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Lactic Acid Bacteria Isolates from Pla-som, Their Antimicrobial Activities and Fermentation Properties in Pla-som

Sirilak Sanpa[a*], Sirikarn Sanpa[a] & Maitree Suttajit[b]

[a] *Division of Microbiology and Parasitology, School of Medical Sciences, University of Phayao, Phayao, 56000 Thailand*
[b] *Division of Biochemistry, School of Medical Sciences, University of Phayao, Phayao, 56000 Thailand*

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Abstract

Pla-som is a traditional fermented fish product widely consumed in Thailand. However, its hygienic quality and test consistency are often uncontrollable. To minimize the risk of fermentation failure, the use of selected starter culture could result in quality control of the end product. In this study, lactic acid bacteria (LAB) were isolated from eight samples collected from different Pla-som producers in Phayao City using De Man Rogosa Sharpe (MRS) agar. The cell-free supernatant of isolated strains was determined for the antibacterial activity against food borne pathogens (*Escherichia coli, Staphylococcus aureus, Bacillus cereus, Clostridium perfringens, Shigella* sp., and *Vibrio* sp.). In order to exhibit the presence of proteinacious bacteriocin produced by LAB, the crude supernatant of isolated strains were inactivated with proteolytic enzymes, pepsin and trypsin. The results showed that the antibacterial activity of the 14 from 55 bacterial isolates was diminished by the enzymes. Strain ST2 and TT3 were selected based on the highest antimicrobial activity on tested pathogenic bacteria and identified by 16S rRNA sequence analysis as *Lactobacillus paraplantarum* and *Pediococcus pentosaceus,* respectively. Therefore, they were used as starters in Pla-som fermentation. Lower pH value and higher acidity were also observed during the fermentation period. The addition of selected starter cultures significantly decrease fermentation time due to a combination of pH reduction and acid production. The sensory evaluation of the fish product with the mixed starter had the highest overall satisfaction score as tested in 30 volunteers.

Introduction

Pla-som is a traditional fermented fish widely consumed in the northern part of Thailand (Sikhiram, 2018). It is made from fish, sugar, salt, garlic and cooked rice and fermented using natural microbial flora (Hwanhlem et al., 2011). The traditional production of Pla-som was based on spontaneous fermentation due to

* Corresponding Author
e-mail: s.sanpa@gmail.com

the development of the microflora that is naturally present in the raw materials. The fermentation provides a combination of reduced pH and produced organic acid, mainly lactic acid (Saithong et al., 2010; Pringsulaka et al., 2012; Riebroy et al., 2008).

LAB are generally accepted as safe microorganisms that play an important role in food fermentation and preservation either by the presence of natural microflora or the addition of starter cultures under controlled conditions (Paludan-Miiller et al., 1999). The preservative effect exerted by LAB is mainly due to the production of lactic acid. LAB also produces antimicrobial compounds such as hydrogen peroxide, reuterin and bacteriocins (Yang et al., 2012). Bacteriocins are antimicrobial peptides against other bacteria (Yang et al., 2014). In recent years, bacteriocin producing LAB were find to have a potential use as safe additives for food preservation (Diop et al., 2007). Nisin, a bacteriocin produced by *Lactococcus lactis,* is the most thoroughly studied and applied as a commercial additive to certain foods (Woraprayote et al., 2016). Other bacteriocins such as pediocin, may also have potential applications in foods, though they are not currently approved as antimicrobial food additives (Porto et al., 2017).

The traditional fermented fish production has the public health problem of poor hygiene due to microbial contamination during the process, leading to a failure in GMP standardization (Pumipan & Inmaung, 2016). The quality of Pla-som products show the inconsistency of acidity, test, and fermentation time as reported by Chompuming (Chompuming et al., 2010). To minimize the risk of fermentation failure, using a selected starter culture could shorten the fermentation time and resulting in quality control of the end product (Saithong et al., 2010; Visessanguan et al., 2006).

This study aims to search LAB isolated from Pla-som fermented products and to determine their inhibitory effects on food-borne pathogens. Therefore, the LAB was isolated and evaluated for their antimicrobial effects on food borne pathogens including *Escherichia coli, Staphylococcus aureus, Bacillus cereus, Clostridium perfringens, Shigella* sp. and *Vibrio* sp. (Phugan et al., 2018). The effective bacterial strains were selected and used in Pla-som fermentation in which the fish products were finally evaluated for sensory by the testers.

Materials and methods

1. Isolation of lactic acid bacteria

Lactic acid bacteria (LAB) were isolated from eight samples collected from different Pla-som producers in Phayao City. Twenty-five grams of Pla-som samples were added to 225 ml of sterilized 0.1% (w/v) peptone solution. The diluted sample was cultured on de Man Rogosa Sharp (MRS) agar containing 0.4% (w/v) bromocresol green and incubated at 30°C for 48 h (Pringsulaka et al., 2012; Kargozari et al., 2015). The anaerobic cultivation was done by using an anaerobic jar. Colonies that changed the medium color from green to yellow were collected and streaked on MRS agar for purification. Each of the isolated colonies was tested for catalase and Gram strain, then the isolates that were catalase negative and Gram- positive were maintained in MRS broth with 20% glycerol at -20°C.

2. Antimicrobial activity and enzymatic testing of bacteriocin producing LAB

The selected strains were inoculated into fresh MRS broth and incubated at 30°C in anaerobic condition. After 24 h of cultivation, centrifuged at 10,000 rpm for 10 min, the cell free supernatant was collected for antibacterial activity against food-borne pathogens (*E. coli, S. aureus, B. cereus, C. perfringens, Shigella* sp., and *Vibrio* sp.). The agar wells diffusion method was used to determine the antimicrobial activity of LAB strains. The food borne pathogens grown in nutrient broth at 37°C was adjusted to NO 0.5 McFarland standards and spread over the surface of a nutrient agar plate with a sterile cotton swab. The plates were allowed to dry and a sterile cork borer with diameter of 6 mm was used to cut wells in the agar. Each well was filled with 50 µl of the culture supernatant obtained from the LAB strains. After incubation at 37°C for 24 h, the plates were observed for a zone of inhibition around the well. The experiment was carried out in triplicates, lactic acid with pH 2.3 was used as a positive control and MRS broth without bacterial culture was used as a negative control.

To confirm the production of a proteinaceous bacteriocin, cell free supernatant displaying antimicrobial potential after acid neutralization and H_2O_2 elimination was treated with 1 mg/ml of proteolytic enzymes, including pepsin and trypsin (Sigma-Aldrich Corporation, USA) at 37°C for 2 h (Herreros et at., 2005). The cell free supernatant without proteolytic enzymes treatment, was used as a positive control. Antimicrobial activity of the treated cell free supernatant was determined by agar

diffusion bioassay as described above (Diop et al., 2007; Elayaraja et al., 2014).

3. Identification of selected LAB

The selected LAB strains were identified using morphological and biochemical tests and 16S rRNA sequence analysis (Diaz et al., 2013). The selected LAB was characterized by Gram staining, cell morphology, catalase reaction, motility, oxidative-fermentative test, methyl red test, Voges-Proskauer test, gelatin degradation, indole test, citrate test and carbohydrate fermentation. The strains were identified to the species by the scheme devised by Schillinger & Lucke (1989) for lactobacilli with several modifications. The morphology of the isolates was determined under x1,000 magnification using phase-contrast microscopy. The DNA extraction method for 16S rRNA sequencing and sequence data analysis was done as previously described (Anyogu et al., 2014). Genomic DNA was extracted from the isolates using the Microbial DNA Isolation kit. Universal primers F44 (5'RGTTYGATYMTGGCTCAG-3') and R1543 (5'-GNNTACCTTKTTACG ACTT-3') were used for the amplification of the 16S rRNA gene by PCR. A homology search of the sequences was conducted using the BLAST program at the NCBI database.

4. Pla-som preparation and sensory evaluation

The formula of Pla-som consists of 920 g of fish meat, 10 g of garlic, 30 g of salt and 40 g of steamed rice. Pla-som fermentations were carried out with 4 treatments: (1) without inoculation of starter culture (control), (2) inoculation with *Lactobacillus paraplantarum* ST2, (3) inoculation with *Pediococcus pentosaceus* TT3 and (4) inoculation with mixed starter culture (*L. paraplantarum* ST2 and *P. pentosaceus* TT3). In treatment 2-4, the starter cultures were added to the final concentration of 5 log of CFU/g. The mixture of each treatment was transferred into a plastic bag and incubated at room temperature (30±2°C) for 4 days. Their acidity and pH value were recorded during fermentation period. The fermented Pla-som sample was cultured on MRS agar and total plate count agar to determine lactic acid bacteria and total viable bacteria, respectively.

After the 4-day fermentation, all samples were fried in palm oil at a cooking temperature. The sensory evaluation was tested by 30 volunteers using a 5 point Hedonic scaling modified from Seo et al. (2009) and Tinakorn Na Ayutthaya et al. (2018) (1 = dislike extremely, 5 = like extremely). The testers were asked to evaluate the 4-day fermented samples for color, odor, flavor, texture and overall acceptance.

5. Statistical analysis

Statistical analysis was performed using One-way Analysis of Variance (ANOVA) and T-tests for a statistical significance of $P<0.05$ by using SPSS software program version 23. All data are reported as means ± standard deviations (SD).

Results and discussion

1. Isolation of lactic acid bacteria (LAB) and their antimicrobial activity

Lactic acid bacteria (LAB) were isolated from 8 Pla-som samples made in Phayao Province. De Man Rogosa Sharpe (MRS) agar containing 0.4% (w/v) bromocresol green was used as a preliminary screening medium for LAB. There were 55 isolated strains that changed the medium color from green to yellow. Among them, 47 isolates were gram-positive rod or cocci, non-spore forming and negative for catalase test; after that, the isolates were selected for further experiments. These 47 isolates were then analyzed for the antimicrobial activity against food-borne pathogens (*E. coli, S. aureus, B. cereus, C. perfringens, Shigella* sp. and *Vibrio* sp.). The results showed that 33 isolates could inhibit all six food-borne pathogens. After acid neutralization and H_2O_2 elimination, pepsin and trypsin were used for testing of proteinaceous bacteriocin. The cell-free supernatant without proteolytic enzyme treatment was used as a positive control. There were 14 isolates (NL5, TP3, TP4, TP6, TP9, TT3, TT2, MT3, MT4, ST5, ST2, ML3, ML6, and ML1) that lost their antibacterial activity with proteolytic enzymes (Table 1). The results suggested that these 14 strains were able to produce proteinaceous bacteriocin.

During the production of fermented food, LAB played an important role in the formation of flavor and texture and the control of spoilage and pathogenic microorganisms (Gao et al., 2014; Michalak et al., 2018). Among the 14 strains, ST2, which showed strong and broad antibacterial activities against various bacterial strains, was then selected as the starter of Pla-som production. In the fermentation process, various lactic acid bacteria play different roles for improvement of the products; therefore, strain TT3, which is the only one member of Pediococci group, was also selected as starter of Pla-som production.

2. Identification of lactic acid bacteria (LAB)

The selected strains from antimicrobial activity and enzymatic test of bacteriocin-producing LAB were

Table 1 Inhibitory effect of LAB on six food-borne pathogens after treatment with proteolytic enzymes, pepsin and trypsin

Isolate code	Proteolytic enzyme	Inhibition zone (mm)					
		B. cereus	*C. perfringens*	*E. coli*	*S. aureus*	*Shigella* sp.	*Vibrio* sp.
NL 5[ab]	Control	11.5	14	12	12	12	10.5
	Pepsin	9.5	12	10.5	9.5	12	10
	Trypsin	10	12.75	10.5	10	11	7.5
TP 3[abc]	Control	7	14	10	11	11.5	13.5
	Pepsin	-	9.5	10	-	10.5	10.75
	Trypsin	7	14	10	9	10.5	10
TP 4[abc]	Control	9.5	10.75	12	13	11.5	13
	Pepsin	-	11.5	12	-	11.25	10.25
	Trypsin	9	-	11.25	-	11	10.5
TP 6[bc]	Control	10	10.5	12	14	11.5	12.5
	Pepsin	9	9	12	-	-	-
	Trypsin	8.5	-	9	-	9	-
TP 9[bc]	Control	11	11.75	13.5	14	12.5	13.5
	Pepsin	-	-	11.75	-	-	-
	Trypsin	10.5	17.5	10	-	-	-
TT 3[bc]	Control	7.5	14.5	12.5	8.75	9.5	11.25
	Pepsin	-	-	12	8	9	-
	Trypsin	6.75	-	12	8.17	9.25	-
TT 2[c]	Control	9.5	11	12	12.5	12.75	14.25
	Pepsin	9.25	-	-	8	-	-
	Trypsin	7.5	10.5	-	-	9	-
MT 3[bc]	Control	9.5	12	12.25	12.5	12.25	14.25
	Pepsin	9	9	9	-	8	-
	Trypsin	8	-	10	-	9	-
MT 4[abc]	Control	9.5	12	12.5	14	11	15.75
	Pepsin	-	9.5	12.5	9	10.5	-
	Trypsin	9	-	12	12	9.5	-
ST 5[abc]	Control	10	12.5	13.5	12.5	12	13.5
	Pepsin	9.5	10	13	10.5	11.75	-
	Trypsin	9	12	12.75	9	11.75	-
ST 2[a]	Control	21	20.5	13	16	12	10.5
	Pepsin	12.5	13.75	12.75	-	12	10.5
	Trypsin	11	13	13	9.5	10.75	10
ML 3[abc]	control	9.5	12.5	12	12	11	11.5
	Pepsin	9.5	12.5	11	11	8.75	11
	Trypsin	9	11.75	11	10	10	11
ML 6[abc]	Control	10	13.5	13	10.5	12	12.75
	Pepsin	8.25	8.75	-	10	10	-
	Trypsin	10	10	12	8.5	-	12
ML 1[abc]	Control	9.5	12	11.5	13	10	10
	Pepsin	9	9	10.75	10	9.5	9.5
	Trypsin	8	12	11	10	10	-

Remark: Mean from triplicate determinations.
Different superscripts in the same column indicate significant differences ($p < 0.05$).

determined for characteristics of Gram staining, cell morphology, catalase reaction, motility, OF test, methyl red test, Voges-Proskauer test, gelatin degradation, indole test, citrate test and carbohydrate fermentation. The strains were confirmed by molecular identification of 16S rRNA (approximately 1,500 bp) sequence, which showed a high similarity more than 99% to *Lactobacillus pentosus, L. paraplantarum, L. plantarum,* and *Pediococcus pentosaceus* (Table 2).

It was reported that *L. paraplantarum* and *P. pentosaceus* could also be isolated from rice bran, pickles, sourdough, fermented sausages and olive fermentation brine (Parente

Table 2 Biochemical and physiological characteristic of LAB and their molecular identification by 16S rRNA sequence

Isolate code	Characteristic														Identification based on 16s rRNA sequencing (%identity)
	Gram stain	Shape	Catalase	Motility	OF test	Methyl red	VP	Gelatin	Indole	Citrate	Carbohydrate fermentation				
											Glucose	Sucrose	Lactose	Manitol	
NL5	Positive	Rod	-	-	+/+	+	-	-	-	-	+	+	+	+	*Lactobacillus pentosus* (99.93)
TP3	Positive	Rod	-	-	+/+	+	-	-	-	-	+	+	+	+	*Lactobacillus pentosus* (99.93)
TP4	Positive	Rod	-	-	+/+	+	-	-	-	-	+	+	+	+	*Lactobacillus plantarum* subsp. *plantarum* (100)
TP6	Positive	Rod	-	-	+/+	+	-	-	-	-	+	-	-	+	*Lactobacillus pentosus* (99.93)
TP9	Positive	Rod	-	-	+/+	+	-	-	-	-	+	-	-	+	*Lactobacillus pentosus* (99.93)
TT2	Positive	Rod	-	-	+/+	+	-	-	-	-	+	+	+	+	*Lactobacillus plantarum* subsp. *plantarum* (99.92)
TT3	Positive	Cocci	-	-	+/+	+	-	-	-	-	+	+	+	+	*Pediococcus pentosaceus* (99.74)
MT3	Positive	Rod	-	-	+/+	+	-	-	-	-	+	+	+	+	*Lactobacillus plantarum* subsp. *plantarum* (99.87)
MT4	Positive	Rod	-	-	+/+	+	-	-	-	-	+	+	+	+	*Lactobacillus plantarum* subsp. *plantarum* (99.87)
ST5	Positive	Rod	-	-	+/+	+	-	-	-	-	+	+	+	+	*Lactobacillus paraplantarum* (99.87)
ST2	Positive	Rod	-	-	+/+	+	-	-	-	-	+	+	+	+	*Lactobacillus paraplantarum* (99.87)
ML3	Positive	Rod	-	-	+/+	+	-	-	-	-	+	+	+	+	*Lactobacillus pentosus* (99.93)
ML1	Positive	Rod	-	-	+/+	+	-	-	-	-	+	+	+	+	*Lactobacillus paraplantarum* (99.87)
ML6	Positive	Rod	-	-	+/+	+	-	-	-	-	+	+	+	+	*Lactobacillus pentosus* (99.93)

Table 3 The changes in total viable count (TVC) and lactic acid bacteria (LAB) in natural fermentation and inoculation with starters

Fermentation Time	0 h		24 h		48 h		72 h		96 h	
	TVC (log cfu/g)	LAB (log cfu/g)	TVC (log cfu/g)	LAB (log cfu/g)	TVC (log cfu/g)	LAB (log cfu/g)	TVC (log cfu/g)	LAB (log cfu/g)	TVC (log cfu/g)	LAB (log cfu/g)
Without starter	7.6 ± 0.1^{a}	3.6 ± 0.1^{b}	7.6 ± 0.1^{a}	4.9 ± 0.1^{b}	7.7 ± 0.2^{a}	5.7 ± 0.2^{b}	7.7 ± 0.1^{a}	6.2 ± 0.2^{b}	7.7 ± 0.3^{a}	6.9 ± 0.1^{b}
Strain ST2	8.1 ± 0.4^{ab}	4.1 ± 0.1^{a}	7.5 ± 0.1^{ab}	5.3 ± 0.3^{a}	7.1 ± 0.4^{ab}	6.5 ± 0.3^{a}	6.9 ± 0.3^{ab}	7.1 ± 0.2^{a}	6.9 ± 0.2^{ab}	6.8 ± 0.3^{a}
Strain TT3	7.5 ± 0.3^{b}	4.5 ± 0.2^{ab}	7.1 ± 0.2^{b}	5.1 ± 0.2^{ab}	6.5 ± 0.3^{b}	6.3 ± 0.2^{ab}	6.9 ± 0.2^{b}	6.8 ± 0.3^{ab}	6.9 ± 0.1^{b}	6.9 ± 0.3^{ab}
Mixed starter culture	7.8 ± 0.2^{ab}	4.6 ± 0.1^{a}	7.5 ± 0.3^{ab}	5.8 ± 0.1^{a}	7.1 ± 0.1^{ab}	6.9 ± 0.3^{a}	6.8 ± 0.3^{ab}	7.1 ± 0.3^{a}	6.8 ± 0.3^{ab}	6.8 ± 0.2^{a}

Remark: Mean ± SD from triplicate determinations
Different superscripts in the same column indicate significant differences ($p < 0.05$).

et al., 2010). *Lactobacillus paraplantarum* L-ZS9 is a probiotic starter isolated from fermented sausage and it is a great producer of class II bacteriocins (Liu & Li, 2016; Zhang et al., 2016). There are many *Pediococcus* strains that produce pediocin, an effective antilisterial bacteriocin (Porto et al., 2017). However, the use of these two bacteria strains, *L. paraplantarum* and *P. Pentosaceus,* as the starter in Pla-som fermentation has been scarcely studied and reported.

3. Pla-som fermentation and sensory evaluation

According to the highest pathogen inhibition, *L. paraplantarum* ST2 and only one member of

Pediococci group, *P. pentosaceus* TT3, were used as starters for Pla-som production. In all Pla-som fermentation with and without inoculation of the starter culture, the numbers of LAB are significantly different (Table 3). Comparison between with and without inoculation of the starter culture, the total viable count in Pla-som without the starter inoculation is significantly higher than Pla-som with the inoculation of the starter culture. On the other hand, the viable lactic acid bacteria in Pla-som without the starter is significantly lower than Pla-som with the starter culture. The result suggest that *L. paraplantarum* ST2 and *P. pentosaceus* TT3 remarkably inhibited the growth of contaminating bacteria as reported by previous studies (Hwanhlem et al., 2011; Visessanguan et al., 2006; Tasaku et al, 2017).

The initial pH and titratable acidity of all the samples were 6.0 and 0.35%, respectively. During the fermentation process, all samples inoculated with the starter culture, both of single culture or mixed culture, exhibited lower pH and higher acidity than natural fermentation without the inoculated starter culture. These samples showed a significant difference from 48 h of fermentation onwards (Fig.1). The manufacturing time of each production could be shortened from 72-96 h to within 48 h and the consistency of acidity and pH levels could be controlled resulting in the qualified Pla-som products, which would be commercially advantageous.

The acceptability of Pla-som inoculated with LAB starter culture was determined by comparing the results of colour, odour, flavour, texture and overall acceptance. The results showed that Pla-som products inoculated with LAB starter were not significantly different from natural fermentation or without the inoculated starter (Table 4). The sensory evaluation was determined with 4-day fermented products, Pla-som inoculated with the mixed starter culture showed the highest overall satisfaction rating of 3.54 from 5.00 satisfaction score.

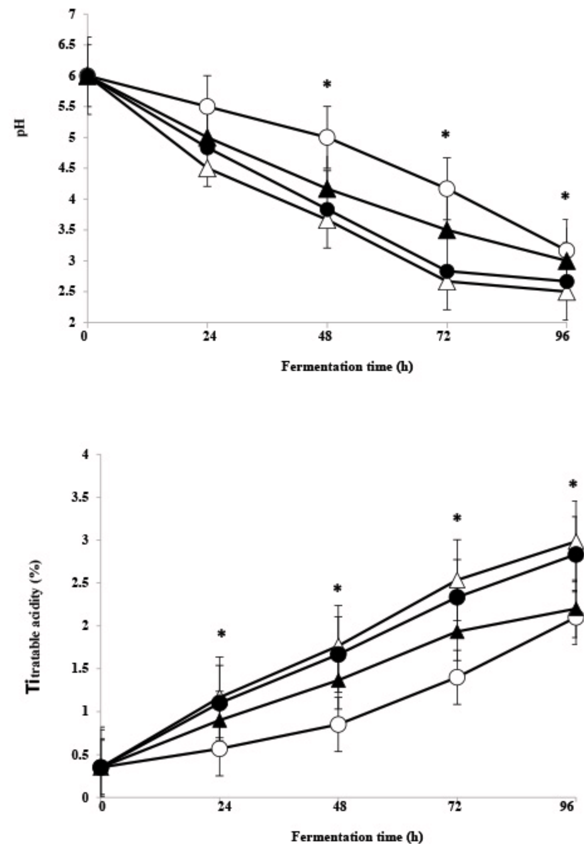

Fig. 1 pH (A) and titratable acidity (B) during Pla-som fermentation. Symbol: open circle, natural fermentation (control); open triangle, inoculated with *L. paraplantarum* ST2; closed triangle, inoculated with P. pentosaceus TT3; and closed circle, inoculated with mixed starter culture.
Error bars represent standard errors, and an asterisk denotes a significant difference between the events within a condition ($p<0.05$).

Finally, both bacterial strains, *L. paraplantarum* ST2 and *P. pentosaceus* TT3, isolated from this study are relatively safe. They were isolated from many food types and were identified as the unharmful species for consumers. The antimicrobial activity of ST2 and TT3 strains against food-borne pathogens contaminated in

Table 4 Sensory characteristics of Pla-som after 4 day fermentation.

	Colour	Odour^{ns}	Flavour	Texture	Overall acceptance
Without starter	3.71± 0.09^{a}	4.10±0.17	3.89±0.19^{a}	2.54±0.11^{b}	3.48±0.06^{a}
Strain ST2	3.37±0.07^{b}	4.20±0.12	3.28±0.25^{bc}	3.03±0.18^{a}	3.47±0.07^{a}
Strain TT3	3.27±0.13^{b}	3.89±0.10	3.23±0.28^{c}	2.94±0.19^{ab}	3.20±0.11^{b}
Mixed starter culture	3.48±0.17^{ab}	3.94±0.10	3.83±0.17^{ab}	3.09±0.13^{a}	3.54±0.14^{a}

Remark: Mean ± SD from triplicate determinations
Different superscripts in the same column indicate significant differences ($p < 0.05$).
ns; not significant

raw materials and food products is demonstrated as a good feature of the strains, which can be further developed as a standard starter used in the fermentation process. Furthermore, the standardization of Pla-som production by ST2 and TT3 strains as a mixed starter culture would definitely upgrade the spontaneous Pla-som fermentation traditionally practiced by the local population.

Conclusion

In this study, lactic acid bacteria (LAB) were isolated from 8 Pla-som samples in Phayao Province. Antimicrobial activity of the bacterial isolates against food-borne pathogens was analyzed. After acid neutralization and H_2O_2 elimination, bacteriocin, which is proteinaceous compound, was positively tested by using pepsin and trypsin. The bacterial strains, ST2 and TT3, were experimentally selected as the starters in Pla-som fermentation. The results show that the pH of the culture inoculated with such selected starters rapidly decreased and the acidity increased within 48 h. The manufacturing time of each production could be shortened from 72-96 h to within 48 h. Besides, the bateriocin in selected bacterial strains was also able to stop pathogenic microorganisms in Pla-som fermentation. Our findings are beneficial to the development and standardization of the Pla-som production from the household level to the reliable SME level.

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