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Screening of Lactic Acid Bacteria as Potential Probiotics in Beef Cattle

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Abstract: The objectives of this study were to isolate, select and identify lactic acid bacteria (LAB) for the probiotic properties in cattle. Small, large intestines and feces samples were collected from 6 healthy Native x Brahman cattle from the animal farm division of Faculty of Agriculture, Khon Kaen University, Thailand. All samples were cultivated using a modified De Man, Rogosa and Sharp (MRS) agar supplemented with 0.3% $CaCO_3$ (w/v). Bacterial colonies which showed clear zone surrounding their colonies were selected to a further test of the basic probiotic properties including acid and bile tolerance test, anti-pathogenic bacteria. Safety features such as antimicrobial susceptibility were also tested using the disk diffusion method. The results showed that the concentration of LAB from small intestine, large intestine and feces were 5.15 x 10^7, 5.85 x 10^7 and 1.25 x 10^{12} CFU/g, respectively. Twenty seven out of 86 isolates tolerated to pH 3 and 15 isolates tolerated to bile salt. Fifteen out of these acid-and bile-tolerant isolates showed the ability to inhibit *Escherichia coli* ATCC 25923 and *Salmonella* Typhimurium. All acid and bile tolerant isolates were highly sensitive to penicillin, erythromycin, tetracycline and vancomycin. In contrast, 11 and 4 isolates resisted to streptomycin and gentamicin, respectively. However, only one isolate (F30) resulted in resisting to low pH, bile salt and anti-pathogenic bacteria. This isolate was identified using 16S rRNA gene sequencing as *Streptococcus* sp., [closely related to *Streptococcus infantarius* (99.93%)] and shown as a potential probiotic in cattle.

Key words: Probiotic, lactic acid bacteria, anti-pathogenic bacteria, beef cattle

INTRODUCTION

Antibiotics were first added to feed in order to protect animals against infection and promote their growth rate. These functions led to wide usage of probiotics as a feed additive. However, a safety issue arose concerning transmission of antibiotic resistant bacteria. The use of antibiotics in animal feed had gradually declined since 1990 and was completely banned since January 2006 under the directive of the European Union countries. Moreover, use of antibiotics in animal feed may cause residues in meats and have a negative effect on consumer health. Therefore, many researchers are interested to study a new way to substitute using herbs and probiotic microorganisms for antibiotics (Puphan *et al*., 2013). Probiotics are live micro-organisms which can survive and pass through the gastrointestinal tract providing benefits to the host, either in humans (Gilliland, 1990) and animals (Fuller, 1989) by improving absorption in the digestive system (Chukeatirote, 2003), stimulating the immune system and promoting macrophage activity (Fuller, 1993). They are also known to enhance growth rates, inhibit intestinal pathogens and have anti-diarrhea properties (Crittenden *et al*., 2005). In addition, some researchers even had cholesterol level reduction with probiotics in lamb (Lubbadeh *et al*., 1999), humans and swine (Gilliland *et al*., 1985). Most probiotics are lactic acid bacteria due to these groups are tolerance to acid and bile salt. They can survive in the gastrointestinal tract which is the critical property of a probiotic (Cebeci and Gurakan, 2003). Current use of probiotics in ruminants is not widespread in Thailand. Therefore objectives of this study were to screen lactic acid bacteria from cattle to use as probiotics in animal feed for alternative of antibiotic use.

MATERIALS AND METHODS

Samples collection and isolation of lactic acid bacteria: Fresh samples of small intestine, large intestine and feces were collected from six healthy Native x Brahman cattle at the slaughterhouse in Khon Kaen. Fresh samples were kept in air-tight plastic bags and were also kept cold. The samples were divided into portions of 5 grams that were dissolved into 45 milliliters of solvent Maximum Recovery Diluents (MRD) (Oxoid Inc., Hampshire, UK) and agitated for 30 s. Serial 10-fold 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 added 0.3% (w/v) $CaCO_3$ using the pour

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plate method. The incubation was carried out aerobically for two days at 37°C as described by ISO-15214 (1998). Bacterial colonies were enumerated by acid production which showed a clear zone surrounding from the plate that grew 30-300 colonies. Bacterial concentrations were calculated (volume of bacterial colonies x dilution factor) and expressed as colony forming unit per gram of sample (CFU/g). The different morphologies of bacterial colonies from each sample were picked up and stored in a modified freezing medium (Trypticase Soy Broth (Criterion™), 0.6% yeast extract and 20% glycerol) at -20°C for further testing.

Selection of lactic acid bacteria as probiotic property: Eighty-six LAB isolates were determined for principle probiotic properties such as acid and bile acid tolerance, antimicrobial susceptibility test and anti-pathogen activity test.

Acid tolerance test: One hundred microliters of stock cultures were transferred to MRS broth (Oxoid) and incubated at 37°C overnight, then sub-cultured into 10 ml of fresh MRS broth and incubated again for 24 h. The cultures were centrifuged at 20,000 rpm for 10 min, the pellets were washed twice in sterile phosphate buffered saline (PBS, pH 7.2; Sigma) and resuspended in 1 ml of PBS. Each strain was diluted 1/100 in PBS at pH 3. Hydrochloric acid (HCl) was used to adjust the pH of the PBS. After 1, 3 and 6 h incubation, viable bacterial counts were determined by plating serial dilution (in Maximum Recovery Diluents, MRD; Oxoid) on MRS agar followed by incubation under microaerophilic conditions (in anaerobic jar) at 37°C for 48 h. All tests were carried out in duplicate.

Bile tolerance test: The selected LAB isolates from the acid tolerance test were tested in the bile tolerance test using the method modified by Gilliland *et al.* (1984). Briefly, 100 µl of stock cultures were grown in 10 ml of MRS broth (Oxoid) incubated at 37°C for 24 h. Fresh LAB were transferred to MRS broth (control) and MRS broth containing bile salts of cattle (7% concentration) following by incubation under microaerophilic conditions at 37°C for 3 h. Finally, the percentages of survival by the pour plate method in MRS agar following by incubation under microaerophilic condition (in anaerobic jar) at 37°C for 48 h were measured. All tests were carried out in duplicate.

Anti-pathogen activity test: Agar spot was used to test antibacterial activity of LAB using method modified by Makras and Vuyst (2006). Stock isolates were cultured in 10 ml of MRS broth and incubated at 37°C for 24 h. After that 10 µl of fresh LAB (10^8 CFU/ml) were dropped onto MRS agar and then left to dry. This was followed by incubation under microaerophilic conditions (in anaerobic jar) at 37°C for 24 h. Bacterial strains were used as indicator organisms for antibacterial activity screening *Escherichia coli* ATCC 25922 and *Salmonella Typhimurium* (Salmonella was isolated from pig's guts and obtained from Department of Veterinary Public Health, Faculty of Veterinary Medicine, Khon Kaen University). Bacterial strains indicators were cultured in nutrient broth incubating at 37°C for 24 h. and the turbidity adjusted to 0.5 MC Farland (10^8 CFU/ml) using 0.85% NaCl. Next, they were diluted with soft nutrient agar until 10^6 CFU/ml and transferred in suspension to soft nutrient agar on LAB that was grown in MRS agar and incubated at 37°C for 24 h. Inhibition of pathogenic bacteria efficiency was measured by diameter of inhibition zone (clear zone) using a ruler. The inhibition efficiency was calculated by diameter of inhibition zone divided by the diameter of colony of LAB. The diameter 1.1 to 1.9 means low, 2 to 2.9 means moderate and more than 3 cm means high, respectively.

Antimicrobial susceptibility test: The selected LAB isolates (acid tolerance test and bile tolerance test) were tested for anti-microbial susceptibility by a modified minimal disc diffusion method (Bauer *et al.*, 1966) using LSM agar (Klare *et al.*, 2007). Eleven antibiotics were chosen for testing: (I) β-Lactam group, inhibitors of cell wall synthesis: penicillin G 10 µg; (ii) gram-positive spectrum: erythromycin 15 µg, vancomycin 30 µg, (iii) broad spectrum:, tetracycline 30 µg; (iv) inhibitors of protein (v) synthesis: aminoglycosides gentamicin 10 µg, streptomycin 10 µg. All antibiotic discs (diameter = 6 mm) were obtained from Oxoid (Oxoid, Hampshire, England).

Each LAB isolate was cultured (1% inoculums) at 37°C in MRS broth and incubated anaerobically for 18 h. The culture solution was dipped using sterile cotton swap and swabbed in three directions on MRS agar plate. Standard discs of antimicrobial agents were seeded in the plates and incubated anaerobically at 37°C for 48 h. The diameter of the antibiotics inhibition zone was measured using a ruler. All antibiotics were tested in duplicate. Inhibition zone diameters in millimeters were measured inclusive of diameter of disc. The results were expressed as sensitive (S = 21 mm); intermediate (I, 16 to 20 mm) and resistant (R = 15 mm), respectively (Vlkova *et al.*, 2006).

Species level identification of selected LAB isolates: Species identification (by full 16S rDNA sequencing methods) of selected LAB isolate was subsequently submitted to the National Center for Genetic Engineering and Biotechnology (BIOTECH), Thailand.

RESULTS AND DISCUSSION

Sample collection and isolation of lactic acid bacteria: The LAB was successfully isolated from the samples using a selective medium of MRS agar with modified by adding 0.3% (w/v) $CaCO_3$. The clear zone appearance

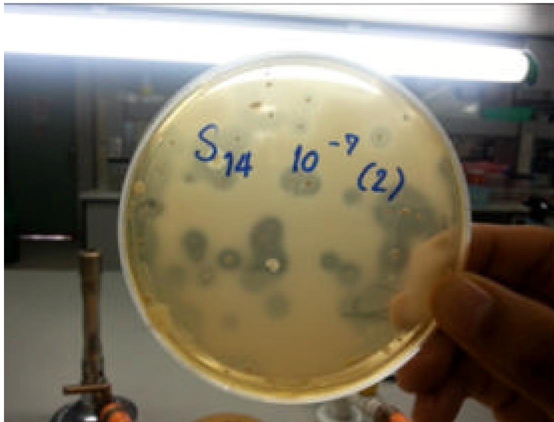

Fig. 1: Bacterial colonies with clear zone (arrow) grew on MRS agar + 0.3% $CaCO_3$

surrounding bacterial colonies are shown in Fig. 1. These clear zone colonies guaranteed to be LAB due to their lactic acid producing properties. Some researchers had attempted to develop the selective medium for lactobacilli (Jackson *et al*., 2002; Tharmaraj and Shah, 2003) but in this study, the selective medium (MRS agar) was developed easily to isolate the LAB. The results showed that 86 isolates were collected and LAB concentration from small intestine, large intestine and feces of cattle were 5.15 x 10^7, 5.85 x 10^7 and 1.25 x 10^{12} CFU/g, respectively as shown in Table 1.

Selection of lactic acid bacteria as probiotic property: Acid tolerance test: All isolates were tested for acid tolerance at pH 3 for 0, 3 and 6 h after incubation. The result found that at 3 h, 27 isolates survived more than 50% and only 21 isolates survived more than 70%. Survival rates decrease linearly (57%) when incubated at 6 h as shown in Table 3. Testing acidic tolerance was made to imitate the animal's natural stomach conditions, which normal pH is about 0.9. While receiving food the pH goes up to 3. Therefore, isolating LAB may die if they are not resistant to acidic conditions (Erkkila and Petaja, 2000). Wheeler and Noller (1997) reported that ruminants fed high starch (48.5%) had pH ranged in the gastrointestinal tract by 2.5-6.2; rumen pH 5.8±2.5, abomasums pH 2.5±1.4, small intestine pH 6.2±1.7 and rectum pH 6.2±1.7, respectively. Our low pH-resisting LAB were similar to research of Sornplang (2009) who isolated LAB from native chicken gut and tested for acidic conditions. They found that after incubation at pH 3 for 3 h, only 11 of 65 isolates had a survival rate more than 80%. The capability of acid tolerance of LAB are dependent on bacterial cell wall characteristics (Hood and Zotolla, 1998; Madureira *et al*., 2005). The cell wall of gram-positive bacteria is thicker than that of gram-negative bacteria consisting of peptidoglycan, teichoic acid and colonic acid. Peptidoglycan is a polymer structure of disaccharide pentapeptide containing with two sugars, N-acetyl glycocyamine (NAG) and N-acetyl muramic acid (NAM) and 4-amino group linked to NGA and NMA with a peptide bond. Peptidoglycan thickness of gram-positive bacteria such as *L. helveticus* was approximately 20-80 nm (Firtel *et al*., 2004; Johnson *et al*., 2006). Moreover, acid tolerance is dependent on the activity of enzyme H^+-ATPase (controlling transportation of proton through the cell wall), type of culture medium and culture conditions (Hood and Zotolla, 1998; Madureira *et al*., 2005).

Bile tolerance test: All 27 isolates selected from the acid test were screened by testing in 7% bile for 3 and 6 h. The results showed that 15 isolates had lived and the survival rate ranged from 25-81% after incubation for 3 h (Table 2). The survival rate in the bile salt condition is one of the main criteria for *in-vitro* selection of potentially probiotic bacteria and microbes (Hawaz, 2014). Because the bacterial cell wall is comprised mainly of phospholipids, bile salt which is an emulsifier and solubilizes the lipid that can damage the bacterial cells (Musikasang, 2008). In addition, morphology such as *Lactobacillus acidophilus* M92, in smooth form, had a higher survival rate than the notched form one (Suskovie *et al*., 2000). The different bile composition of each animal was one of the main factors affecting the survival of bacteria. For an example, bile from cattle containing trihydroxy conjugated was less effective in inhibiting bacterial growth than dihydroxy conjugated from swine (Musikasang, 2008).

Anti-pathogen activity test: The 15 isolates resisting to acidity and bile salt were tested for anti-pathogenic activity including *Escherichia coli* ATCC 25923 and *Salmonella* Typhimurium. The Inhibition of pathogenic bacteria efficiency was measured by diameter of inhibition zone (clear zone). The results showed that all isolates can inhibit *E. coli* ATCC 25923 and *Salmonella* Typhimurium. Only 2 isolates, F30 and S7, had high effectively inhibit to *E. coli* ATCC 25923; while, efficiency of inhibiting *Salmonella* Typhimurium of LAB was moderate as shown in Table 3. This result was consistent with Oyarzabal and Conner (1995) who reported that LAB can constrain *Salmonella* spp. and *Escherichia coli*. The ability to inhibit pathogens was clearly by Sarela *et al*. (2000) who explained that LAB produce some substances inhibiting the growth of pathogenic bacteria such as lactic acid, acetic hydrogen peroxide and bacteriocin.

Antimicrobial susceptibility test: Antibiotic susceptibility test of LAB using the disc diffusion method was shown in Table 4. The results showed that 15 isolates were

Table 1: Number of colony at various dilutions (CFU/g)

	Small intestine[1]				Large intestine[1]				Feces[1]			
Cattle	10^5	10^6	10^7	Count	10^5	10^6	10^7	Count	10^5	10^6	10^7	Count
1	62	4	0	6.2×10^6	nc	71	1	6.2×10^6	44	2	0	4.4×10^{10}
2	55	10	6	5.5×10^6	47	2	0	5.5×10^6	331	60	0	6.0×10^{11}
3	nc	52	8	5.2×10^7	50	7	0	5.2×10^7	nc	250	0	2.5×10^{12}
4	42	0	0	4.2×10^6	308	34	1	4.2×10^6	nc	nc	39	3.9×10^{12}
5	nc	209	4	2.1×10^8	82	3	0	2.1×10^8	nc	40	0	4.0×10^{11}
6	nc	30	0	3.0×10^7	nc	241	18	3.0×10^7	80	6	0	8.0×10^{10}
Average				5.1×10^7				5.1×10^7				1.2×10^{12}

[1]Count with triplicate plates. NC: Means numerous colonies

Table 2: Survival (%) after incubation at pH 3 and in 7% fresh cattle bile at pH 8

	pH 3			Isolates	pH 8 (7% fresh cattle bile)		
Isolates	0 h	3 h	6 h		0 h	3 h	6 h
S2	100	80	55	S2	100	43	0
S3	100	71	54	S3	100	40	9
S6	100	75	45	S6	100	65	23
S7	100	76	59	S7	100	52	0
S10	100	50	25	S10	100	68	20
S19	100	81	76	L3	100	70	30
S20	100	84	72	L6	100	45	0
S22	100	70	67	L7	100	55	25
L1	100	71	45	L18	100	25	0
L3	100	86	44	L22	100	77	28
L6	100	72	60	F17	100	54	0
L9	100	62	31	F20	100	70	30
L10	100	79	67	F25	100	72	23
L12	100	80	71	F26	100	60	32
L13	100	75	58	F30	100	81	45
L 18	100	79	33				
L20	100	75	61				
L22	100	87	70				
F4	100	80	72				
F5	100	88	74				
F6	100	76	63				
F12	100	75	62				
F17	100	80	71				
F20	100	66	23				
F25	100	80	62				
F26	100	60	45				
F30	100	86	65				

S: Small intestine, L: Large intestine, F: Feces

Table 3: Efficiency inhibitor and bacteriocin activity of selected LAB

	Efficiency inhibitor	
Isolates	*E. coli* ATCC 25923	*Salmonella* Typhimurium
S2	++	+
S3	++	++
S6	++	++
S7	+++	+
S10	+	++
L3	+	+
L6	+	++
L7	++	++
L18	++	++
L22	++	++
F17	++	++
F20	++	++
F25	+	++
F26	++	++
F30	+++	++

(+) Means efficiency inhibitor more than 1, (-) means no bacteriocin production. Low: +, Moderate: ++, High: +++, S: small intestine, L: large intestine, F: Feces

Table 4: Antibiotic susceptibility of selected LAB

	No. of isolated LAB		
Antibiotics	Resistant (R)	Moderately (M)	Susceptibility (S)
Penicillin	0	0	15
Erythromycin	0	0	15
Tetracycline	0	0	15
Vancomycin	0	0	15
Streptomycin	11	1	3
Gentamicin	4	0	11

susceptible to antibiotics; penicillin, erythromycin, tetracycline and vancomycin, 11 and 4 isolates were resistant to streptomycin and gentamicin, respectively. These results were consistent with Musikasang (2008) who reported that most of the LAB are resistant to penicillin, tetracycline and erythromycin. The LAB-isolated from native chicken feces was also resistance to antibiotics (amino glycoside group) such as kanamycin and streptomycin (Sornplang and Leelavatcharamas, 2010). This is a natural property of LAB having intrinsic resistance and acquired resistance (Ammor *et al*., 2007). However, there were no reports presented regarding gene transfer from LAB resistance to antibiotics to the normal micro-flora in the gastrointestinal tract (Mathur and Singh, 2005).

Species level identification of selected LAB isolates: Full 16S rDNA sequencing of F30 revealed that F30 is *Streptococcus* sp. similar to *Streptococcus infantarius* (99.93%). This species had been reported and was used as probiotics in the European Union (Anadon *et al*., 2006).

Conclusion: We concluded that F30 isolate had appropriate properties for use as a probiotic. It had a high survival rate in the conditions of low pH and bile concentration of 7%, can inhibit pathogenic bacteria *Escherichia coli* ATCC 25923 and *Salmonella* Typhimurium and did not show resistance to antibiotics, an acquired resistance but showed an intrinsic resistance to streptomycin and gentamicin (aminoglycoside group). Therefore, the isolate F30 can be further developed for use as a probiotic in cattle feed.

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