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Probiotic Properties of Lactic Acid Bacteria Isolated from Fermented Food

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***Abstract*—**The objectives of this study were to isolate LAB from various sources, dietary supplement, Thai traditional fermented food, and freshwater fish and to characterize their potential as probiotic cultures. Out of 1,558 isolates, 730 were identified as LAB based on isolation on MRS agar supplemented with a bromocresol purple indicator&$CaCO_3$ and Gram-positive, catalase- and oxidase-negative characteristics. Eight isolates showed the potential probiotic properties including tolerance to acid, bile salt & heat, proteolytic, amylolytic & lipolytic activities and oxalate-degrading capability. They all showed the antimicrobial activity against some Gram-negative and Gram-positive pathogenic bacteria. Based on 16S rDNA sequence analysis, they were identified as *Enterococcus faecalis* BT2 & MG30, *Leconostoc mesenteroides* SW64 and *Pediococcus pentosaceous* BD33, CF32, NP6, PS34 & SW5. The health beneficial effects and food safety will be further investigated and developed as a probiotic or protective culture used in Nile tilapia belly flap meat fermentation.

***Keywords*—**Lactic acid bacteria, pathogen, probiotic, protective culture.

I. Introduction

LACTIC ACID BACTERIA (LAB) are microorganisms that play a major role in the production of fermented foods and have been used widely to improve food safety with additional health benefits. Many LAB are able to promote fermentation by utilization of nutrients available in food materials and produce a variety of substances such as organic acids, aromatic compounds, health benefit-substances, etc [1]. In addition, several LAB species are regarded as probiotics, live microorganisms which when administered in adequate amounts confer a health benefit on the host [2], [3]. However, such effects are likely strain dependent [4]. Many antimicrobial peptides are produced by LAB and their applications for the inhibition of food spoilage and pathogenic bacteria without toxicity has received much more attention [5]. Thus, selection of probiotics is primarily considered on their stability, safety and health benefits.

Nile tilapia belly flap meat contains a high quantity of organic matters such as amino acids, saturated & unsaturated fatty and several minerals [6]. Fermentation of Nile tilapia belly flap meat with LAB probiotics could be very feasible since this food material was rich in nutrients for LAB growth. The ability of LAB to produce several health-benefit substances was also very useful to the consumers. Therefore, fermentation of Nile tilapia belly flap meat with LAB probiotics could provide the potential approach for the development of fermented food rich in healthy LAB by-products. The objectives of this study were to isolate and identify LAB from various sources and to characterize their potential probiotic properties.

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II. Materials and Methods

A. Isolation of LAB

LAB were isolated from dietary supplement, traditional Thai fermented fish (Pla-som, Naem-Pla, Spawn, Fish sausage), Thai fermented pork, Nile tilapia and Mae Kong fish. Briefly, 10g of sample were added into 90ml 0f 0.85% normal saline, homogenized using a blender, spread on the MRS (de Man, Rogosa and Sharpe) agar with 0.004% bromocresol purple and 1% $CaCO_3$ and incubated at 30°C for 48hrs.

B. Morphology and Biochemical Tests

Preliminary identification was based on Gram staining, catalase, and oxidase tests. Carbohydrate fermentation was tested in MRS containing a bromocresol purple indicator and different sugar including glucose, lactose, maltose, fructose, sucrose, mannitol, sorbitol and inulin. Gas production was detected via Durham tube. For temperature tolerance, LAB isolates were dropped on the MRS agar and incubated at 30, 37, 42 and 50°C, 24-48hrs.

C. Potential Probiotic Properties

Proteolytic, lipolytic and amylolytic activities were tested by streaking on the selective agar medium containing skim milk, tributyrin and various types of starch (Soluble, Glutinous rice, Rice and Tapioca starch) and incubated at 30°C, 7 days. After flooded with iodine solution for 15-30min, amylolytic activity was detected by clear zone surrounding the culture.

1. Acid Tolerance: LAB isolates were dropped on MRS agar adjusted to pH 4, 5 and 6 and incubated at 30°C, 24-48 hrs.
2. Bile Salt Tolerance: LAB isolates were dropped on MRS agar containing 0.3 and 5% (w/v) bile salts and incubated at 30°C, 24-48hrs.
3. Oxalate-Degrading: LAB isolated were dropped on MRS agar containing 10mM disodium oxalate or oxalic acid dehydrate in the absence of glucose and incubated at 30°C, 24-48hrs.

4. Antimicrobial Activity: LAB isolates were tested for antimicrobial activity against several bacterial pathogens by growing the LAB on MRS, overlaying with LB agar and swabbing with pathogen cultures. After incubation at 30°C, 48 hrs, the inhibition zone was measured [3].

D. Molecular Identification of LAB

All 8 selected isolates were identified by PCR using two pairs of primers, universal *E. coli* primers, 20F (5′-GAG TTTGATCCTGGCTCA-3′) and 1500R (5′-GTTACCTTG TTACGACTT-3′) and LAB specific primers, LB-F (5′-AGAAGAGGACAGTGGAAC-3′) and LB-R (5′-TTACAAACTCTCATGGTGTG-3′).

E. Growth of LAB in Nile Tilapia Belly Flap Meat

A 100g of ground Nile tilapia belly flap meats were inoculated with 1mL *Pediococcus pentosaceous* NP6 or *Leconostoc mesenteroides* SW64containing 10^6 cells in the absence and presence of 1% NaCl incubated at 30°C. After 2 days, numbers of viable LAB were selected and counted by a spread plate on the MRS agar supplemented with appropriate antibiotics.

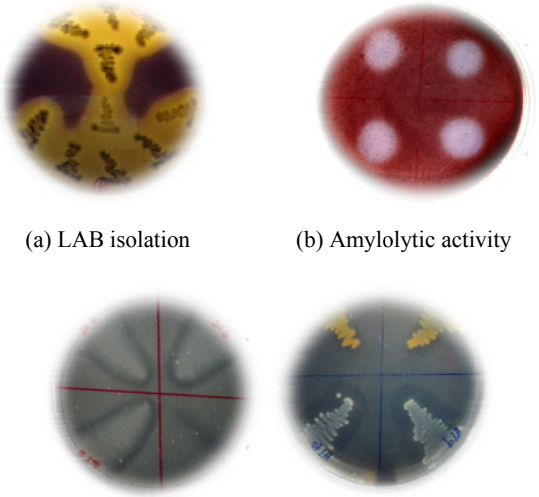
III. Results and Discussion

Total of 1,558 yellowed colonies surrounding with clear zone (Fig. 1 (a)) on MRS medium supplemented with bromocresol purple and $CaCO_3$were selected from dietary supplement, fermented foods and freshwater fish. Based on the Gram-positive staining, catalase-negative and oxidase-negative results, 730 isolates were potentially identified as LAB.

Eight isolates were selected according to the following probiotic properties; enzymatic activity including amylolytic (Fig. 1 (b)), proteolytic (Fig. 1 (c)), and lipolytic (Fig. 1 (d)) activities, tolerance to the strong acid conditions at pH 2 up to 10 and 24 hours in MRS broth and prebiotic inulin broth, respectively (Table I). They were all tolerant to 0.3-5% bile salt and 1-14% NaCl and able to ferment several sugars: lactose, maltose, fructose, sucrose, mannitol, sorbitol andinulin detected by the changing of broth color from purple to yellow and gas in the Durham tube (Fig. 2 (a)). In addition, they showed γ-hemolytic activity suggesting that they do not lyse red blood cell and could be safe for further applications (Fig. 2 (b)). They were heat-resistant and able to grow up to 50°C. Their oxalate-degrading capability also indicated the potential health benefit for the reduction of stone formation.

These 8 selected isolates were identified by 16S rDNA as *Enterococcus faecalis* (BT2, MG30), *Leconostoc mesenteroides* SW64 and *Pediococcus pentosaceous* (BD33, CF32, NP6, PS34, SW5). As shown in Table II, the antimicrobial activity showed that they inhibited growth of some pathogenic bacteria including Gram-negative *Pseudomonas aeruginosa* ATCC27853 (*PA*), *Salmonella typhimurium* ATCC13311 (*ST*), *Vibrio cholera* non 01/0139 (*VC*), *Escherichia coli* ATCC25922 (*EC*), and Gram-positive *Bacillus cereus* ATCC 11778 (*BC*) and *Staphylococcus epidermidis* ATCC12228 (*SE*).

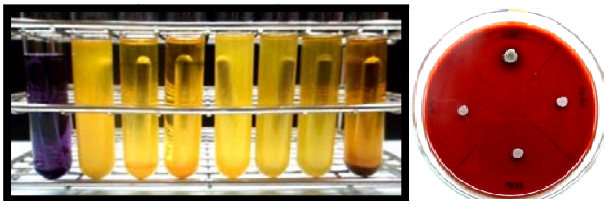
When NP6 and SW64 were inoculated on the ground Nile tilapia belly flap meats and incubated for 2 days, the bacterial cells were still slightly detected both in the absence or presence of NaCl (Fig. 3). The results indicated that these two strains were able to grow and utilize complex nutrients available in fish meat. The raw-fermented sausages have been shown as the suitable environment for the proper growth and survival of probiotic *Lactobacillus casei* LOCK0900 [7]. Thus, the LAB isolates used in this study may be suitable for preparation as the probiotic cultures or starters in raw-meat that can be potentially used in food applications.



(a) LAB isolation (b) Amylolytic activity

(c) Proteolytic activity (d) Lipolytic activity

Fig. 1 (a) Isolation of LAB on MRS agar supplemented with bromocresol purple and $CaCO_3$. (b) Amylolytic activity on starch agar. (c) Proteolytic activity on skim milk agar. (d) Lipolytic activity on tributyrin agar



(a) (b)

Fig. 2 (a) Carbohydrate fermentation and gas production. (b) γ-hemolytic activity on Columbia blood agar

TABLE I
Acid Tolerance and Enzyme Activity of LAB

Strain	MRS*	Inulin*	Enzyme activity**
E. faecalis MG30	10	0	L
E. faecalis BT2	10	6	A, P, L
P. pentosaceous BD33	3	6	A, P
P. pentosaceous CF32	5	6	L, P
P. pentosaceous NP6	5	24	L
P. pentosaceous PS34	3	24	L, P
P. pentosaceous SW5	5	24	L
L. mesenteroides SW64	4	0	L

*Media in whichsurvival time (Hour) are measured at pH2
**A: Amylolytic activity, L: Lipolytic activity, P: Proteolytic activity

TABLE II
ANTIBACTERIAL ACTIVITY OF LAB AGAINST GRAM-NEGATIVE AND GRAM-POSITIVE PATHOGENIC BACTERIA

Strain	*PA*	*ST*	*VC*	*EC*	*BC*	*BS*	*SE*
E. faecalis MG30	-	-	-	-	-	-	-
E. faecalis BT2	+	+	+	-	+	+	+
P. pentosaceous BD33	+	+	+	-	+	+	+
P. pentosaceous CF32	+	+	+	-	+	+	+
P. pentosaceous NP6	+	+	+	-	++	-	+
P. pentosaceous PS34	+	+	+	-	+	-	++
P. pentosaceous SW5	+	+	+	-	++	-	+
L. mesenteroides SW64	+	+	+	-	-	-	-

PA: Pseudomonas aeruginosa ATCC27853
ST: *Salmonella typhimurium* ATCC13311
VC: *Vibrio cholera* non 01/0139
EC: *Escherichia coli* ATCC25922
BC: *Bacillus cereus* ATCC 11778
SE: *Staphylococcus epidermidis*ATCC12228

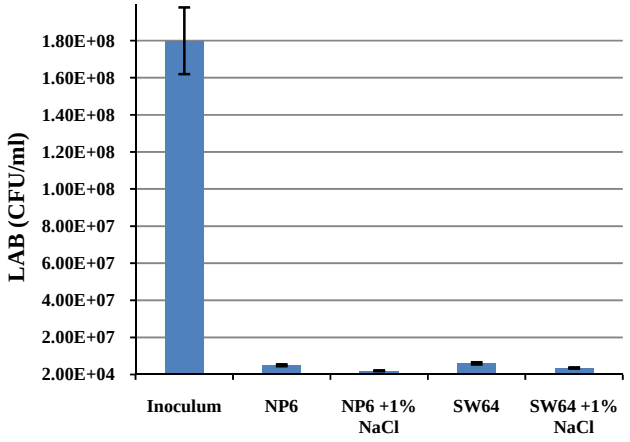


Fig. 3 Growth of *P. pentosaceous* NP6 and *L. mesenteroides* SW64 in ground Nile tilapia belly flap meats

IV. CONCLUSIONS

A total of 730 LAB isolates were isolated and selected from various traditional Thai fermented foods and freshwater fish. Eight isolates showed the most potential probiotic properties. They are tolerant to heat, acid, bile salt, and NaCl suggesting the ability to survive through the stringent conditions in the stomach and small intestine. The production and secretion of the extracellular enzymes, amylase, lipase or protease indicate their capability to utilize various nutrients as carbon or nitrogen sources. The antibacterial activity against some pathogenic bacteria demonstrates the production of bioactive substances that could be used to inhibit growth of spoilage and pathogenic microorganisms contaminated in food products. Based on several properties of *P. pentosaceous* observed in this study which are known as the bacteriocin-producing bacteria, these species could be further developed as the probiotic or protective cultures used in the Nile tilapia or fish meat fermentation.

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