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Selection of Lactic Acid Bacteria Isolated from Thai Traditional Fermented Foods to use as Starter Cultures Based on Antioxidant Activity and no Histidine Production

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Abstract: Four strains of Lactic Acid Bacteria (LAB), isolated from various Thai traditional fermented foods, were investigated on free radical scavenging activity of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic Acid (ABTS). Antioxidant activity by all isolated LAB in Intracellular Cell Free Extracts (ICFE) was higher than in intact cells. The LAB strains FF and FB, isolated from fermented foods (fish and sliced beef), their (ICFE) showed the highest radical- scavenging activity for DPPH and ABTS at 65% and 60%, respectively for the former strain. The detection of amino acid decarboxylase of LAB were investigated by growing on decarboxylase medium and quantitatively evaluated by confirmation of amine forming capacity using an HPLC procedure. In this work, all tested LAB strains were tyramine producer; however, the weakest strain was FF followed by strain FB. In contrast, all of them did not produce histamine. Based on 16S rRNA genes, strain FF was identified as *Lactobacillus fermentum*, while strain FB was *Lactobacillus plantarum*. Both are potential starter cultures for producing fermented meat sausages.

Keywords: *Antioxidant, histamine, Lactobacillus fermentum, Lactobacillus plantarum, tyramine*

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I. INTRODUCTION

Antioxidant substances that inhibit or prevent the rate of reaction of cellular oxidisable substrates by scavenging free radical and Reactive Oxygen Species (ROS). The pollution, several normal metabolic processes and UV radiation have been associated with the induction of high level of ROS. DPPH and ABTS radicals are widely used as relatively quick method for evaluating free radical-scavenging activity [1]. Some LAB strains have been found to impart important function such as antioxidant activities. Therefore, antioxidants from natural sources are more desirable. It has long known that some lactobacilli

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possess antioxidative activity and are able to decrease the risk of accumulation of ROS [1, 2, 3, 4].

Biogenic Amines (BA) are organic substances derived through decarboxylation of amino acids such as tyramine and histidine. Tyramine and histamine are the most serious BA in the fermented foods due to their toxicological effects in human include, increasing or decreasing blood pressure, headaches, flushes, nausea and cardiac palpitations and particularly relevant for food safety. It is also reported that high level of tyramine (100800 mg/kg) in foods is known to be toxic; while recommended histamine level in fermented foods i.e., fermented meat should not exceed 100 mg/kg. In addition, the most frequent risk of BA is their high concentrations accumulated in fermented foods of uncontrolled microbial activity as no starter cultures [5, 6, 7].

The production of BA has been found in several groups of microorganisms, mainly yeasts, gram-negative and gram-positive bacteria. Gram-positive bacteria, especially Lactic Acid Bacteria (LAB), being mainly responsible for the production of BA in fermented foods [8]. Some LAB such as *Lactococcus lactis* subsp. *cremoris, Streptococcus thermophilus and Lactobacillus delbrueckii subsp. bulgaricus* produce tyramine; however, they did not produce histamine [6, 9].

The aims of this study were to select LAB isolated from variety of Thai traditional fermented foods, that were able to produce high antioxidative activity based on scavenging activity on DPPH and ABTS radicals, and investigating biogenic amine production (tyramine and histidine) with the aim to use them as starter cultures for producing fermented meat sausages.

II. MATERIALS AND METHOD

A. Selection of LAB Strains

To isolate LAB, a 50 g of each fermented sample was added into 450 ml of normal saline (0.85% NaCl) and mixed well by using a stomacher for 30 sec. Supernatant was collected and plated on MRS agar containing 0.004% bromcresol purple and 0.02% sodium azide, and incubated at 35°C for 48 h. Four LAB strains (FS, FM, FB and FF), isolated from Thai fermented foods; shrimp, mushroom, sliced beef and fish respectively, were used in this study as they are a good producer for γ-Aminobutyric Acid (GABA). These strains were maintained in MRS supplemented with 20% glycerol at -20°C. Each strain was subcultured at least three times consecutively using a 1% (v/v) inoculum in MRS broth at 37°C for 18 h before use.

B. Antioxidant Activity of the LAB in Vitro

1) Preparation of cells and cell free extracts: Four LAB strains used were grown in MRS broth at 35°C for 18 h. The bacterial cell were harvested by centrifugation (8,000g, 4°C for 10 min), for the preparation of intact cells, cells pellets were washed three times with phosphate buffer solution (PBS: 0.85% NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4) and resuspended in PBS. For the preparation of intracellular cell-free extracts, cell pellets were washed twice with deionized water and re-suspended in deionized water to make the bacterial cell counts to 107 108 and 109 CFU/ml. The cell free extracts were prepared by the method of [3]. The cells (107, 108 or 109 CFU/ml) were lysed by incubating with 1 mg/ml lysozyme at 37°C for 30 min followed by ultrasonic disruption. The sonication was performed for 5 min (50 sec on/ 10 sec off pulse; at 60% amplitude). To obtain supernatants as the cell-free extracts of the LAB strains; the cell debris was removed by centrifugation at 10,000 g, 4°C for 10 min.

2) DPPH radical-scavenging assay: The DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical-scavenging activity was measured according to the method as described by [3]. Briefly, DPPH solution (0.4 mmol/L) was mixed with 100 μl of intact cells or intracellular cell free-extracts (107, 108 or 109 CFU/ml). The control included only deionized water and DPPH solution. The blanks as contained only ethanol and the cell, after which the mixture was incubated at room temperature in the dark for 30 min. The absorbance of the mixture was measured in triplication at 517 nm. The scavenging ability was calculated as:

DPPH radical-scavenging activity (%) = [1 - (Asample - Ablank) / Acontrol] x 100.

3) ABTS radical-scavenging activity: The ABTS radical-scavenging activity of the LAB strains was determined according to the method of [10] ABTS was dissolved in water to 7 mM with 2.45 mM aqueous solution of potassium persulphate and the mixture was conducted in the dark at room temperature for 16 h prior to use. As ABTS solution was diluted with ethanol an absorbance of 0.70 $\pm$ 0.02) at 734 nm and equilibrated at 30°C exactly 6 min after initial mixing. A 1 ml of different concentration cell free extracts was mixed with 4 ml of ethanolic solution of ABTS and absorbance was measured at 734 nm using a spectrophotometer after 6 min. The ABTS activity was calculated by using formula:

ABTS radical-scavenging activity (%) = [1 - (A_c - A_s) / A_c] x 100.

Where A_c is the absorbance of the blank without cell free extract, and A_s is the absorbance in the presence of the extract.

C. *Tyrosine- and Histidine-decarboxylase Activity of Isolated LAB Strains*

Four strains of LAB as previously mentioned were used in this study. In order to promote the enzyme induction before the actual screening test. They were subcultured 5 to 7 times in MRS broth containing 0.1% of each precursor amino acid (all from ACROS OGANICS, United Kingdom), comprising tyrosine free base or histidine monohydrochloride, in addition to supplementation with pyridoxal-5-phosphate in the medium (at 0.005%). Bromocresol purple was used as pH indicator at 0.006%. The pH was adjusted to 5.3 and the medium was autoclaved for 10 min at 121°C [11]. All isolates were inoculated in duplicate on the Decarboxylase Agar Medium (DCM) with and without amino acid (negative control, tyrosine and histidine) and incubated for 4 days at 35°C. Positive result was recorded when a purple color occurred or amino acid precipitate disappeared around the colonies.

D. *HPLC Confirmation of an Ability of the Isolated LAB to Produce Biogenic Amine*

A 0.2 ml of each LAB grown in MRS broth containing precursors, amino acids and pyridoxal-5-phosphate, was inoculated into decarboxylase broth containing either 0.5% tyrosine or 0.25% histidine. After incubating at 35°C for 4 days under anaerobic conditions by overlaying with paraffin, culture broths were centrifuged at 10,000 g 4°C for 5 min. The derivatization proceed as follows: 500μl of supernatant (or standard) was mixed with 100 μl of saturated Na_2CO_3 (pH adjusted to 11.2), 2 ml of internal standard (1, 7-diaminoheptane; Sigma-Aldrich) was added and the mixture was vortexed for 1 min. Derivatization proceeded for 40-45 min in dark at 40°C [12]. Then, an aliquot (200μl) of NH4OH was added into the tube to stop the reaction and incubated at room temperature for 20 min. The solid residue was dissolved in 1 ml of acetonitrile (ACN). Separation after 1, 7-diaminoheptane derivertization was carried out by gradient elution with milli Q water/acetonitrile on a Nova Pack C18 column (150mm x 3.9 mm, particle size 4μm) at the flow rate of 0.8 ml/min and measured using a photometric UV/Vis detector at 254 nm.

E. *Identification of the Isolated LAB*

The genomic DNA was extracted using the Power Soil DNA kit (MOBIO, USA) from 2 ml of the MRS culture broth of each LAB. The 16S rDNA gene was amplified from a single isolated colony using 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) primers. The PCR amplification was carried out in a final volume of 25 *mu*l. The reaction volume consisted of 10 mM of each primer, 1 U of Taq DNA polymerase, DNA template (approx. 10 ng) and iProof^{TM} High-Fidelity Master Mix (BIORAD, CA) in a thermal cycler (T100^{TM}, BIO-RAD, Singapore). Amplicons were obtained with a PCR cycling program of 95°C for 5 min, 30 cycles at 95°C for 30 sec, 60°C for 2 min, 72°C for 1 min and a final 5 min extension at 72°C [13]. The PCR products were separated in 1.5% agarose gel and observed on a Geldoc/UV transilluminator. The purified PCR products were sequenced with primer 520F (5′-CAG C (A/C)G CCG CGG TAA T(A/T)C-3′) using an automate DNA sequencer (3100-Avant Genetic Analyzer, ABI, Carlsbad, CA, USA). The purified PCR products were sequenced using an automate DNA sequencer at First Base Laboratories SdnBhd, Malaysia. The partial 16S rDNA sequences were compared with GenBank database on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) by BLASTN. CLUSTAL W was used for multiple alignments and a phylogenetic tree was constructed using MEGA 6 [14].

F. *Statistical Analysis*

All experiments were performed in triplicate ($n = 3$). Means and standard deviations are presented. One-way Analysis of Variance (ANOVA) was used to analyze data and statistical differences between means were compared with Duncan's multiple comparison test at $p < 0.05$.

III. RESULTS

A. *Antioxidant Activity of LAB Stains in Vitro*

1) DPPH radical-scavenging activity: The results of DPPH radical-scavenging activity with the intact cells and intracellular cell free extracts of the tested LAB strains are shown in Fig. 1. Four LAB strains demonstrated DPPH scavenging activity from the cell density ranged from 107 to 109 CFU/ml and increased with increasing concentration of cells. Strains FF and FB demonstrated maximum DPPH scavenging activity of intracellular cell-free extracts as 65% and 61% respectively, at concentration of 109 CFU/ml compared with strains FM and FS as only 55% (Fig. 1A, 2A). This activity of intracellular cell-free extracts was higher than that of intact cells.

2) ABTS radical-scavenging activity: Results of ABTS radical-scavenging activity of the four LAB strains were generally similar to those observed for DPPH radical-scavenging activity. The activity of ABTS shows the maximum absorbance at 734 nm in ethanol. As shown in Fig. 1B, the intracellular cell-free extracts of FF and FB strains with cell concentration at 109 CFU/ml reached up ABTS scavenging activity to 60% and 58% respec-

tively, (Fig. 1B). The intact cells of FF and FB strains at 109 CFU/ml showed ABTS scavenging activity 50% and 48% respectively, (Fig. 2B). It means that the values of intact cells and intracellular cell-free extract of FF and FB strains for ABTS scavenging activity were significantly different ($p < 0.05$).

B. Tyrosine- and Histidine-decarboxylase Activity of Isolated LAB

Positive reaction for, the histidine- and tyrosine-decarboxylase activities were recorded as a purple color occurred or amino acid precipitate disappeared around the colonies in the decarboxylase agar medium. A negative result is indicated by the change of color to yellow. Table 1 shows all isolated LAB strains gave a positive result for only tyrosine-decarboxylase activity when tested by the screening procedure. In contrast, no production of histamine for all strains tested.

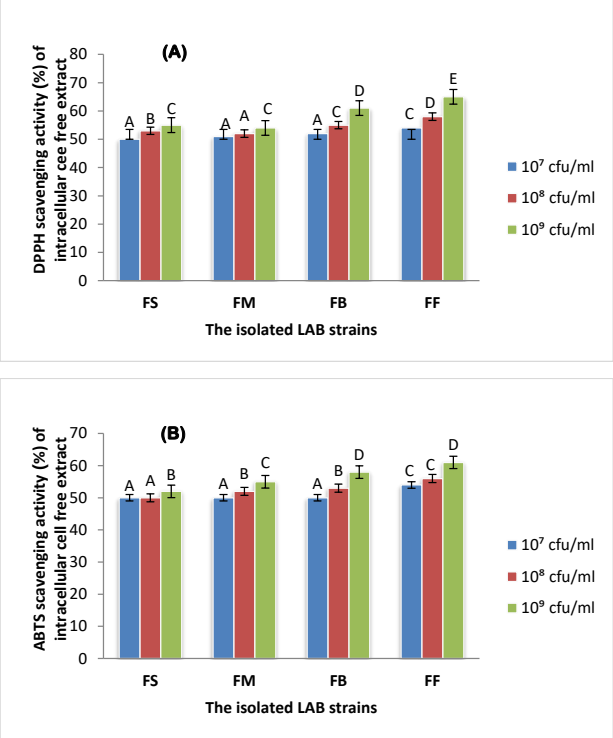

Fig. 1. Antioxidant activities of intracellular cell free extracts of the isolated LAB strains (A) DPPH free radical-scavenging activity, (B) ABTS radical-scavenging activity. FS, isolated from fermented Shrimp, FM, isolated from fermented mushroom, FB isolated from fermented sliced beef, FF, isolated from fermented fish. Results are mean ± *S.D.* ($n = 3$).

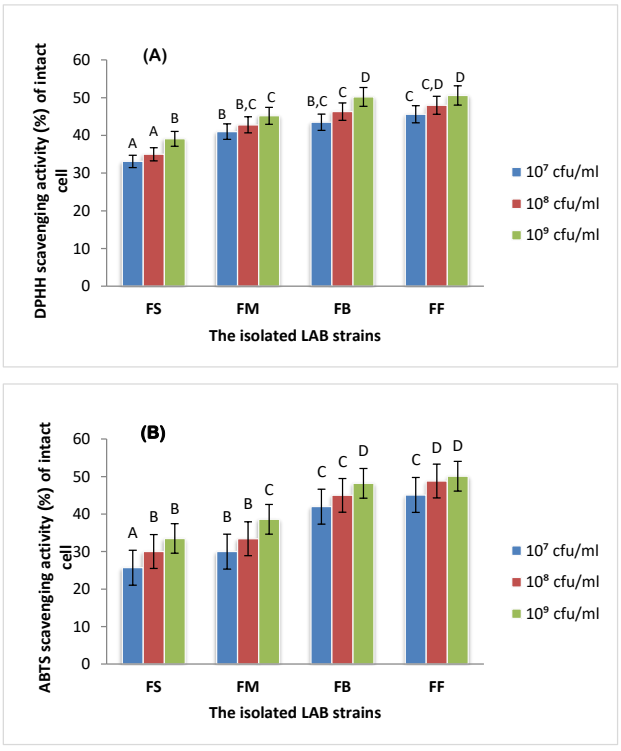

Fig. 2. Antioxidant activities of intact cell of the isolated LAB strains (A) DPPH free radical-scavenging activity, (B) ABTS radical-scavenging activity. FS, isolated from fermented shrimp; FM, isolated from fermented mushroom; FB isolated from fermented sliced beef; FF, isolated from fermented fish. Results are mean ± *S.D.*

C. Confirmation of Biogenic Amine by HPLC

Four LAB strains were confirmed their production of BA (tyramine and histidine) by HPLC analysis. Table 2, shows the quantitive production of tyramine in four LAB strains with the highest found in strains, FS and FM at 37.72 mg/l and 37.43 mg/l, respectively. However, the results of strains FF showed the lowest tyramine production (14.42 mg/l), and strain FB was 17.44 mg/l. In addition, no histamine production was detected in all supernatants of the FS, FM, FB and FF strains.

Based on the results strains FF and FB were selected, this is because they could be considered as good starter cultures for controlling BA production in the fermented meat sausages.

TABLE 1
BIOGENIC AMINE (BA) PRODUCTION BY THE ISOLATED LAB STRAINS: DETERMINATION WAS MADE AFTER CULTIVATION AT 37°C FOR 4 DAYS ON DECARBOXYLASE AGAR MEDIUM (DCM)

LAB strain	BA production on decarboxylase agar medium	
	Tyramine	Histamine
FS	+	-
FM	+	-
FB	+	-
FF	+	-

+, - : positive and negative results of biogenic amine production

TABLE 2
CONTENTS OF BIOGENIC AMINES (BA) PRODUCED BY THE ISOLATED LAB STRAINS AFTER CONFIRMATION BY HPLC

LAB strain	Biogenic amine production (mg/l)	
	Tyramine	Histamine
FS	37.72 ± 1.05^a	nd
FM	37.43 ± 1.08^a	nd
FB	17.44 ± 0.88^b	nd
FF	14.42 ± 0.24^c	nd

Mean and its standard deviation of three determinations are presented.
Values with the different lowercase letters in the same column are significantly different at $p < 0.05$. nd: not detectable

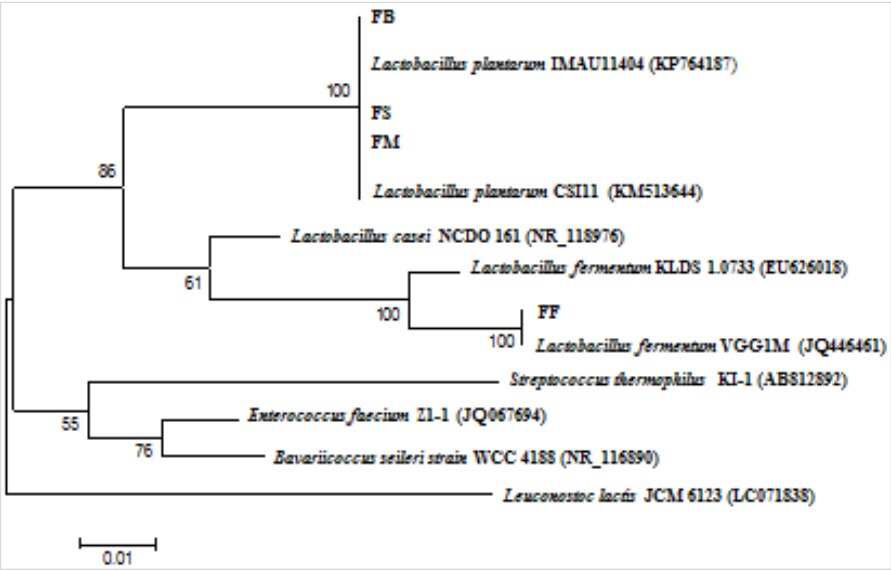

Fig. 3. Phylogenetic tree of four LAB strains based on 16S-rRNA gene sequences analysis. The percentage of bootstrap is indicated for each node. Scale bar represents the number of substitutions per site.

D. Bacterial Identification

Results of phenotypic identification corresponded well with genotypic results (Fig. 3). The 16S rDNA sequence analysis of strains FS, FB and FM were 100% similarity to *Lactobacillus plantarum* and strain FF was 100% similarity to *L. fermentum*. Based on the Genbank database, comparison with strain FB was isolated from a fermented sliced beef had the closest relative to *L. plantarum* (KP764187). On the other hand, strains FS (isolated from fermented shrimp), and FM (isolated from

fermented mushroom) were very close to *L. plantarum* (KM513644). And strain, FF (isolated from fermented fish) was very close to *L. fermentum* (JQ446461).

IV. DISCUSSION

Based on the antioxidant activity and biogenic amine production, *L. fermentum* FF isolated from fermented fish and *L. plantarum* FB isolated from fermented slice beef, were the most suitable LAB for consideration to be used as starter cultures (Fig. 1 and 2, Table 2). It is well recognized that some LAB strains show antioxidant activities and are able to decrease the risk of accumulation of ROS [2, 3, 15]. Both intact cells and intracellular cell free extracts were used to measure the ability of DPPH and ABTS radical- scavenging activity. The effect of antioxidant on DPPH radical-scavenging is encountered aproton-donating substance such as an antioxidant, the radical would be scavenged and the absorbance is reduced [10]. This scavenging shows a maximum absorbance at 517 nm in ethanol and change in color from purple to yellow [1]. The results revealed that the DPPH radical-scavenging activity of all 4 isolated LAB strains increased the activity with follow up increasing concentration of cells in the range of 107 109 CFU/ml (Fig. 1A, 2A).

Results of ABTS radical-scavenging activity of the isolated LAB from various fermented foods were generally similar to those observed for DPPH radical-scavenging activity. For instance, at intracellular cell free extract concentration at 109 CFU/ml, strains FS, FM, FB and FF exerted ABTS radical scavenging activity of 52-61%. The antioxidative activities of all LAB isolated from Thai traditional foods were dependent on the cell concentration (Fig. 1A and 1B), and this is in agreement with the report by [3]. The radical scavenging activities from cell free extracts may be a result of intracellular antioxidant enzymes [16]. The results indicate that they are potent beneficial LAB.

However, among the four screened strains, *L. fermentum* FF showed the greatest probiotic potential due to its high free radical scavenging activity all of DPPH and ABTS. The results of this study indicate that potent LAB strains have antioxidative ability.

Biogenic amine production has been most extensively studied with focus to tyramine and histamine. This is because tyramine and histamine are considered as the most toxic and particularly relevant for food safety. Results in this study are in agreement with the fact that fermenting LAB is occasionally associated with tyramine formation (Tables 1 and 2) [11].

The positive tyramine decarboxylase activity of four isolated LAB were continuously maintained over time. (Table 2). Both LAB strains FF and FB were produced small amounts of tyramine with no production of histidine. Hence, they could be considered as safe for using as starter cultures [17].

V. CONCLUSION

In conclusion, this study obtained *L. fermentum* FF and *L. plantarum* FB that showed strong antioxidant activity (DPPH and ABTS radical-scavenging activity) and no production of histamine with weak tyramine production. Hence, both LAB strains could be considered as potential starter cultures for producing functional foods, particularly fermented meat sausages.

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