PE8.08 Screening of bacteriocin-like inhibitory substances from lactic acid bacteria for fermented meat starter culture 55.00

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***Abstract*—six hundred-thirty isolates of Lactic acid bacteria (LAB) were isolated from intestinal content of ducks, Thai native chickens, broilers, Japanese quails, pigs, cows and buffalo. There were five isolates produced bacteriocin-like inhibitory substance (BLIS). Three isolates (D4, D8 and D9) of ducks produced BLIS. Cell free supernatant (CFS) of D4 and D9 displayed antibacterial activity against *Lactobacillus sakei* subsp. *sakei* JCM 1157^T, *Lb. sakei* TISTR 890, *Lb. plantarum* ATCC 14917, *Leuconostoc mesenteroides* subsp. *mesenteroides* JCM 6124^T, *Leu. mesenteroides* TISTR 942, *Listeria innocua* ATCC 33090^T, *Brochotrix campestris* NBRC 11547 ^{T}, *Pseudomonas fluorescens* JCM 5693 and *P. fluorescens* TISTR 358. Moreover, CFS of D4 demonstrated more spectrums against *Lactococcus lactis* subsp. *cremoris* TISTR 1344, *Enterococcus feacalis* JCM 5803^T, *En. feacalis* TISTR 888 and *Bacillus coagulans* JCM 2257. However, CFS of D8 exhibited antibacterial activity against only *Lb. sakei* subsp. *sakei* JCM 1157^T, *Leu. mesenteroides* subsp. *mesenteroides* JCM 6124^T, *L. innocua* ATCC 33090^T and *P. fluorescens* JCM 5693^T. CFS of two isolates (B27 and B42) of broilers presented antibacterial activity against *Lb. sakei* subsp. *sakei* JCM 1157^T. In order to inhibitory activity, D4, D8 and D9 were selected to investigate for probiotic properties. It was found that all isolates were grown and their CFS showed antibacterial activity at pH range of 3-10, 0.3-0.9% bile salt and 1-8% NaCl when used *Lb. sakei* subsp. *sakei* JCM 1157^T and *L. innocua* ATCC 33090^T as an indicator strains.**

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I. INTRODUCTION

MEAT starter culture contained living or resting microorganisms that develop the desired metabolic activity in the meat [11]. It initiates rapid acidification of the raw meat batter and lead to improve tastier, safer and healthier of the end product. This is used for the production of fermented sausages. [4]. Recently, bacteriocin-producing LAB have received enormous application in food industry including fermented meat products [3,6]. Fermented sausages are non heated meat products, which may be suitable carrier for probiotics into human gastrointestinal tract [11]. A probiotic is a culture of living microorganisms mainly LAB [10] and LAB are normal residents of the gastrointestinal tracts [2]. The use of probiotic bacteria as fermenting agents in meat products is beginning to develop and the idea of using antimicrobial peptides such as bacteriocins [11]. In order to act as a probiotic, a strain must be able to survive in the acidic conditions in the stomach and resist the bile acid at the beginning of small intestine [10]. In addition, salts act as one of the first hurdles against the growth of unwanted microorganisms. It also induces the solubility and diffusion of myofibrillar proteins from muscle forming a gel between meat and meat as well as meat and fat particles of the raw sausage material. Salt (NaCl 2.5-3.0% (w/w), initial value) is also an important flavor component of the end product [11]. Therefore, the tolerant of BLIS-producing strains and BLIS production in the low pH, high concentration of NaCl and the presence of bile salt in MRS were studied.

II. MATERIALS AND METHODS

A. Isolation of LAB

LAB were isolated from intestinal content of ducks, Thai native chickens, broilers, Japanese quails, pigs, cows and buffalo. Twenty-five grams of sample was

homogenized in 225 ml peptone (0.1%) water and ten fold serially dilution was carried out. 0.1 ml of each dilution was spread on MRS (De Man, Rogosa and Sharpe broth; Merck, Germany) agar containing 0.5% $CaCO_3$ and subsequently incubated under anaerobic condition (candle jar) at 37°C for 48 h. Only clear zone producing colonies were selected and stored in 30% glycerol at -80°C for further study.

B. Screening of LAB strains for antibacterial activity

Six hundred-thirty isolates were examined for antibacterial activity by spot-on-lawn test [1]. CFS of LAB strain was adjusted to pH 6.8-7 and subsequently sterilized by boiling for 5 min. Antibacterial activities of BLIS was tested by spotting 10 µl of CFS onto the surface of agar plate which was overlaid with 5 ml of soft agar (1% w/v) seeded with 10 µl of freshly-grown indicator strains (about 10^7 cfu/ml). The antibacterial activity was expressed in an arbitrary unit (AU/ml). AU was calculated as (1000/10)D where D was the dilution factor [7]. List of indicator strains, media and their growth conditions was shown in Table 1.

C. Effects of pH, bile salt and NaCl on bacteriocin production

NaCl concentration 1-8%, bile salt 0.3, 0.6 and 0.9% were added to MRS broths. Similarity, effect of pH on production of bacteriocin was studied by adjusting pH of MRS broth to pH range of 2-10. BLIS production was evaluated by spot-on-lawn against *Lb. sakei* subsp. *sakei* JCM 1157^T and *L. innocua* ATCC 33090^T. The growth of LAB strains were determined by observing turbidity of cell growth.

III. RESULTS AND DISCUSSION

A. Screening of bacteriocin-producing LAB

Lactic acid Bacteria were isolated from animal intestines of ducks (108 isolates), Thai native chickens (74 isolates), broilers (45 isolates), Japanese quails (100 isolates), pigs (150 isolates), cows (69 isolates) and buffalo (30 isolates). Their antibacterial activities were determined. Among of these isolates, CFS (pH 6.8-7) of five LAB which three isolates (D4, D8 and D9) were distributed from ducks and another two (B27 and B42) were from broiler. Their CFS displayed antibacterial activity against indicator strains as shown in Table 1. This experimental result has shown the similar trend to another research that *Lb. salivarius* K4 and K7 isolated from broiler intestines produced BLIS. CFS of *Lb. salivarius* K4 showed antibacterial activity against *Lb. sakei* subsp. *sakei* JCM 1157^T, *Leu. mesenteroides* subsp. *mesenteroides* JCM 6124^T, *L. innocua* ATCC 33090^T, *En. feacalis* JCM 5803^T, *Br. campestris* NBRC 11547^T and *Bacillus coagulans* JCM 2257^T. Meanwhile, CFS of *Lb. salivarius* K7 revealed antibacterial activity against only *Lb. sakei* subsp. *sakei* JCM 1157^T, *Leu. mesenteroides* subsp. *mesenteroides* JCM6124^T and *B. coagulans* JCM 2257^T [8,9]. This paper is the first report on isolating BLIS-producing LAB from duck intestines.

B. Effects of pH, bile salt and NaCl on growth and BLIS production

As refer to board spectrum activity against indicator strains, therefore three isolates of D4, D8 and D9 were selected for probiotic properties. The results revealed that the growth and BLIS production of D4 and D9 were observed at wide pH range of 2-10 and 0.3%-0.9% bile salt in MRS broth. The highest antibacterial activity was shown at pH 7-9 and at 1% NaCl (Table 2). Moreover, both isolates could grow and produced BLIS at high concentrations of NaCl (8%).

D8 could tolerate and produced BLIS in pH range of 3-10, 0.9% bile salt and 1-8% NaCl. In addition, the turbidity and antibacterial activity of D8 were unable to observe in pH 2. However, BLIS production of D4, D8 and D9 decreased as increasing salt concentration and low pH (Table 2). It has been reported that NaCl interfered both cell growth and bacteriocin production [5]. Our experimental results have shown the similar trend to another research done by Adisorn *et al*. [10] who reported that *P. pentosaceus* TISTR 536 and *P. pentosaceus* M13, isolated from Thai ferment meat could grow in MRS broth under pH 4-8 and survived in MRS broth under pH 2 and 3 at 37°C. Moreover, both strains could also tolerate to the high concentration of bile salt in MRS up to 0.6 and 1.0%, respectively [10]. Also, *Lb. salivarius* K4 isolated from chicken intestines could grow and produce bacteriocin in wide pH range of 3-10 and up to 4% NaCl in MRS broth [8]. It can be concluded that these BLIS-producing LAB (D4, D8 and D9) isolates revealed probiotic properties that may

enable to use as starter culture for functional fermented meat products.

IV. CONCLUSION

Five BLIS-producing LAB isolated from animals intestinal content showed antibacterial activity against many indicator strains. Three isolates, D4, D8 and D9 were selected to study for probiotic properties. All strains could grow and produce BLIS under low pH and high concentration of NaCl and bile salt. Therefore, these strains would be potentially to be use as fermented meat starter culture.

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REFERENCES

[1] Ennahar, S., Sonomoto, K. & Ishizaki, A. (1999). Class IIa bacteriocins from Food Preservation. J. Biosci. Bioeng. 6,705-716.

[2] Erkkilä, S., & Petäjä, E. (2000). Screening of commercial meat starter cultures at low pH and in the presence of bile salts for potential probiotic use. Meat Sci, 55, 297-300.

[3] Huot, E.J., Meghrous, C., Barrena-Gonzalez, C., & Petitdemange, H. (1996). Bacteriocin J46, a new bacteriocin produced by *Lactococcus lactis* subsp. *cremoris* J46: isolate characterization of the protein and its gene. Anaerobe, 2, 137-145.

[4] Leroy, F., Verluyten, J., & De Vuyst, L. (2006). Functional meat starter cultures for improved sausage fermentation. Int. J. Food. Microbiol, 106, 270-285.

[5] Neysens, P., Messens, W and De Vuyst. L. (2003). Effect of sodium chloride on growth and bacteriocin production by *Lactobacillus amylovorus* DCE 471. Int. J. Food Microbiol, 88, 29-39.

[6] Noonpakdee, W., Santivarangkna, C., Jumriangrit, P., Sonomoto, K., & Panyim, S. (2003). Isolation of Nisin-producing *Lactococcus lactis* WNC 20 strain from *Nham*, a traditional Thai fermented sausage. Int. J. Food Microbiol. 81: 137-145.

[7] Parente, E., Brienza C., Moles M. and Ricciardi A. (1995). A comparison of methods for the measurement of bacteriocin activity. J. Microbiol. Methods 22: 95-108.

[8] Pilasombut, K., Wajjwalku, W., Nitisinprasert, S., Swetwiwathana, A., Zendo, T., Nakayama, J. Sonomoto, K., & Sakpuaram, T. (2005). Screening and characterization of bacteriocin producing lactic acid bacteria isolated from chicken intestine. Kasetsart J. (Nat. Sci.) 39, 612-621.

[9] Pilasombut, K., Sakpuaram, T.,Wajjwalku, W., Nitisinprasert, S., Swetwiwathana, A., Zendo, T., Fujita, K., Nakayama, J., & Sonomoto, K. (2006). Purification and amino acid sequence of a bacteriocin produced by *Lactobacillus salivarius* K7 isolated from chicken intestine. J. Sci. and Technol. Vol. 28 (Suppl. 1): 121-132.

[10] Swetwiwathana, A., Pilasombut, K., & Sethakul, J. (2008). Screening of bacteriocin-producing lactic acid bacteria isolated from Thai fermented meat for probiotic prospect. J. Biotechnol, 136s,717-742.

[11] Työppönen, S., Petäjä, E. & Mattila-Sandholm, T. (2003). Bioprotectives and probiotics for dry sausages. Int. J.Food. Microbiol, 83, 233-244.

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Table 1 Antibacterial activity of Lactic acid bacteria

Indicator strains	Media	°C	Antibacterial activity (AU/ml)						
			D4	D8	D9	B27	B42	K4	K7
Lactic acid bacteria									
Lactobacillus plantarum ATCC 14917	MRS	30*	200	-	400	-	-	-	-
Lactobacillus sakei subsp. *sakei* JCM 1157^{T}	MRS	30*	51200	6400	6400	-	-	3200	1600
Lactobacillus sakei TISTR 890	MRS	37*	100	-	400	-	-	ND	ND
Lactococcus lactis subsp. *cremoris* 1344	MRS	30*	800	-	-	-	-	-	-
Leuconostoc mesenteroides subsp.*mesenteroides* JCM 6124 ^{T}	MRS	30*	400	100	400	-	-	100	200
Leuconostoc mesenteroides TISTR 942	MRS	30*	800	-	400	-	-	ND	ND
Enterococcus faecalis JCM 5803 ^{T}	MRS	37*	400	-	-	100	400	200	-
Enterococcus faecalis TISTR 888	MRS	37	400	-	-	-	-	ND	ND
Other bacteria									
Bacillus coagulans JCM 2257 ^{T}	TSB-YE	37	100	-	-	-	-	1600	400
Bacillus coagulans TISTR 1447	TSB-YE	37	-	-	-	-	-	ND	ND
Listeria innocua ATCC 33090 ^{T}	TSB-YE	37	6400	800	800	-	-	800	-
Brochotrix campestris NBRC 11547 ^{T}	TSB-YE	26	800	-	400	-	-	800	-
Pseudomonas fluorescens JCM 5693 ^{T}	TSB-YE	26	800	100	100	-	-	ND	ND
Pseudomonas fluorescens TISTR 358	TSB-YE	26	-	-	100	-	-	ND	ND
Staphylococcus aureus TISTR 118	TSB-YE	37	-	-	-	-	-	ND	ND
Streptococcus sp. TISTR 1030	MRS	30	-	-	-	-	-	ND	ND
Aeromonas hydrophila TISTR 1321	NB	30	-	-	-	-	-	ND	ND

ATCC = American Type Culture Collection, Rockville, Md
JCM = Japanese culture of Microorganism,Wako, Japan
NBRC = National Institute of Technology and Evaluation (NITE) Biological Resource Center
TISTR = Thailand Institute of Scientific and Technological Research
* = Anaerobic condition
\- = No antibacterial activity
ND = Non detect
NB = Nutrient broth
TSB-YE = Tryptic soy broth + 0.6%Yeast extract
K4 and K7 = *Lb. salivarius* K4 and K7

Table 2 Effects of pH, bile salt and NaCl on growth and BLIS production of D4, D8 and D9 against *Lb. sakei* subsp. *sakei* JCM 1157^T and *L. innocua* ATCC 33090^T

Treatment	Antimicrobial activity (AU/ml)					
	D4		D8		D9	
	Lb. sakei	*L. innocua*	*Lb. sakei*	*L. innocua*	*Lb. sakei*	*L. innocua*
Effect of pH						
Control (pH7)	51200	6400	6400	800	6400	800
pH 2	400	-	-	-	400	-
pH 3	400	-	200	-	400	-
pH 4	400	-	400	-	400	100
pH 5	12800	1600	1600	-	400	800
pH 6	12800	3200	3200	400	800	800
pH 7	51200	6400	6400	400	6400	800
pH 8	51200	6400	12800	800	6400	800
pH 9	25600	6400	12800	800	12800	400
pH 10	1600	200	12800	400	12800	400
Effect of bile salt						
0.3%	400	200	200	200	400	200
0.6%	400	200	200	200	400	200
0.9%	400	200	200	200	400	200
Effect of NaCl						
1%	25600	1600	400	400	6400	400
2%	6400	800	400	100	1600	200
3%	3200	200	200	100	1600	100
4%	1600	200	400	-	1600	100
5%	1600	200	200	-	1600	100
6%	1600	200	100	-	800	100
7%	1600	200	100	-	800	100
8%	1600	200	100	-	100	100

- = No antibacterial activity