PE9.24 An in-vitro screening of isolated bacteriocin-producing lactic acid bacteria from Thai fermented meat for probiotic prospect 163.00

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***Abstract* - A probiotic is a culture of living microorganisms mainly lactic acid bacteria (LAB) or bifidobacterium. The colonisation of these strains in human and animals' gut prevents the growth of harmful bacteria by competitive exclusion and by the production of organic acids and antimicrobial compounds such as bacteriocins. In order to act as a probiotic, a strain must be able to survive the acidic conditions in the stomach and resist the bile acids at the beginning of small intestine. Since there are many LAB strains, which isolated from thai fermented meat products and revealed to produce bacteriocins. Thus, the survival or tolerant in the low pH and the presence of bile salts in MRS of 6 strains of bacteriocin-producing LAB isolated from traditional thai fermented meat products such as pediocin PA-1 producer from Nham (*Pediococcus pentosaceus* TISTR 536) and Mum (*P. pentosaceus* M 13), pediocin-like producer from meat-rice sausage (*Lactobacillus plantarum* RS54), plantaricin W producer from Nham pla (*Lb. plantarum* NF3) and nisin Z producers from Nham (*Lactococcus lactis* strain N100 and N190) was studied. The results revealed that both strains of *P. pentosaceus* TISTR 536 and *P. pentosaceus* M 13 could grow in MRS broth under pH 4 – 8 and survive in MRS broth under pH 2 and 3 at 37^{o}C for 18 h, while the other strains showed no survival under pH 2 and 3 at the same incubated condition. Both of *P. pentosaceus* TISTR 536 and *P. pentosaceus* M 13 could also tolerate to the high concentration of bile salt in MRS broth (pH 8.0) up to 0.6 and 1.0 % respectively. These 2 strains implied the resistance to a wide range of clinical important antibiotic such as Gentamicin, Penicillin G Sulphametho-xazole and Oxacillin. The natural anti-biotics resistance of these LAB, especially the bacteriocin-producing strain *P. pentosaceus* TISTR 536 and M 13, may enable the development of antibiotic/ probiotic combination therapies for such conditions as diarrhea and gastro-intestinal tract infection when using these strains as functional foods or probiotics in traditional thai fermented meat products.**

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I. INTRODUCTION

The acid and bile tolerances are two fundamental properties that indicate the ability of a probiotic microorganism to survive the passage through the gastrointestinal tract, resisting the acidic conditions in the stomach and the bile acids at the beginning of small intestine (1, 2). The survival of bacteria in gastric juice depends on their ability to tolerate low pH. The pH of excreted HCl in stomach is 0.9. However, the presence of food raises the pH value to the level of pH 3. After the ingestion of food, it takes 2-4 h for the stomach to empty (3). Bile salts, which act as detergent-like substance, are synthesized from cholesterol in the liver, stored in the gall bladder, and released into the small intestine after ingestion of a fatty meal. This detergent is critical to microorganisms since their cell membranes are composed of lipids and fatty acids. However, some microorganisms are able to reduce this detergent effect by their ability to hydrolyse bile salts by bile salts hydrolase enzyme (BSH) and thus to decrease their solubility (3). BSH activity has been found in many species including *Lactobacillus* (4). However, the resistance to bile salts varies a lot between the *Lactobacillus* species and even between strains, and the mechanism is still unknown (3). The mean of bile salts as a critical concentration for the screening of a resistant probiotic strain is considered to be 0.3 % w/v (1, 5).

Since there are many LAB strains, which were isolated from thai fermented meat products (Nham) and revealed to produce bacteriocin-like inhibitory substances (BLIS) (6). Thus, the aim of this study was to select BLIS-producing strains isolated from thai fermented meat products which could survive or tolerate the low pH and the presence of bile salts in MRS broth for potential probiotics use in human and animals. In addition, antibiotic susceptibility test of all studied LAB was also included in this study.

II. MATERIALS AND METHODS

Microorganisms **:** (type of bacteriocins)
- *Lactococcus lactis* N100 and N190 isolated from Nham (nisin Z)
- *Lactobacillus plantarum* NF3 isolated from fermented fish (Nham pla) (plataricin W)
- *Lb. plantarum* RS54 isolated from fermented meat-rice sausage (pediocin-like)
- *Pediococcus pentosaceus* M 13 isolated from Mum (pediocin PA-1)
- *P. pentosaceus* TISTR 536 isolated from Nham (pediocin PA-1)

Medium **: MRS broth** (Merck) was used for culturing of all LAB strains, **MRS agar + 0.5 % $CaCO_3$** was used for LAB viable cells count and **MRS agar without $CaCO_3$** was used for study on antibiotic susceptibility for each studied LAB.

Effect of pH on the growth of LAB **:** A modified method of Erkkilä and Petäjä (3) was applied in this study. Approximately 10^6 cfu/ml aliquot of an overnight LAB cultured was inoculated into an experimental series of 10 ml MRS broth with the following pH : pH to 2, 3, 4, 5, 6 and 8 (adjusted using 8 M HCl and 5 N NaOH). The tubes were incubated at 37^o C for 18 h. The viable organisms in each pH broth were counted after 18 h of incubation on MRS agar + 0.5 % $CaCO_3$ and incubated for 48 h at 30^o C.

Effect of bile salts on the growth of LAB **:** A modified method of Gilliland et al. (4) and Erkkilä and Petäjä (3) was used in the study. Approximately 10^6 cfu/ml aliquot of an overnight LAB cultured was inoculated into an experimental series of 10 ml MRS broth (pH 8.0) with the concentration of 0, 0.3, 0.6 and 1.0 % bile salts. All tubes were incubated at 37^o C for 18 h. The viable organisms of each bile salts concentration in MRS broth were counted after 18 h of incubation on MRS agar + 0.5 % $CaCO_3$ and incubated for 48 h at 30^o C.

Antibiotic susceptibility test of LAB **:** Antimicrobial susceptibility of all studied LAB was determined by the disk-diffusion method with 9 clinically important anti-biotics from Oxoid (Gentamicin, Clinda-mycin, Cephalothin, Tetracycline, Chloram-phenicol, Penicillin G, Sulphamethoxalzole, Oxacillin and Erythromycin) in accordance with the standard of the NCCLS (7).

III. RESULTS AND DISCUSSION

Effect of pH on the growth of all studied LAB

An initial load of 10^6 cfu/ml of each strain from 3 isolated strains of potent bacteriocin-producing LAB from Nham [*P. pentosaceus* TISTR 536, *Lc. lactis* strain N100 and N190], Mum [*P. pentosaceus* M13] meat-rice sausage [*Lactobacillus plantarum* KMITL-QU 54] and Nham pla [*Lb. plantarum* NF3] were studied for ability to grow and tolerate acid in MRS broth under various pH (2 – 8) for 18 h at 37^o C (Table 1). The results revealed that at pH 4 the number of cell of both strains of *Lc. lactis* (N100 and N190) and *Lb. plantarum* (RS 54) still remained at inoculated level, but all strains could grow well in MRS broth under pH 5 - 8. *P. pentosaceus* TISTR 536 and M13 isolated from Nham and Mum, respectively, could grow in MRS broth under pH 4 – 8. Both strains of *P. pentosaceus* TISTR 536 and M13 could tolerate to the broth under pH 3 (a log cycle reduction of cell number) and pH 2 (cell number reduced from 10^6 cfu/ml to 10^2 cfu/ml) for 18 h of incubation at 37^o C, while the rest of 4 strains of *Lb. plantarum* (NF3 and RS54) and *Lc. lactis* (N 100 and N 190) showed no growth under pH 2 at the same incubation period.

The results revealed that the strains of *P. pentosaceus* TISTR 536 and M13 proved to be most acid tolerant. Little or no effect was seen at pH 3 with both strains and the cells still remained under pH 2 for 18 h. The results are similar to those obtained by Erkkilä and Petäjä (3) and Goldin et al. (8), who regarded *L. curvatus* (RM10), *P. acidilactici* (P2) and *Lactobacillus* GG as potentially probiotics. By this regard, it is implied that these 2 strains can be acted as probiotics.

Effect of bile salts on the growth of LAB

Effect of bile salts on the growth of all studied LAB (Table 2) revealed that all studied LAB strain could tolerate to 0.3 % of bile salts in MRS broth. *P. pentosaceus* TISTR 536 and M13 exerted the most tolerant to the higher concentration of bile salts (0.6 and 1.0 %). No effect was seen in MRS broth with 0.3 % bile salts after 18 h of incubation at 37^o C, while the cell number of other strains were reduced to 2-3 log cycle in the same bile salts concentration. There was no growth showed in the series broth of 0.6 and 1.0 % bile salts concentration for both strains of *Lc. lactis* (N100 and N190) and *Lb. plantarum* (NF3 and RS54). Regarded to the earlier report of Gilliland et al. (5) who stated that 0.3 % bile salts was the critical concentration for probiotics screening tolerant strains. Thus, all LAB strains in this study implied to be probiotics. However, from the aforementioned

results of acid tolerant and high concentration of bile salts tolerant, both strains of *P. pentosaceus* (M13 and TISTR 536) tend to be the best probiotics for human and animal when compared to the other strains.

Antibiotic susceptibility test of LAB

For antibiotic susceptibility test (Table 3), it was revealed that all studied LAB could resist to Sulphamethoxazole. *Pediococcus pentosaceus* TISTR 536 and M13 were the most resisted to a panel of tested antibiotics. The strain resisted to Gentamicin, Penicillin G, Sulphamethoxalzole and Oxacillin. Four strains of *Lb. plantarum* (NF3 and RS54) and *Lc. lactis* (N100 and N190) resisted only to Tetracycline and Sulphamethoxalzole.

IV. CONCLUSION

The study implied that two strains of *P. pentosaceus* TISTR 536 isolated from Nham and M13 from Mum can be used for humans and animals as probiotic traits, due to the survival of both strains in the acidic conditions as presence in the stomach and resist the bile salts as presence at the beginning of small intestine. The natural resistance of these LAB to a wide range of clinically important antibiotics may enable the development of antibiotic/probiotic combination therapies for such conditions as diarrhea and gastro-intestinal tract infection, when using these strains as probiotic products.

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Table 1 : Number of lactic acid bacteria (initial load of 10^6 cfu/ml) after incubation for 18 h at 30° C in MRS broth at various pH

Strain	pH 2	pH 3	pH 4	pH 5	pH6	pH 8
M13	100	1.7×10^5	$> 10^8$	$> 10^8$	$> 10^8$	$> 10^8$
TISTR 536	100	2.5×10^5	$> 10^8$	$> 10^8$	$> 10^8$	$> 10^8$
N100	0	14	1.0×10^6	$> 10^8$	$> 10^8$	$> 10^8$
N190	0	10	1.1×10^6	$> 10^8$	$> 10^8$	$> 10^8$
RS54	0	2.0×10^3	1.2×10^6	$> 10^8$	$> 10^8$	$> 10^8$
NF3	0	31	$> 10^8$	$> 10^8$	$> 10^8$	$> 10^8$

Table 2 : Number of lactic acid bacteria (initial load of 10^6 cfu/ml) after incubation for 18 h at 30° C in MRS broth pH 8 with 0, 0.3, 0.6 and 1.0 % of bile salts

Strain	bile salts concentration (%)			
	0	0.3	0.6	1.0
M13	$> 10^8$	6.0×10^6	1.2×10^4	140
TISTR 536	$> 10^8$	7.2×10^6	2.5×10^4	170
N100	$> 10^8$	5.5×10^4	0	0
N190	$> 10^8$	2.4×10^4	0	0
RS54	$> 10^8$	2.7×10^3	0	0
NF3	$> 10^8$	3.2×10^3	0	0

Table 3 : Antimicrobial susceptibility of LAB

Strain	antibiotic								
	P	OX	TE	DA	E	SXT	C	KF	CN
M13	R	R	I	I	S	R	S	S	R
TISTR 536	R	R	I	S	S	R	S	S	R
N100	S	S	R	S	I	R	S	S	I
N190	S	S	R	S	S	R	S	S	I
RS54	S	S	R	I	S	R	S	S	I
NF3	S	S	R	I	S	R	S	S	I

I = intermediate, R = resistant, S = sensitivity, P =Penicillin G, OX = Oxacillin, TE = Tetracycline, DA = Clindamycin, E = Erythromycin, SXT = Sulphamethoxalzole, C = Chloramphenicol, KF = Cephalothin, CN = Gentamycin