-fermented fish product samples were collected at a factory at Long Thanh hamlet, Tan Long commune, Nga Nam town, Soc Trang province, Vietnam. Three indicator strains, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Vibrio parahaemolyticus* BV016 (obtained from the Molecular Laboratory, Biotechnology Research and Development Institute, Can Tho University, Vietnam).

Isolation and characterization of the LABs

Briefly, the collected samples were homogenized, then 10 g of the sample was enriched with 90 mL of MRS broth. After 24 h, 50 µL of the culture was spread on MRS agar plate and incubated at 37 °C for 24 h. The isolates were differentiated based on their morphological and biochemical characteristics (Mohankumar and Murugalatha, 2011).

Anti-bacterial activity assay

The bacterial isolates were cultured in MRS broth at 37 °C for 24 h. After that, 10 µL of aqueous culture (10^6 CFU/mL) were spotted onto the surface of MRS agar plates by micropipette (diameter of 1 cm) and incubated for 12 h at room temperature. The suspensions of three bacterial indicator strains were mixed well with LB 0.6% agar medium and poured on a previously incubated MRS agar Petri dish. The inhibition zones were observed in 24-36 h. A diameter wider than 4 mm is considered positive (Ngo *et al.*, 2011).

Bacteriocin anti-bacterial activity

Preparation of crude bacteriocin: LAB was cultivated in MRS broth (10^6 CFU/mL). The enriched culture was centrifuged at 8,000 rpm for 15 min at 4 °C. The cell-free supernatant (used as crude bacteriocin) was obtained and adjusted the pH to 6.5 by 0.1 M NaOH (Elayaraja *et al.*, 2014).

Eighty (80) µL of crude bacteriocin was added to each well of the MRS agar plate previously inoculated with indicator bacteria. Then, the plate was incubated at 35 °C and inhibition zones were recorded after 24 h. All assays were performed in triplicate.

Identification of LAB isolates

The LAB isolates that showed remarkable antimicrobial ability were chosen for 16S rRNA sequencing. The target gene was amplified by PCR, using the following primers (27F: 5'-AGAGTTTGATCCTGGCTC-3'; 1492R: 5'-TACGGTTACCTTGTTACGACT-3') (Weisburg *et al.*, 1991).

PCR analysis was conducted by Abbasiliasi *et al*. (2012) with some modifications. PCR reactions were performed in a volume reaction of 50 µL, containing 27 µL of BiH_20, 20 µL of Master Mix 2×, 1 µL of Forward primer and Reverse primer in each and 1 µL DNA template. PCR cycles were set up with one initial denaturation step of 5 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 45 sec of annealing at 55 °C and extension at 72 °C for 80 sec, with final extension step at 72 °C for 5 min. The amplification process was performed in C1000 thermocycler (Bio-rad, USA). PCR products were separated by 2% agarose electrophoresis for 45 min at 50 V and the bands were visualized by Run-Safe stain (Cleaver Scientific, United Kingdom). Amplicons with clear bands and no nonspecific products were submitted to Macrogen, Korea, for sequencing. The raw sequence was interpreted by Bioedit software version 7.2.1. The quality value checks to confirm the accuracy of the sequencing procedure and unidentified nucleotide was verified based on the chromatogram. BLAST tool on the National Centre for Biotechnology Information (NCBI) was used to perform similarity searches of sequencing data.

Statistical analysis

All the values are indicated as means ± SD of three replications. The results were statistically processed by the ANOVA method using Minitab 16 software. Tukey's test was implemented to assess the significant difference among values with a significance level of $P<0.05$.

RESULTS AND DISCUSSION

Isolation and characterization from sour-fermented fish product

Four strains of LAB were isolated from samples of sour-fermented fish products, namely MCR1, MCR2, MCR3 and MCR4. They were initially identified based on colony morphology, cytology and biochemical characteristics, as listed in Table 1. Particularly, these strains grown on MRS medium had a round shape, milky white colony colour, entire margin, convex elevation and diameter 1-2 mm (Figure 1A). Moreover, the MCR2 strain had spherical-shapes, while MCR1, MCR3 and MCR4 had rod cell shapes (Figure 1B). According to biochemical testing results, four LAB strains were Gram-positive, catalase and oxidase negative, and CaCO3 degradable. Those

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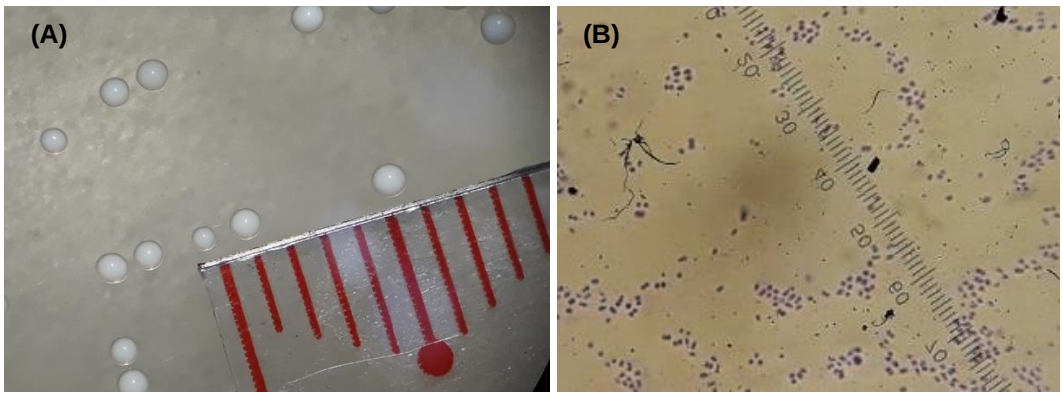

Figure 1: (A) Stereoscopic microscopy of MCR1 isolate of the colony; (B) Microscope view of MCR1 illustrated Gram-positive at 400× magnification.

Table 1: Characteristics of colony morphology, characteristics of cytology and biochemical characteristics of isolates.

Isolate code	Colony morphology	Cell shape	Spore forming	Gram staining	$CaCO_3$ degradation	Catalase	Oxidase
MCR1	Circular, cream, smooth, entire margin	Rod	-	+	+	-	-
MCR2	Circular, milky white, smooth, entire margin	Cocci	-	+	+	-	-
MCR3	Circular, cream, smooth, entire margin	Rod	-	+	+	-	-
MCR4	Circular, cream, smooth, entire margin	Rod	-	+	+	-	-

Note: (-) negative; (+) positive.

Table 2: The inhibitory effect of LAB isolates against *Escherichia coli*, *Staphylococcus aureus* and *Vibrio parahaemolyticus*.

Bacterial isolates	Inhibition zones (mm)		
	E. coli	*S. aureus*	*V. parahaemolyticus*
MCR1	$10.67^{ab} \pm 2.07$	$11.50^{a} \pm 1.05$	$5.25^{d} \pm 0.50$
MCR2	$11.67^{a} \pm 1.86$	$10.50^{a} \pm 1.05$	$9.25^{ab} \pm 0.50$
MCR3	$8.83^{b} \pm 1.87$	$9.67^{b} \pm 0.52$	$7.75^{c} \pm 0.50$
MCR4	$9.50^{ab} \pm 1.23$	$10.00^{a} \pm 1.27$	$8.50^{ab} \pm 0.57$

Note: Each treatment was replicated three times. Means ± SD followed by the same letter is not significantly different at 5% level in the same column using Tukey's test.

characteristics were similar to other LAB strains described by Ngo *et al.* (2011) and Rajoka *et al.* (2017).

Anti-bacterial ability

The LAB strains have effectively inhibited the growth of *E. coli* and *S. aureus*, with the appearance of clear inhibition zones after the period of incubation (Figure 2). Additionally, MCR2 and MCR1 strains showed the strongest inhibition against *E. coli* (11.67 mm) and *S. aureus* (11.50 mm), whereas MCR3 had the weakest affected both strains (8.83 and 9.67 mm, respectively) (Table 2). According to Li *et al.* (2020), *Lactobacillus rhamnosus* could inhibit multidrug-resistant *E. coli* in mice. Moreover, the *L. rhamnosus* SHA113 strain had strong inhibitory effects on *S. aureus in vitro* and did not cause significant side effects when tested in mice Li *et al.* (2020).

Bacteriocin anti-bacterial activity

The anti-bacterial ability of crude bacteriocin solution against *E. coli* and *S. aureus* was evaluated using the agar well diffusion method (Figure 3). The results showed that all four crude bacteriocins solutions from LAB isolated in this study could inhibit *E. coli* and *S. aureus* (Table 3). The bacteriocins solution from MCR2 and MCR4 displayed the strongest ability to inhibit the growth of *E. coli*, with inhibition zones diameter were 6.75 mm and 6.00 mm, respectively. The MCR2's bacteriocins exhibited the strongest inhibitory activity on *S. aureus* growth, with an inhibition zone diameter of 10.50 mm.

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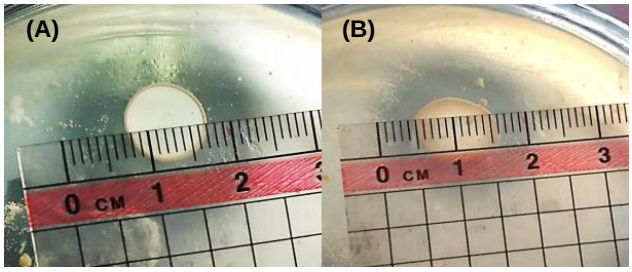

Figure 2: The inhibitory effect of LAB isolates against indicator strains by spot method. LAB isolates inhibited the growth of (A) *Escherichia coli* and (B) *Staphylococcus aureus*.

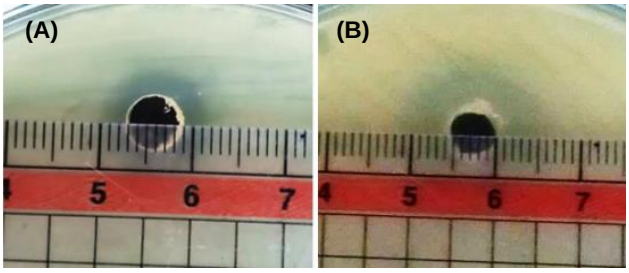

Figure 3: The result of the crude bacteriocin-producing ability of LAB isolates. (A) Bacteriocin solution from MCR2 inhibited *E. coli*, (B) Bacteriocin solution from MCR2 inhibited *S. aureus*.

Table 3: The inhibitory effect of bacteriocin against *Escherichia coli* and *Staphylococcus aureus*.

Bacterial isolates	Inhibition zones (mm)		
	E. coli	*S. aureus*	*V. parahaemolyticus*
MCR1	$4.25^{b} \pm 0.96$	$5.00^{bc} \pm 0.82$	-
MCR2	$6.75^{a} \pm 0.96$	$10.50^{a} \pm 1.29$	-
MCR3	$4.25^{b} \pm 0.50$	$3.75^{c} \pm 1.26$	-
MCR4	$6.00^{a} \pm 0.82$	$6.00^{a} \pm 0.82$	-

Note: Each treatment was replicated three times. Means ± SD followed by the same letter is not significantly different at 5% level in the same column using Tukey's test. -: NA value.

However, the inhibitory effect of MCR3 bacteriocin was the lowest, with a diameter of the inhibition zone of 3.75 mm. In general, MCR2 was the most efficient LAB isolate, retarding the growth of both indicator strains. Previous studies proved the anti-bacterial effects of bacteriocins from LAB, such as *Lactobacillus shellorum* MN047 producing bacteriocin BM173 could inhibit *E. coli* and *S. aureus* (Qiao *et al.*, 2021), *Lactobacillus plantarum* SHY 21-2 produced plantaricin LP 21-2 which inhibited *E. coli*, *S. aureus* and *Saccharomyces cerevisiae* (Peng *et al.*, 2021). Bacteriocin from LAB isolates, however, did not affect the growth of *V. parahaemolyticus*, although it was regulated by those strains in the prior assay. Nguyen (2017) reported that four LAB strains LABT3.1, RP5.4.1, T4.2 and RP5.5.1 did not produce inhibition zones against *Vibrio parahaemolyticus* when tested with bacteriocin solution from LAB, but the inhibition of those isolates could be due to acid secretion, as *V. parahaemolyticus* would have reduced virulence to the host in culture with pH<4. Moreover, Vignolo *et al.* (2000) reported that *Listeria monocytogenes* and *L. innocua* were resistant to nisin, lactocin 705 and enterocin CRL35 and suggested that resistance to bacteriocin could result from altered bacterial membrane composition, destruction of bacteriocin by proteases or altered receptors.

Identification of LAB strains

Among the four LAB isolates from the sour-fermented fish product, the MCR2 had the remarkable inhibitory effect against indicator bacterias and it was chosen for 16S rRNA gene sequencing. After submitting the 16S rRNA gene sequences to GenBank, the MCR2 strain was identified as *Pediococcus acidilactic* with accession numbers KF806471. Abbasiliasi *et al.* (2012) isolated *Pediococcus acidilactic* with the capability of producing bacteriocin, which had the potential in food preservation applications.

CONCLUSION

The sour-fermented fish product contained four LAB strains. All but *V. parahaemolyticus* were able to suppress *E. coli* and *S. aureus*. A further MCR2 strain was identified as *Pediococcus acidilactic*, which had the highest anti-bacterial activity among all four isolates. By and large, these LAB isolates were considered as probiotic prospects. There is a need to do further *in vitro* and *in vivo* studies to better understand the properties of probiotics and their safety evaluations.

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