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Antiaflatoxigenic Effect of Lactic Acid Bacteria Isolated From Some Thai Fermented Foods

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ABSTRACT

Eighty – seven isolates of lactic acid bacteria (LAB) were isolated from some Thai fermented foods. They comprised *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Lactococcus*, *Streptococcus*, and *Enterococcus*. Each of these isolates was tested for its antifungal activity against *Aspergillus flavus*. Of eighty – seven LAB isolates, the two isolates with high antifungal activity were chosen for further identification. They were identified as *Lactobacillus plantarum* and *L. brevis*. The effects of LAB supernates on growth and aflatoxin production in culture of *A. flavus* grown in malt extract broth for 14 days at 25°C were also studied. Both of them showed inhibitory ability on growth and aflatoxin production in cultures.

Key words: lactic acid bacteria, Thai fermented foods, antiaflatoxigenic

INTRODUCTION

Aflatoxins are secondary metabolites produced by *Aspergillus flavus*, *A. flavus subsp. parasiticus*, and *A. nomius* in various foods and agricultural commodities. Aflatoxins have been shown to be toxigenic, carcinogenic, mutagenic, and teratogenic to different species of animals (Campbell and Stoloff, 1974). Aflatoxin B_1 is the most potent hepatocarcinogen in many animal species (Chu, 1977). Aflatoxins have been reported to be produced in cereal grains, peanuts, tree nuts, figs, seeds and fermented products including cheese and fermented meats such as salami, sausage, and country cured hams (Gourama and Bullerman, 1995). Therefore, the presence of aflatoxins in foods presents a potential hazard to human health.

Lactic acid bacteria (LAB) are gram positive, non – sporulating microaerophilic bacteria whose main fermentation product from carbohydrates is lactate. They comprise both cocci and rods. LAB are commonly found in foods including fermented meat, vegetables, fruits, beverages and dairy products (De Vuyst and Vandamme, 1994). They are also of paramount importance in food technology because of their contribution to flavour and aroma development and to spoilage retardation. The inhibition of growth of food spoiling bacteria can be due to one or more of the antibacterial substances produced by LAB namely organic acids, hydrogen peroxide and proteinaceous substances with a bactericidal or bacteriostatic mode of action, such as bacteriocins (Vandenbergh, 1993). The antibacterial effects of LAB and their metabolites are well documented and have been extensively

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investigated, but more research on the antifungal effects is needed (Klaenhammer, 1988; Stonsaovapak *et al.* , 1994).

The present study was undertaken to demonstrate the inhibition of aflatoxitgenic fungi by LAB isolated from some Thai fermented foods, in order to reduce the health hazard of aflatoxins. The inhibition of toxigenic molds by LAB could be of great public health significance.

MATERIALS AND METHODS

Mold culture

The organism used in this study was *Aspergillus flavus*, obtained from the Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. It was maintained on slants of potato dextrose agar (PDA ; Merck, Darmstadt) at 4°C until further use.

Preparation of spore suspension

Culture of the fungi was grown on PDA slants for 7 to 10 days at 25°C until well sporulated. The spores were harvested by adding 10 ml of sterile water and aseptically dislodging the spores with a sterile inoculating loop. Spore suspensions were aseptically filtered through sterile cheesecloth to remove mycelial debris. The spore suspensions were further adjusted with sterile water to give a final spore concentration of approximately 10^4 spores / ml. The spore concentration was determined on PDA plates using standard pour plate technique at 25°C for 2 to 3 days. The PDA used in this study was not acidified, and the pH of this medium after sterilization was 5.6 ± 0.2

Bacterial strains and culture conditions

LAB used in this study were isolated from locally available Thai fermented foods including fish and shellfish, pork, vegetables, and rice (Table 1). 25 g of each sample was added to 225 ml of sterile phosphate buffer pH 7.0 and thoroughly homogenized for 1 minute in a stomacher. 0.1 ml of the appropriate dilutions were spread onto the surface of MRS plates (MRS medium; Merck, Darmstadt) and incubated for 3 days at 30°C. Colonies of LAB were randomly selected from MRS plates. They were propagated and maintained in MRS broth.

Preparation of culture supernatants

For preparation of culture supernatants, the randomly selected isolates LAB were grown in MRS broth at 30°C for 18 h without shaking. Cells were removed by centrifugation at 8,000 rpm for 10 minutes, followed by filtration of the culture supernatants through a 0.45 μm pore size filter (Millipore).

Table 1 Thai fermented foods used in the experiment.

Vegetable	Fish and shellfish	Pork	Rice
Pak-Sian-Dong	Pla-Som	Nham	Khao-Mak
Naw-Mai-Dong	Pla-Paeng-Daeng	Sai-Krok-Prieo	
Pak-Kard-Dong	Pla-Ra		
Tua-Ghog-Dong	Nam-Bu-Du		
Sa-Tau-Dong	Kung-Som		
Hom-Dong	Tai-Pla		
	Kung-Jom		
	Hoi-Dong		

Screening of antifungal activity by LAB

Approximately 10^4 spores / ml of the *A. flavus* (indicator organisms) were used for testing the antagonistic activity. *A. flavus* was spread with a swab on PDA plates, 5 µl of LAB culture supernantant was spotted on freshly prepared lawn of *A. flavus* (Mayr – Harting *et al*., 1972). After incubated plates for 3 – 5 days at 25°C, the plates were checked for inhibition zones. The culture supernatant which were qualified as positive were neutralized to pH 7 with 0.1 N NaOH and were tested again for antifungal activity. LAB that had high antifungal activity were further identified and were used in inhibition test.

Identification of LAB

LAB were identified by gram staining, catalase test, cell morphology, growth at selected temperatures, thermal resistance, and carbohydrate fermentation pattern (Weiss, 1992).

Inhibition test of fungal growth and aflatoxin production

Tests for inhibition of fungal growth and aflatoxin production were assayed in flask cultures. One millilitre of the final spore suspension containing about 10^4 spores / ml of *A. flavus* was inoculated in 125 ml conical flasks containing 10 ml of malt extract broth (Merck, Darmstadt). Different concentrations of selected LAB supernate were added to the flask. The flask cultures were then incubated for 14 days at 25°C. After 14 days of incubation, the survival of mold growth were determined on PDA plates. Duplicate samples were taken for each assay, and experiments were replicated three times to reduce variability

Determination of approximate aflatoxin content in the cultures were carried out according to the method reported by Bullerman *et al*., (1977). Flasks were samples aseptically by removing duplicate portions of 0.1 ml of broth for aflatoxin determination. The culture filtrate was extracted with 5 ml of chloroform by liquid / liquid extraction. Total aflatoxin content was then determined by measuring UV absorption at 362 nm using a spectrophotometer and calculating total aflatoxin content using the molar extinction coefficient of 21,800 reported for aflatoxin B (Asao *et al*., 1963). The approximate aflatoxin content was calculated from three replicate experiments.

RESULTS AND DISCUSSION

Eighty – seven isolates of lactic acid bacteria were isolated from locally available Thai fermented foods including vegetables, fish and shellfish, pork and rice. The genus of all LAB isolates were identified according to the method described by Weiss (1992). The results showed that LAB isolated from Thai fermented foods were *Lactobacillus* (73 isolates), *Pediococcus* (6 isolates), *Leuconostoc* (2 isolates), *Lactococcus* (2 isolates), *Streptococcus* (2 isolates), and *Enterococcus* (2 isolates) (Table 2).

The results showed that many kinds of LAB can be found in Thai fermented foods. It was also shown that Lactobacillus was the predominant genus among LAB isolated in this study. This finding agreed with those of Charernjiratrakul and Rodpradit (1997), who studied on the isolation and identification of lactic acid bacteria from Thai fermented foods.

Each of these isolates were tested for antifungal activity against *A. flavus* by using spot-on-lawn assay. The result showed that of the 87 LAB isolates studied, 47 isolates did not show any inhibitory ability against *A. flavus*. Twenty – three isolates had low inhibitory ability producing inhibition zones of less than 6 mm. Ten isolates were considered to have low inhibitory ability too, by producing 6-10 mm inhibition zones. Five isolates were considered to have moderate inhibitory ability producing 10-18 mm inhibition zones. Two isolates had high inhibitory ability producing inhibition zones of more than 20 mm (Table 3).

Table 2 The genus of lactic acid bacteria isolated from Thai fermented foods.

Genus	Number of LAB	
	Isolates	%
Lactobacillus	73	83.90
Pediococcus	6	6.90
Leuconostoc	2	2.30
Lactococcus	2	2.30
Streptococcus	2	2.30
Enterococcus	2	2.30

Table 3 Antifungal activity of lactic acid bacteria isolated from Thai fermented foods against *A. flavus.*

Number of isolates	*A. flavus*
	Inhibition zone
47	-
23	+
10	++
5	+++
2	++++

- = no inhibition zone
+ = inhibition zone of less than 6 mm
++ = inhibition zone of 6 – 10 mm
+++ = inhibition zone of 10 – 18 mm
++++ = inhibition zone of more than 20 mm

When the pH of the culture supernatants of the two isolates which gave high inhibitory were adjusted to 7 with 0.1 N NaOH before testing their antifungal activity, the supernatants remained their ability to inhibit the growth of *A. flavus*. It indicated that fungal inhibition was not due to the lactic acid produced.

Antifungal activities of LAB had been reported by some investigators (Batish *et al*., 1991), and the inhibitory compound was polypeptide.

Two isolates of LAB with high inhibitory ability were morphologically, physiologically, and biochemically characterized and identified according to the method described by Weiss (1992). The result showed that L1 isolate identified as *Lactobacillus plantarum* and L2 isolate was *L. brevis* (Table 4).

The effects of L1 and L2 supernates on growth and aflatoxin production in culture of *A. flavus* grown in malt extract broth for 14 days at 25°C were presented in Table 5. No growth or aflatoxin could be detected in the culture to which 10% of L1 supernate had been added, whereas slightly growth and trace of aflatoxin production were obtained when 10% of L2 supernate was added to the cultures.

It has also been reported previously that Lactobacillus cell-free supernatant inhibited the production of aflatoxin (Karunaratne *et al*., 1990). It was shown that the aflatoxin inhibition was probably due to an inhibitory metabolite other than hydrogen peroxide and low pH. The aflatoxin reduction was due to low-molecular-weight inhibitory compounds. Partial purification and characterization of the inhibitor showed that it was a heat-stable compound.

From the available literature and also this study on the effect of LAB on mold growth and mycotoxin production, it would appear that LAB have the potential as biological control agents in foods to prevent mold growth. The antifungal biopreservatives have the potential to constitute suitable food preservatives that are safe, effective, and acceptable to consumers, regulatory agencies,

Table 4 Morphological, physialogical, and biochemical characteristics of the 2 isolates of lactic acid bacteria having high inhibitory ability.

Characteristics	Lactic acid bacteria isolates	
	L1	L2
Morphology	Rod	Rod
Gram stain	+	+
Catalase test	-	-
Growth at 15°C	+	+
Gas production	-	+
Hydrolysis of arginine	-	+
Fermentation of		
Arabinose	-	+
Cellubiose	+	-
Esculin	+	-
Galactose	+	+
Gluconate	+	+
Glycerol	-	-
Inulin	-	-
Lactose	+	+
Maltose	+	+
Mannitol	+	-
Melezitose	+	-
Melibiose	+	+
Raffinose	+	-
Rhamnose	-	-
Ribose	+	+
Salicin	+	-
Sorbitol	+	-
Sucrose	+	-
Trehalose	+	-
Xylose	-	+
	L. plantarum	*L. brevis*

and the food industries. Many reports showed that the inhibition of mycotoxins by LAB was due to factors other than acidity, and there is a strong indication that some inhibitory compounds are protein in nature. It is recommended that in future studies more research is needed to purify and identify inhibitory compounds.

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Table 5 Growth and aflatoxin production of *A. flavus* grown in malt extract broth containing inoculum level of 10^4 spores / ml and inhibitor (selected LAB supernate) after 14 days of incubation at 25°C.

Inhibitor	Concentration (%)	*A. flavus*	
		Log CFU/g	Aflatoxin (µg/ml)
Control		6.80 ± 0.02[a]	2.75 ± 0.10[a]
L1 supernate	1	4.02 ± 0.03	1.84 ± 0.04
(*L. plantarum*)	5	2.28 ± 0.01	0.08 ± 0.02
	10	NG[b]	ND[c]
L2 supernate	1	5.00 ± 0.03	2.02 ± 0.06
(*L. brevis*)	5	3.28 ± 0.08	1.25 ± 0.04
	10	1.20 ± 0.01	1.08 ± 0.01

[a] Values are mean ± SD, n = 3

[b] NG, no growth

[c] ND, not detected

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