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Antibiotic Resistance of Lactic Acid Bacteria Isolated from a Fermented Fish Product, *Pla-chom*

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ABSTRACT

Lactic Acid Bacteria (LAB) have been isolated from various fermented food products in Thailand. However, the antimicrobial susceptibility of the microbes in fermented food products are a matter of concern in several countries. The aim of this study was to determine the antimicrobial susceptibility of LAB strains isolated from the Thai traditional fermented small fish product, *pla-chom*. The concentrations of LAB were also studied. The antimicrobial susceptibility and resistance of 10 representative LAB strains to 8 antibiotics were investigated using the disk diffusion method. The antibiotics used for the tests included penicillin, ampicillin, erythromycin, tetracycline, vancomycin, streptomycin, sulfamethoxazole-trimethoprim and metronidazole. The average concentration of LAB, when the product was fermented at 30°C for 3 days, was 2.5×10^6 CFU g^{-1} of product. The inhibition zone diameters of all antibiotics were between 0 and 30 mm for all the LAB strains isolated and tested. All 10 LAB isolates were resistant to vancomycin, streptomycin, sulfamethoxazole-trimethoprim and metronidazole. The LAB strains were highly resistant to tetracycline (7 isolates), to penicillin (6 isolates), but showed low resistance to ampicillin (2 isolates). All 10 LAB isolates were sensitive to erythromycin. Three LAB isolates were sensitive to all four antibiotics (penicillin, ampicillin, erythromycin and tetracycline), which are commonly used in the treatment of bacterial infections in humans and animals. These three LAB isolates could possibly be used as starter cultures for this fermented product.

Key words: Antibiotic resistance, Commensal bacteria, fermented small fish product, *pla-chom*

INTRODUCTION

Thai traditional fermented foods are produced by natural fermentation of various foods, included fishery products (*nam-pla*, *ka-pi*, *bu-du*), fermented fish (*pla-ra*, *pla-som*, *pla-chao*, *som-fak*, *pla-chom*), meat products (*nham*, *sai-krog-prieo*, *mum*) and plant products (*naw-mai-dong*, *phak-gard-dong*, *miang*, *khao-mak*, *khanom-jeen*) (Tanasupawat, 2009). *Pla-chom* is most popular in the north-eastern part of Thailand. It is produced from small raw fish mixed with ground roasted gelatinous rice (*khaao khuaa*), salt and garlic. *Pla-chom* is typically fermented at room temperature between 28-30°C for 3 days.

Lactic Acid Bacteria (LAB) are a large group of beneficial bacteria that produce lactic acid as an end product of food fermentation. Currently, there is concern about the possible spread of antibiotic resistance from antibiotics used for the inhibition of pathogenic microorganisms, or from beneficial microbes occurring naturally in foods. LAB from fermented products may act as a reservoir of antibiotic resistance genes that could be transferred to pathogenic bacteria or normal flora in the gastrointestinal tract of humans and animals (Florez *et al*., 2005). Antibiotic resistance of LAB is comprised of two characteristics: (1) natural or intrinsic resistance, being nontransmissible; (2) acquired resistance, usually caused from bacterial mutation and possibly carrying plasmid encoding of antibiotic resistance genes and which may potentially be transmissible to other bacteria (Courvalin, 2006). There is little information about the antibiotic susceptibility of commensal bacteria such as LAB isolated from fermented food products and no data exist about antibiotic susceptibility in LAB from the fermented small fish product, *pla-chom*. The present study investigated the antibiotic susceptibility profiles of LAB isolated from *pla-chom* products.

MATERIALS AND METHODS

Sample collection and isolation of Lactic Acid Bacteria (LAB): In February 2011, twenty samples of the fermented fish product, *pla-chom* were collected from local markets in the area of amphur Muang, Khon Kaen province. *Pla-chom* is made from small raw fish. Ten kilograms of fish were mixed with garlic, salt and ground gelatinous rice and then fermented at 30°C for 3 days. Ingredient proportions of the *pla-chom* product are shown in Table 1. Samples were kept in air-tight plastic bags at 4°C. To find the volume LAB, the samples were divided into 5 g portions that were dissolved into 45 mL of Maximum Recovery Diluent solvent (Oxoid Inc., Hampshire, UK) and agitated for 30 sec. Serial tenfold dilutions from the homogenate were made according to ISO-6887-1 (1999) and plated in De Man Rogosa and Sharpe (MRS) agar (De Man *et al*., 1960), with modification by adding 0.4% (w/v) $CaCO_3$ using the pour plate method. Incubation was carried out aerobically for 2 days at 37°C as described in ISO-15214 (1998). Bacterial colonies were enumerated in terms of their growth (30-300 colonies) by the appearance of the surrounding acid-produced clear zone on the plate. Bacterial concentrations were calculated (volume of bacterial colonies x dilution factor) and expressed as colony- forming units per gram of sample (CFU g^{-1}). The different morphologies of bacterial colonies from each sample were gathered and stored in a modified freezing medium (Criterion™ tryptic soy broth, 0.6% yeast extract and 20% glycerol) at -70°C for further testing.

Cell morphologies of bacteria were checked using Gram staining. The isolates were tested for a catalase reaction. Rod and coccal cells of bacteria determined to be catalase negative and Gram positive were characterized as LAB (Salminen *et al*., 2004) and were selected for further testing.

Antimicrobial susceptibility test: The representative LAB isolates were further tested for antimicrobial susceptibility by the disk diffusion method (Bauer *et al*., 1966). Eight antibiotics

Table 1: Ingredients used in *pla-chom* processing

Ingredients	Total weight
Small fish (raw)	10 kg
Garlic	500 g
Salt	100 g
Ground roasted gelatinous rice	50 g

commonly used in treating human or animal infections (European Food Safety Authority, 2008) and from different antibiotics classes were chosen for the test. They were penicillin G (10 μg), ampicillin (10 μg), erythromycin (15 μg), tetracycline (30 μg), vancomycin (30 μg), streptomycin (10 μg), sulfamethoxazole-trimethoprim (25 μg) and metronidazole (50 μg). All antibiotic disks (diameter = 6 mm) were obtained from Oxoid. The antimicrobial susceptibility test was similar to that described by Charteris *et al.* (1998). Briefly, each LAB isolate was inoculated with 10^8 CFU (turbidity of 0.5 Mac-Farland standard) at 37°C in MRS broth and incubated anaerobically for 18 h. A sterile cotton swab was dipped in each culture solution and swabbed in three directions on a Mueller-Hinton agar plate. All antibiotic disks were seeded in the plates and incubated anaerobically at 37°C for 48 h. The diameters of antibiotic inhibition zones were measured using a ruler under a colony-counter apparatus (Weiss-Gallenkamp, UK) and expressed in mm which included the diameter of the antibiotic disk. Antimicrobial susceptibility was interpreted according to the cut-off levels proposed by Charteris *et al.* (1998), with strains considered resistant if inhibition zone diameters were equal to or smaller than 19 mm for penicillin G and ampicillin, 14 mm for vancomycin and tetracycline and 13 mm for erythromycin. Inhibition zone diameters equal to or smaller than 8 mm for streptomycin, sulfamethoxazole-trimethoprim and metronidazole were considered to indicate resistance, according to the cut-off levels (with minimal modifications) of Reddy *et al.* (2007). All antibiotics were tested in duplicate.

RESULTS AND DISCUSSION

Lactic acid bacteria isolation: Lactic acid bacteria were successfully isolated from *pla-chom* using a selective medium of MRS agar modified by adding 0.4% (w/v) $CaCO_3$. A clear zone appeared surrounding the bacterial colonies, caused by a reaction to the colonies'acid production. The colonies were confirmed to be LAB by cell morphology, Gram staining and catalase reaction. Previous researchers had attempted to develop a selective medium for lactobacilli (Jackson *et al.*, 2002). In this study, a selective medium (MRS agar) was developed to easily isolate LAB. Characteristics of 14 bacterial isolates and concentrations of 10 representative LAB strains are shown in Table 2 and 3, respectively.

Table 2: Characteristics of 14 bacterial isolates from the fermented fish product, *pla-chom*

Bacteria isolates	Cell form	Gram straining	Catalase
PC1	Rods	+	-
PC2	Rods	+	-
PC3	Rods	+	-
PC4	Rods	+	-
PC5	Rods	+	-
PC6	Rods	+	-
PC7*	Rods	-	-
PC8	Rods	+	-
PC9*	Rods	+	+
PC10	Rods	+	-
PC11	Rods	+	-
PC12*	Cocci	+	+
PC13	Rods	+	-
PC14*	Rods	-	-

+: Positive reaction, -: Negative reaction, *: Isolates were excluded from the basic properties of LAB

Table 3: Concentration of 10, representative LAB strains isolated from the *pla-chom*

	Average number of colony[1] at various dilutions			
No. of sample	10^{-3}	10^{-4}	10^{-5}	Average LAB count (CFU g^{-1})
10	nc[2]	310	25	2.5×10^{6}

[1]count with triplicate plates, [2]numerous colonies

Table 4: Interpretative zone diameters (mm) of 8 antibiotics used on 10 representative LAB strains selected from the fermented fish product, *pla-chom*

	Antibiotics							
Bacteria strains	P (10 μg)	AMP (10 μg)	E (15 μg)	TE (30 μg)	VA (30 μg)	S (10 μg)	SXT (25 μg)	MTZ (50 μg)
PC1	26 (S)	28 (S)	23 (S)	17 (S)	0 (R)	0 (R)	0 (R)	0 (R)
PC2	17 (R)	17 (R)	23 (S)	12 (R)	0 (R)	0 (R)	0 (R)	0 (R)
PC3	30 (S)	30 (S)	26 (S)	17 (S)	0 (R)	0 (R)	0 (R)	0 (R)
PC4	19 (R)	23 (MS)	24 (S)	14 (R)	8 (R)	7 (R)	0 (R)	0 (R)
PC5	17 (R)	28 (S)	24 (S)	14 (R)	0 (R)	0 (R)	0 (R)	0 (R)
PC6	17.5 (R)	20 (MS)	25.5 (S)	8 (R)	0 (R)	8 (R)	0 (R)	0 (R)
PC8	28 (S)	28 (S)	25 (S)	18 (S)	0 (R)	0 (R)	0 (R)	0 (R)
PC10	29 (S)	29 (S)	26 (S)	17 (S)	0 (R)	0 (R)	0 (R)	0 (R)
PC11	17.5 (R)	21 (MS)	23 (S)	13 (R)	0 (R)	7 (R)	0 (R)	0 (R)
PC13	18 (R)	17 (R)	25 (S)	8 (R)	0 (R)	0 (R)	0 (R)	0 (R)

Susceptibility expressed as: (R): Resistant, (MS): Moderately susceptible, (S): Sensitive. P: Penicillin, AMP: Ampicillin, E: Erythromycin, TE: Showed pinpoint colonies within the inhibition zone tetracycline, VA: Vancomycin, S: Streptomycin, SXT: Sulfamethoxazole-trimethoprim and MTZ: Metronidazole

Antimicrobial susceptibility: Lactic acid bacteria such as *Lactobacillus* sp., *L. farciminis* and *L. pentosus* have been isolated from *pla-chom* (Tanasupawat *et al*., 1998) but no data exist about their antibiotic susceptibility. In this study, the results of antimicrobial susceptibility are shown in Table 4. All LAB isolates showed intrinsic mechanisms of resistance to vancomycin, sulfamethoxazole-trimethoprim, metronidazole and aminoglycoside antibiotics (streptomycin). Although LAB strains are species-dependent for intrinsic resistance to sulfamethoxazole-trimethoprim (Klare *et al*., 2007), all LAB strains in our study showed such resistance to the drug, which may derive from certain antagonistic components such as p-aminobenzoic acid (PABA) and thymidine (Turnidge and Bell, 2005). The intrinsic resistance to vancomycin demonstrated by the LAB strains in this study was likely due to the presence of D-alanine ligase, an enzyme that can inactivate vancomycin (Elisha and Courvalin, 1995). Some LAB strains in this study showed acquired resistance to penicillin, ampicillin and tetracycline. These strains may have received their antibiotic resistance by spreading from bacteria had survived antibacterial treatment of animal infections. LAB in this study showed lower resistance to penicillin (60%) than in the study by Savadogo *et al*. (2010) who reported that LAB strains isolated from fermented milk in Burkina Faso, Africa, showed 78.94% penicillin resistance. *Lactobacillus* spp. isolated from chicken feces have been reported to have 36.37% tetracycline resistance (Sornplang and Leelavatcharamas, 2010). In addition, there was an isolate (PC1) showed pinpoint colonies within the inhibition zone with tetracycline. These characteristics demonstrated the tendency of the bacteria to mutate and develop to be a resistance to antibiotics (Kipp *et al*., 2004). In this study, LAB isolated from *pla-chom* was highly resistant to tetracycline (70%). Three selected LAB isolates

(PC3, PC8, PC10) were intrinsically resistant to vancomycin, sulfamethoxazole-trimethoprim, metronidazole and streptomycin but were sensitive to all four antibiotics (penicillin, ampicillin, erythromycin and tetracycline), commonly used in the treatment of bacterial infections in humans and animals. Therefore, these three LAB isolates can be safely applied and could possibly be used as starter cultures in this fermented fish product.

CONCLUSION

Although, lactic acid bacteria are a large group of bacteria isolated from traditional fermented small fish product (*pla-chom*) in present study, there are only some strains that are sensitive to antibiotics commonly used in animals. The selection of these bacterial strains used for the starter culture in food fermentation is an alternative that will give consumers confidence and not to eat products containing lactic acid bacteria to be the reservoirs transferred an antibiotic resistance genes to other bacteria. Further research, should be tested for sensitivity to the antibiotics in the other traditional Thai fermented foods that from animal origins.

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