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Evaluation of Lactic Acid Bacteria Isolates for Silage Fermentation Inoculant in Thailand by Using a Modified Pouch Method

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Abstract

A modified pouch method was used to evaluate 13 strains of lactic acid bacteria (LAB) for silage fermentation inoculants to make good-quality silage in Thailand. Among them, strain SP 1-3, isolated from corn silage and tentatively assigned to *Lactobacillus plantarum*, exhibited an inherent tolerance for high incubation temperature and lactic acid. Strains CS 5-5 and KS 1-9, tentatively assigned to *Pediococcus* sp., also exhibited similar properties to strain SP 1-3, but their levels of lactate tolerance were weaker than that of strain SP 1-3. Strain CS 1-8, isolated from TMR silage and assigned to *Pediococcus* sp., grew well at the early stage of silage fermentation (within 24-h), but did not accumulate a large amount of lactate during the long-term fermentation (21 days). Based on these results, laboratory-scale silage of napiergrass inoculated with strain SP 1-3 or CS 1-8 was prepared. The fermentation quality of silage inoculated with both strains obviously improved the amount of lactate produced and reduced the counts of coliform bacteria and yeast. From these results, both strains, SP 1-3 and CS 1-8, were evaluated as favorable silage fermentation inoculants in tropical regions such as Thailand.

Discipline: Animal industry
Additional key words: LAB, coliform bacteria, yeast

Introduction

During the last 10 years, the amount of milk and fermented milk consumption is rapidly increasing in Thailand[8]. However, the self-sufficiency ratio in raw milk production is low, accounting for about 60% of the demand. To increase the amount of raw milk production, the Thai government (Department of Livestock Development) plans some strategies from the viewpoints of the cattle ability (breeding), feeds, cattle management and so on. Among them, it is understood that the feeding of silage is an effective and easy to introduce technique for increasing the amount of raw milk production, in spite of it not having wide use in Thailand. Actually, it was reported that feeding of good-quality silage throughout a year stably increased the average amount of raw milk production in a project by the Japan International Cooperative Agency (JICA)[12]. The average amount of raw milk production recorded in the project was about 1.5 times higher than that of Thailand[8].

The fermentation quality is varying and sometimes becomes poor. Namely, making good-quality silage is not always assured. To cope with this problem, the screening of lactic acid bacteria (LAB) strains suitable for silage-making in Thailand was carried out and some

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thermotolerant LAB strains[5] were isolated from silage. These strains showed peculiar properties adapted to the tropical environment such as growth at high temperature (40−45°C). But the application of these strains for silage-making has not yet begun because they were not evaluated for silage-making in Thailand by using a model fermentation system. Therefore, we tried to construct the evaluation system[8] of LAB strains for silage-making in Thailand by modifying the pouch method[7,10], a laboratory-scale ensiling method. It was developed in Japan for screening of LAB strains adapted to the Japanese climate and natural conditions for silage-making. Successively, we evaluated LAB strains for silage fermentation inoculants to make good quality silage in Thailand by using the modified pouch method. In this paper, we summarize results of the evaluation of LAB strains.

Methods and materials

1. Microorganisms

Thirteen LAB strains were used in this study and their isolation origins are listed in Table 1. *Enterobacter* sp. SG 1-1 T and *Saccharomyces cerevisiae* SG 2-1 Y were also used for the modified pouch method[8] as coliform bacteria (CFB) and yeast, respectively. Further, the following 8 strains were used as target strains to detect the growth inhibition activity: *Enterobacter* sp. SG 1-1 T, *Escherichia coli* TISTR 527, *Salmonella typhimurium* TISTR 292, *Bacillus subtilis* TISTR 025, *Staphylococcus aureus* TISTR 029, *Lactobacillus plantarum* TISTR 541, *Leuconostoc mesenteroides* TISTR 473 and *Enterococcus faecalis* TISTR 927.

2. Detection of growth inhibition activity

Growth inhibition (bacteriocin) activity of the culture filtrate was examined by the paper disk method[6]. Eight bacterial strains including *Enterobacter* sp. SG 1-1 T (CFB) were used as target strains.

3. Modified pouch method

Napiergrass, harvested in Pathum Thani province at the growth stage of about 30 cm height, was cut into about 2 cm lengths, dried at 80°C for 2 days and autoclaved at 121°C for 20 min. Then 1.5% of glucose was added to napiergrass adjusted to 75% of moisture content by adding sterilized water and this material was used for the modified pouch method[7] as the medium. The medium (40 g) was put into an air-tight pouch (plastic bag: double-layer film of nylon and polyethylene, 20 × 30 cm, 0.1 mm thickness. Hiryu KN210, Asahi Kasei Co., Japan) after inoculating silage microorganisms at the designated size, sealed by a vacuum sealer and anaerobically incubated at 45°C for 6, 24 and 504 h (21 days). All experiments were done in duplicate.

4. Silage preparation

Laboratory-scale napiergrass silage was prepared as follows: Napiergrass mentioned in the item of the modi-

Table 1. List of lactic acid bacteria strains used in this study

Strain no.	Origins
CS 1-4, 1-8, 2-1, 2-3 and 2-12	Isolated from a TMR silage collected in Chonburi province (pH 4.02, dry matter content 31.0%)
CS 5-5	Isolated from a corn silage collected in Chonburi province (pH 5.05, dry matter content 18.0%)
KS 1-9	Isolated from dead leaves collected in Nakhon Ratchasima province
LG 4-2	Isolated from a silage made from Luzy grass (harvested in Lopburi province) with 2.0% glucose in the laboratory (pH 4.58, dry matter content 66.7%)
LS 1-20	Isolated from a concentrate feed fermented for 15 days collected in Lopburi province (pH 4.10, dry matter content 77.2%)
LS 2-23	Isolated from a concentrate feed fermented for 30 days collected in Lopburi province (pH 4.00, dry matter content 59.7%)
SP 1-3	Isolated from a corn silage collected in Nakhon Pathom province (pH 3.70, dry matter content 22.0%)
S 6-1	Isolated from a sugar cane silage collected in Khon Kaen province (pH 3.74, dry matter content 43.0%)
ST 10-1	Stock culture of the laboratory (isolated from silage, tentatively assigned to *Lactobacillus pentosus*)

fied pouch method was cut into about 2 cm lengths and put into an airtight pouch together with 1.5% of glucose and an LAB strain. Moisture content of the fresh grass was 88.8% and its suspension in distilled water showed pH 6.01. The pouch was sealed by a vacuum sealer and anaerobically incubated at 45°C. Strains SP 1-3 and CS 1-8 were inoculated at 4×10^6 cfu/g. On 12-h, 1-day, 2-day and 7-day incubation, the pouch was opened and the counts of microorganisms, silage pH and the amounts of lactate and acetate were analyzed. All experiments were conducted in triplicate.

5. Preparation of analysis samples

After incubation, 10 g of silage was used for the determination of dry matter content (105°C for 48 h). Another 10 g of silage was suspended into 30 mL of distilled water and kept at 4°C for 24 h to make a sample solution for acids analysis by using high performance liquid chromatography (HPLC). Further 10 g of silage was suspended in 30 mL of sterilized saline water to make a sample solution for enumeration of microorganisms.

6. Enumeration method of microorganisms

The number of microorganisms was enumerated by the plate culture count method using lactobacilli MRS broth agar (Difco, USA) for LAB, violet red bile agar with lactose (Difco, USA) for CFB and potato-dextrose agar (Nissui Seiyaku Ltd., Japan) for yeast, respectively. The plate was cultured for 24-h at 45°C for LAB, at 37°C for CFB and at 30°C for yeast. Colonies were counted and their numbers were expressed as visible numbers of microorganisms in colony-forming units (cfu) per gram of fresh matter.

7. HPLC

Lactate produced was analyzed by using a HPLC (Thermo Quest Spectra P-100) with an organic acid analysis column Aminex HPX-87H (Bio-Rad, diameter 7.8 mm × length 300 mm) at 45°C. Eight mM of sulfate solution was used as a mobile phase with a speed of 0.5 mL/min and an UV/VIS photometer (Thermo Quest Spectra UV-150) at wave length 230 nm was used as a detector. Tartarate was used as a internal standard.

Results and discussion

1. Preliminary selection of LAB strains using liquid culture

Two hundred and fifteen LAB strains were isolated from 14 silage samples prepared in Thailand. Among them, 13 strains showed pH of culture filtrate less than 4 after 24-h incubation using lactobacilli MRS broth at 45°C, aerobically (data not shown). Therefore, these strains listed in Table 1 were selected for further experiment.

2. Mixed culture of LAB strains with CFB and yeast using the modified pouch method

It was suggested that the inoculum sizes of each silage microorganism have a strong effect on their growth in mixed culture[9]. Therefore, each LAB strain was cultured in a pouch with CFB and yeast at the combined inoculum sizes of 10^5 cfu/g and 10^2 cfu/g, respectively. The counts of microorganisms and the amounts of lactate produced are shown in Table 2 when all of silage microorganisms were inoculated with the same inoculum size at a low level (10^2 cfu/g). All of the strains did not always show a low pH (less than pH 4 in the mixed culture using pouch method). In spite of that, they showed lower pH in the pure culture using MRS broth. Among them, strain SP 1-3 produced a large amount of lactate (1.02 % in fresh silage) and showed low pH after 21-day incubation. Its count of living cells during 21-day incubation was kept at a high level in spite of the presence of a large amount of lactate. These may exhibit the nature of tolerance for lactate in strain SP 1-3. Strains KS 1-9, CS 1-8 and CS 5-5 also produced large amounts of lactate but their counts of microorganisms after 21-day incubation decreased to 10^3–10^4 times lower than that after 24-h incubation. The tolerance for lactate in these strains should be weaker than that of strain SP 1-3. Other strains produced unsatisfactory amounts of lactate. Among them, strains LS 1-20 and LS 2-34 showed peculiar profiles of lactate production. Namely, both strains produced lactate from an early stage of the fermentation (6-h incubation) but the production of lactate by both strains stopped at low levels after 24-h incubation. In addition, all of the LAB strains seemed to have no remarkable inhibition of the growth of CFB or yeast strains.

Table 3 shows the count of microorganisms and the amounts of lactate produced when the inoculum size of LAB increased 10^3 times higher (10^5 cfu/g) than that of CFB and yeast. By increasing inoculum size to 10^5 cfu/g, strain SP 1-3 produced lactate at an early stage of silage fermentation (6-h incubation) and the amount of lactate produced after 21-day incubation was 1.46% in fresh weight of silage. This amount corresponded to the conversion rate of 97.4% from glucose to lactate and was 1.43 times larger than that of the case with inoculum size 10^2 cfu/g (Table 2). These results are understood by the fact that strain SP 1-3 is a homofermentative LAB strain and converts glucose to lactate at an early stage of the silage fermentation (6-h incubation) because of its large inoculum size. It is expected that the living cell numbers

Table 2. Counts of microorganisms in silage with the same level of initial inoculum size

Strain no.	Culture time (h)	Medium pH	Lactate produced (% in fresh silage)	Count of microorganisms (cfu/g)		
				LAB	CFB	Yeast
CS 1-4	6	6.37	< 0.01	9.5×10^4	7.1×10^4	2.3×10^4
	24	4.92	< 0.01	7.6×10^5	1.5×10^5	2.0×10^5
	504	6.44	0.19	1.2×10^2	2.0×10^2	5.6×10^6
CS 1-8	6	6.37	< 0.01	9.0×10^5	4.7×10^5	1.0×10^4
	24	5.81	0.31	2.3×10^6	1.2×10^6	5.0×10^5
	504	4.75	1.03	5.3×10^3	1.2×10^3	4.7×10^5
CS 2-1	6	6.53	< 0.01	4.4×10^4	1.7×10^3	3.9×10^3
	24	4.15	0.39	1.8×10^6	2.0×10^5	4.7×10^5
	504	4.01	0.55	9.0×10^3	1.9×10^4	2.8×10^4
CS 2-3	6	6.45	< 0.01	1.0×10^4	2.8×10^3	2.4×10^3
	24	4.40	0.26	3.1×10^5	1.2×10^5	5.8×10^5
	504	4.35	0.45	6.2×10^4	9.0×10^3	9.0×10^3
CS 2-12	6	6.56	< 0.01	7.5×10^4	4.1×10^3	1.6×10^3
	24	4.67	0.25	4.9×10^5	3.5×10^5	4.5×10^5
	504	4.26	0.64	1.2×10^4	3.0×10^4	6.8×10^4
CS 5-5	6	5.80	< 0.01	7.0×10^5	4.4×10^4	2.1×10^4
	24	5.34	0.25	3.5×10^7	1.2×10^6	1.6×10^6
	504	4.35	0.82	7.5×10^3	5.2×10^3	3.4×10^4
KS 1-9	6	6.33	< 0.01	1.9×10^5	4.2×10^4	5.8×10^3
	24	5.17	0.44	4.2×10^7	3.3×10^6	8.5×10^5
	504	4.85	0.90	8.5×10^4	1.1×10^5	1.8×10^4
LG 4-2	6	7.04	< 0.01	5.4×10^5	4.4×10^4	1.4×10^4
	24	6.00	0.29	1.8×10^7	3.8×10^6	7.2×10^5
	504	4.87	0.73	3.9×10^4	5.3×10^4	2.6×10^4
LS 1-20	6	6.35	0.07	1.7×10^5	4.2×10^4	2.0×10^4
	24	5.84	0.37	4.1×10^6	2.5×10^6	8.7×10^5
	504	5.65	0.46	1.2×10^4	3.9×10^4	2.6×10^3
LS 2-34	6	6.38	0.09	2.3×10^5	7.2×10^5	2.2×10^4
	24	6.20	0.25	3.7×10^6	1.2×10^6	3.6×10^5
	504	5.78	0.26	2.9×10^5	2.4×10^5	3.5×10^3
SP 1-3	6	6.18	< 0.01	3.1×10^5	1.6×10^5	3.8×10^4
	24	4.39	0.39	5.4×10^6	1.4×10^7	4.7×10^6
	504	4.05	1.02	1.1×10^5	3.9×10^5	9.3×10^3
S 6-1	6	6.65	< 0.01	4.3×10^4	3.9×10^4	4.0×10^3
	24	4.66	0.28	1.6×10^6	3.0×10^5	7.7×10^5
	504	3.94	0.65	4.4×10^2	3.7×10^3	3.6×10^4
ST 10-1	6	6.55	< 0.01	1.2×10^4	2.1×10^3	2.0×10^3
	24	4.89	0.32	1.6×10^6	1.7×10^5	1.6×10^5
	504	4.00	0.60	1.4×10^4	1.2×10^3	2.9×10^3

Silage was prepared by using the modified pouch method of which the medium consisted of about 2 cm lengths of cut napiergrass (moisture content 75%) with 1.5% glucose (w/w). The medium inoculated with lactic acid bacteria (LAB), coliform bacteria (CFB) and yeast (each 10^2 cfu/g) was cultured at 45ºC, anaerobically. *Enterobacter* sp. SG 1-1 T and *Saccharomyces cerevisiae* SG 2-1 Y were used as CFB and yeast, respectively.

Table 3. Counts of microorganisms in silage inoculated with a high level of lactic acid bacteria

Strain no.	Culture time (h)	Medium pH	Lactate produced (% in fresh silage)	Count of microorganisms (cfu/g)		
				LAB	CFB	Yeast
CS 1-4	6	5.93	0.17	5.1×10^6	2.4×10^4	1.6×10^4
	24	4.29	0.31	3.7×10^7	1.1×10^5	1.2×10^5
	504	6.26	0.20	4.2×10^2	9.0×10^1	4.6×10^6
CS 1-8	6	5.43	0.61	8.3×10^6	1.5×10^5	1.2×10^3
	24	4.72	1.04	2.0×10^8	2.9×10^6	4.0×10^5
	504	4.50	0.82	1.4×10^5	3.2×10^3	3.0×10^3
CS 2-1	6	4.66	0.23	2.2×10^6	9.5×10^2	1.5×10^3
	24	3.88	0.50	4.8×10^7	5.9×10^5	6.1×10^5
	504	3.72	0.94	3.0×10^4	2.7×10^4	1.3×10^4
CS 2-3	6	4.91	0.20	2.0×10^6	1.6×10^3	6.9×10^2
	24	4.07	0.24	1.6×10^7	9.0×10^4	1.2×10^5
	504	3.99	0.57	8.5×10^4	6.5×10^3	3.7×10^4
CS 2-12	6	6.08	0.09	2.4×10^6	1.5×10^3	1.7×10^3
	24	4.12	0.42	1.4×10^7	2.4×10^5	1.6×10^5
	504	3.90	0.93	1.9×10^4	1.2×10^4	3.0×10^4
CS 5-5	6	5.81	0.26	6.2×10^6	7.1×10^3	1.5×10^4
	24	4.52	0.57	5.0×10^8	8.6×10^5	7.8×10^5
	504	4.20	1.27	4.4×10^4	6.0×10^3	3.2×10^4
KS 1-9	6	5.54	0.46	5.9×10^6	5.0×10^4	4.8×10^3
	24	4.38	1.03	3.3×10^8	1.2×10^6	5.0×10^5
	504	4.47	1.15	1.4×10^6	4.6×10^4	4.5×10^4
LG 4-2	6	6.31	0.35	2.0×10^6	1.9×10^4	2.0×10^4
	24	4.87	0.78	3.9×10^8	1.0×10^6	4.0×10^5
	504	4.63	0.87	5.9×10^4	2.7×10^4	4.3×10^4
LS 1-20	6	5.47	0.21	3.0×10^6	7.3×10^4	3.2×10^4
	24	4.13	0.60	1.3×10^8	1.3×10^6	5.4×10^5
	504	4.29	0.88	3.6×10^5	4.1×10^4	2.2×10^3
LS 2-34	6	5.40	0.21	5.0×10^6	6.3×10^5	2.3×10^4
	24	4.26	0.50	2.0×10^8	1.6×10^6	3.2×10^5
	504	4.24	1.05	7.7×10^5	4.3×10^4	3.0×10^3
SP 1-3	6	4.60	0.40	4.0×10^6	9.7×10^4	9.3×10^3
	24	3.88	0.80	1.7×10^8	2.6×10^7	8.1×10^5
	504	3.84	1.46	8.9×10^6	5.4×10^5	2.2×10^3
S 6-1	6	4.82	0.32	7.6×10^6	3.3×10^4	2.5×10^3
	24	4.03	0.39	1.9×10^7	4.2×10^5	7.9×10^5
	504	3.87	0.89	3.5×10^2	1.8×10^3	5.0×10^4
ST 10-1	6	5.11	0.26	4.1×10^6	6.6×10^3	1.2×10^3
	24	3.88	0.47	3.4×10^7	2.5×10^5	1.4×10^5
	504	3.73	0.98	8.9×10^4	1.4×10^3	2.7×10^3

Silage was prepared by using the modified pouch method of which the medium consisted of about 2 cm lengths of cut napiergrass (moisture content 75%) with 1.5% glucose (w/w). The medium inoculated with 10^5 cfu/g of lactic acid bacteria (LAB) and 10^2 cfu/g of coliform bacteria (CFB) and yeast was cultured at 45ºC, anaerobically. *Enterobacter* sp. SG 1-1 T and *Saccharomyces cerevisiae* SG 2-1 Y were used as CFB and yeast, respectively.

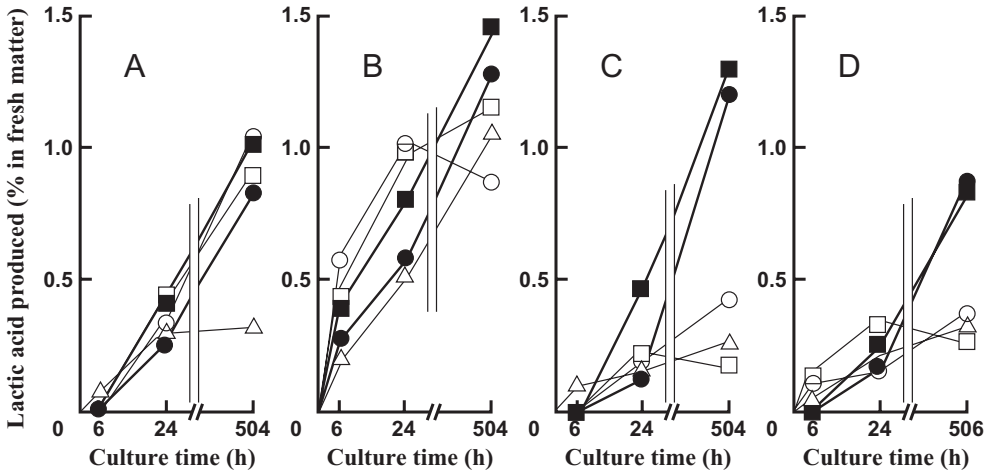

Fig. 1. Time course of lactic acid production by typical isolates of lactic acid bacteria in the modified pouch method
Each lactic acid bacteria (LAB) strain was inoculated with strains of coliform bacteria (CFB) and yeast at various inoculum sizes. Inoculum size (cfu/mL): A; LAB 10^2, CFB 10^2, yeast 10^2. B; LAB 10^5, CFB 10^2, yeast 10^2. C; LAB 10^2, CFB 10^5, yeast 10^2. D; LAB 10^2, CFB 10^2, yeast 10^5. ■ : strain SP 1-3, ● : strain CS 5-5, ○ : strain CS1-8, △ : strain LS 2-34, □ : strain KS 1-9.

of microorganisms in silage except LAB are reduced by increasing the amount of lactate produced and decreasing the pH. However, the counts of CFB and yeast were ambiguously reduced. This may be due to the property[9] of CFB and yeast to utilize lactate as a carbon source and exhibit a tolerance for lactate. Strains CS 5-5 and KS 1-9 also produced large amount of lactate which were 1.3–1.6 times larger than that of these strains inoculated at 10^2 cfu/g (Table 2). On the other hand, the lactate production by strain CS 1-8 was accelerated at the early stage of the silage fermentation and the amounts of lactate after 6-h and 24-h incubation were the largest among the strains tested. However, the amount of lactate produced after 21-day incubation decreased to about 80% of that after 24-h incubation. In the mixed culture of strain CS 1-8 with CFB and yeast, lactate may be transformed to other carboxylic acids[3] such as acetate, pyruvate and so on.

As mentioned above, unique profiles of lactate production were confirmed in 5 isolates, CS 1-8, CS 5-5, KS 1-9, LS 2-34 and SP 1-3. Their time course of lactate production in culture using modified pouch method with various inoculum sizes is shown in Fig. 1. The most important property of LAB strains for silage-making is the ability to produce large amounts of lactate in the silage fermentation process which is a kind of solid mixed and non-sterilized fermentation[11]. This idea is reflected on the modified pouch method. When the inoculum sizes of CFB and yeast, especially in yeast (Fig. 1-D), were higher than that of LAB, the amount of lactate clearly decreased. However, the production of lactate by strains SP 1-3 and CS 5-5 was higher than that by other strains even though the inoculum size of CFB and yeast was 10^3 times higher than that of LAB (Fig 1-C and 1-D). Based on these data, strains SP 1-3 and CS 5-5 are evaluated as excellent strains because they displayed the greatest ability to produce lactate in the modified pouch method. Therefore, strains SP 1-3 and CS 5-5 as well as CS 1-8 and KS 1-9 were cultured to examine their growth properties and favorable culture temperature by using MRS broth. Remaining strains such as CS 2-1, S 6-1 and ST 10-1 might be also evaluated as suitable strains because of their properties to generate low pH from the early stage of silage fermentation (24-h incubation). Namely, the low pH from the early stage of silage fermentation prevents nutrient consumption by undesirable silage microorganisms such as CFB and yeast which are generally abundant in silage prepared in Thailand[8].

3. Pure culture of selected LAB strains in the liquid culture using MRS broth

Amounts of lactate and counts of microorganism of the four LAB strains at various culture temperatures are shown in Table 4. The count of living cells of strain SP 1-3 after 12-h incubation at 35–45°C reached to 10^9 cfu/mL in spite of that the fact that the count of living cells after 6-h incubation was less than 10^7 cfu/mL. Then, it gradually decreased after 48-h incubation at 40 and 45°C. However, the amount of lactate increased in proportion to the rise in incubation temperature. The amount of lactate produced at 45°C was 1.25 times higher than that at 30°C. Namely, this strain may have an inherent tolerance for high temperature (45°C) and tolerance for lactate. These properties are suitable for silage-making in Thailand although this strain has slow growth speed at the

Table 4. Amount of lactate produced and count of cells at various culture temperatures

Strain no.	Culture time (h)	Lactate produced (mg/mL) at (ºC)				Count of LAB (cfu/mL) at (ºC)			
		30	35	40	45	30	35	40	45
CS 5-5	6	0.81	0.76	1.30	0.92	7.8×10^7	5.8×10^7	5.9×10^8	8.1×10^8
	12	3.25	4.47	5.40	6.13	2.5×10^9	2.2×10^9	5.7×10^9	4.1×10^9
	24	6.45	7.74	8.86	6.66	3.9×10^9	3.8×10^9	7.8×10^9	5.9×10^9
	32	10.71	9.27	9.88	9.74	3.5×10^9	3.3×10^9	4.0×10^9	3.8×10^9
	48	8.79	9.43	11.25	8.67	3.0×10^9	3.1×10^9	3.8×10^9	3.5×10^9
CS 1-8	6	0.72	0.72	1.31	1.11	1.5×10^8	1.5×10^8	6.1×10^8	8.4×10^8
	12	5.24	5.32	5.55	5.17	1.6×10^9	2.0×10^9	2.5×10^9	2.9×10^9
	24	8.60	7.98	9.49	9.02	2.9×10^9	2.8×10^9	3.1×10^9	3.2×10^9
	32	8.29	8.53	9.05	8.26	2.7×10^9	2.6×10^9	2.8×10^9	2.7×10^9
	48	7.95	8.12	9.10	7.90	2.7×10^9	2.6×10^9	2.8×10^9	2.6×10^9
KS 1-9	6	0.48	0.55	1.54	0.78	1.4×10^8	1.4×10^8	4.2×10^8	7.3×10^8
	12	4.88	4.08	4.37	5.41	2.5×10^9	2.9×10^9	2.8×10^9	2.6×10^9
	24	7.14	8.00	8.58	8.53	3.0×10^9	3.2×10^9	3.1×10^9	3.6×10^9
	32	8.07	9.18	9.94	9.38	2.4×10^9	2.5×10^9	2.6×10^9	2.8×10^9
	48	9.33	11.75	11.42	10.61	2.5×10^9	2.3×10^9	2.2×10^9	2.4×10^9
SP 1-3	6	0.01	0.30	0.51	0.55	1.5×10^6	5.4×10^6	7.6×10^6	9.2×10^6
	12	0.36	2.46	2.25	1.76	4.5×10^7	1.2×10^9	1.6×10^9	1.1×10^9
	24	7.20	7.25	9.89	9.43	7.9×10^9	5.8×10^9	1.8×10^9	1.8×10^9
	32	8.40	9.44	11.10	12.83	3.8×10^9	3.4×10^9	2.4×10^9	2.3×10^9
	48	11.45	12.93	13.76	14.34	1.5×10^9	1.2×10^9	9.6×10^8	8.5×10^8

Each strain was cultured in lactobacilli MRS broth at 30, 35, 40 and 45ºC, anaerobically.

early stage of silage fermentation.

On the other hand, the living cells of strains CS 5-5, CS 1-8 and KS 1-9 were almost 10^8 cfu/mL after 6-h incubation and reached to 10^9 cfu/mL after 12-h incubation at 30–45°C. Then, the count of living cells was kept at the level of more than 10^9 cfu/mL after 48-h incubation. However, the maximum amounts of lactate produced by these three strains were at 35–40°C and the amounts were only about 66–83% of that by strain PS 1-3.

Time course of lactate production by strains CS 1-8 and SP 1-3 is shown in Fig. 2. Strain CS 1-8 grew well and produced lactate from the early stage of the fermentation (6-h incubation), and amount of lactate produced at 45°C after 12-h incubation was about 5 times bigger than that by strain SP 1-3. However, the amount of lactate produced at 45°C after 48-h incubation was only 55.1%

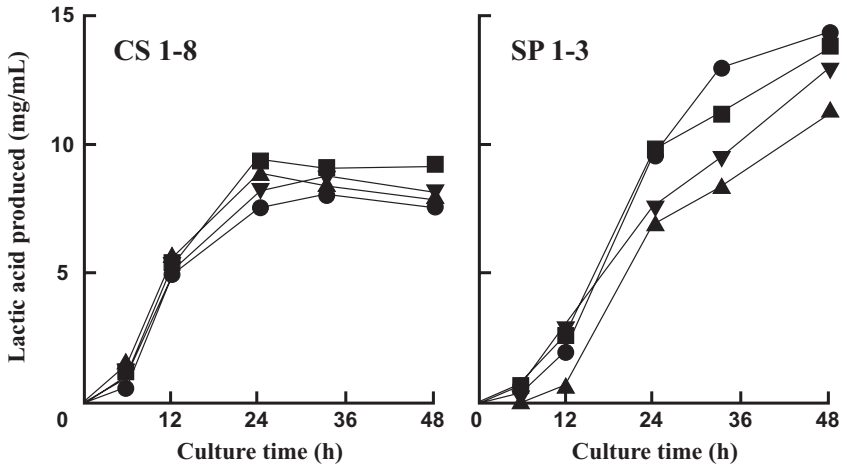

Fig. 2. Time course of lactic acid production by lactic acid bacteria strains CS 1-8 and SP 1-3 at various temperatures
Each lactic acid bacteria strain was cultured in lactobacilli MRS broth.
Each symbol shows culture temperature. ● : 45ºC. ■ : 40ºC. ▼ : 35ºC. ▲ : 30ºC.

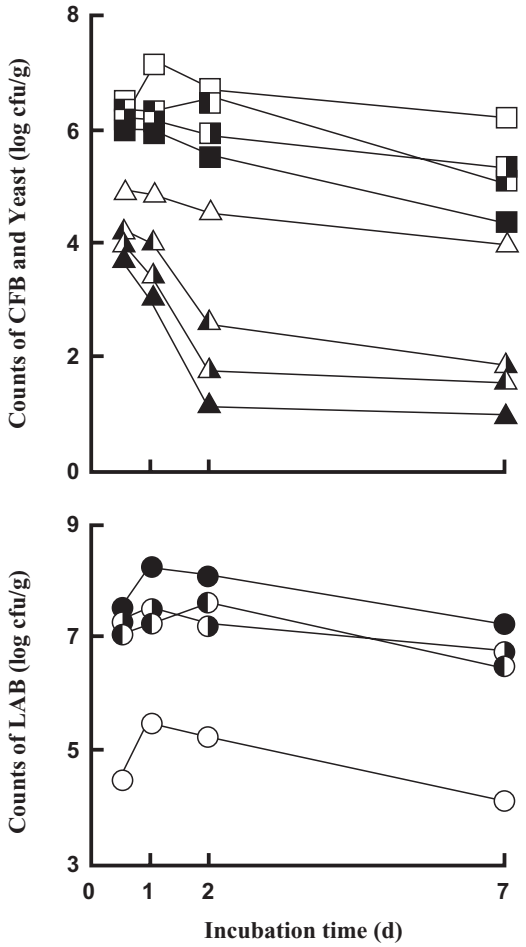

Fig. 3. Changing of the count of microorganisms in silage inoculated with or without lactic acid bacteria strains SP 1-3 and CS 1-8

Each lactic acid bacteria strain was cultured in lactobacilli MRS broth. ○: lactic acid bacteria (LAB). □: coliform bacteria (CFB). △: yeast.
○, □, △: no inoculation of LAB.
◑, ◨, ◭: inoculation of strain SP 1-3.
◐, ◧, ◮: inoculation of strain CS 1-8.
●, ■, ▲: inoculation of the mixture of SP 1-3 and CS 1-8.

of that by strain SP 1-3. Strain CS 1-8 should have a higher growth speed but weak tolerance for lactate.

From these results, the mixture of strain SP 1-3 and strain CS 1-8 should be expected to have quick production from the early stage and a large accumulation of lactate during long-term fermentation.

4. Growth inhibition activity against CFB and yeast by selected LAB strains

Growth inhibition activity of the culture filtrate from selected LAB strains (CS 1-8, CS 5-5, KS 1-9 and SP 1-3) were examined. All of the culture filtrates showed pH 3.8−4.0 and inhibited the growth of five bacteria strains including CFB (*Enterobacter* sp. SG 1-1 T). However, these growth inhibitions seemed to be caused by low pH (pH 3.8−4.0) because the inhibition activity disappeared after pH adjustment to 5.5 (data not shown). This conclusion is supported by no reduction in the counts of CFB using the modified pouch method (Table 2 and 3). Growth inhibition activities of these strains against three LAB strains including *Lactobacillus plantarum* were certainly not detected. However, there is still no change in the evaluation that strain SP 1-3 is an excellently favorable strain for making good-quality silage in Thailand although it shows no growth inhibition activity against CFB (*Enterobacter* sp. SG 1-1 T).

5. Preparation of silage inoculated with strains SP 1-3 and CS 1-8

It was suggested that biological additives including LAB were important to improve the silage quality in also tropical regions[4]. Therefore, the ability of strains SP 1-3 and/or CS 1-8 to improve the silage quality was examined by preparing napiergrass silage. The count of LAB in silage without the inoculation of LAB strains was about 10^4–10^5 cfu/g which was the same level as the count of yeast and 10^2 times lower than that of CFB (Fig. 3). On the other hand, the count of LAB in silage with the inoculation of LAB strain SP 1-3 or strain CS 1-8 was about 10^7 cfu/g and was 10^2–10^3 times higher than that of silage without the inoculation of an LAB strain. At the same time, the counts of CFB and yeast were obviously repressed by the inoculation of an LAB strain. This may be because the count of LAB in napiergrass (originated from nature) is at an insufficient level for good silage fermentation and can not rapidly produce a sufficient amount of lactate.

The pH of silage inoculated with an LAB strain was less than 4.0 after 7-day incubation while that inoculated with no LAB strain was about pH 4.4 (Table 5). The amount of lactate produced in silage without the inoculation of an LAB strain was about 0.72% corresponding to about 48% of glucose added after 7-day incubation (Table 5). However, the amount of lactate produced in silage inoculated with LAB strain SP 1-3 increased to about 1.35%. The amount of lactate produced by strain CS 1-8 was unexpectedly 1.41% corresponding to about 90−94% of glucose added and similar to the result obtained in Table 3. In addition, a sufficient amount of lactate was not produced in the silage inoculated with the mixture of strain SP 1-3 and CS 1-8 after 7-day incubation, unexpectedly. The production of acetate in silage was repressed to less than one-fifth after 7-day incubation by the inoculation of an LAB strain, while in silage with no inoculation of an LAB strain, it was accelerated (Table

Table 5. Amounts of acids produced in napiergrass medium

Exp. no.	Strain inoculated		Incubation time (h)	pH of culture filtrate	Acids produced (% in fresh silage)	
	SP 1-3	CS 1-8			Lactate	Acetate
1	–	–	12	5.72	0.18	0.22
2	–	–	24	5.27	0.34	0.25
3	–	–	48	4.67	0.44	0.33
4	–	–	168	4.40	0.72	0.57
5	+	–	12	5.20	0.39	0.46
6	+	–	24	4.25	0.77	0.21
7	+	–	48	4.05	1.12	0.11
8	+	–	168	3.93	1.35	0.10
9	–	+	12	4.69	0.48	0.23
10	–	+	24	4.32	0.62	0.13
11	–	+	48	4.10	0.88	0.10
12	–	+	168	3.96	1.41	0.09
13	+	+	12	4.67	0.45	0.19
14	+	+	24	4.22	0.53	0.12
15	+	+	48	3.98	0.80	0.08
16	+	+	168	3.98	0.85	0.07

Medium used in this study consisted of about 2 cm lengths of cut napiergrass (moisture content about 75%) with 1.5% glucose (w/w). After inoculation of strains SP 1-3 and-or CS 1-8 (each 4.0×10^6 cfu/g), the medium was incubated at 45ºC in an anaerobic jar.

Table 6. Taxonomic properties of lactic acid bacteria strains selected

Items		Strain no.			
		SP 1-3	CS 1-8	CS 5-5	KS 1-9
Shape		Short rod	Cocci (tetrad)	Cocci (tetrad)	Cocci (tetrad)
Gram stain		+	+	+	+
Optical form of lactic acid produced		DL	DL	DL	DL
CO_2 production from glucose		–	–	–	–
Growth at	37ºC	++	++	++	++
	45ºC	++	++	++	++
	in NaCl 6.5%	+	++	++	++
	pH 4.5	++	++	++	++
	pH 9.6	++	++	++	++
Sugars fermentation	Arabinose	++	++	++	++
	Fructose	++	++	++	++
	Glucose	+	++	++	++
	Lactose	++	+	–	+
	Mannose	++	++	++	++
	Maltose	+	+	–	–
	Raffinose	++	–	–	–
	Rhamnose	+	+	+	+
	Ribose	++	+	–	+
	Sorbitol	++	++	++	++
	Sucrose	–	–	–	–
	Trehalose	++	++	++	++
	Xylose	+	++	++	++

++, +: positive. –: negative.

5). In silage without an LAB strain inoculated, heterofermentative LAB strains originated from nature might grow and produce large amounts of acetate[1]. Results of the silage preparation also suggest that strains SP 1-3 and CS 1-8 were excellently suitable strains for silage-making in tropical regions.

6. Taxonomic study of selected LAB strains

To identify the selected four LAB strains mentioned before, their taxonomic properties were examined according to a reference manual[2]. All of them were Gram-positive, homofermentative and tetrad cocci except strain SP 1-3 which was a short rod. Their sugar fermentation and growth at various culture conditions are summarized in Table 6. From these results, strain SP 1-3 was presumed to be *Lactobacillus plantarum*. The generic and biochemical characteristics should be examined to confirm the scientific name. The three cocci were presumed to be *acidilactici-pentosaceus* group strains of the genus *Pediococcus*. It is also necessary to carry out further experiments of generic and biochemical properties for final determination of their scientific names.

In addition, it has been reported that LAB strains belonging to the genera *Lactobacillus* and *Pediococcus* predominated in tropical silage[13]. Our four strains mentioned above also belonged to both genera, unexpectedly.

Conclusion

1. Thirteen strains of lactic acid bacteria (LAB), preliminarily selected from 215 LAB strains isolated from 14 silage samples prepared in Thailand, were examined for silage fermentation inoculants to make good-quality silage in Thailand.
2. Among them, strains SP 1-3, CS 5-5, CS 1-8 and KS 1-9 were selected as favorable strains.
3. In particular, strain SP 1-3 which was isolated from corn silage and tentatively assigned to *Lactobacillus plantarum* exhibited thermotolerant and lactate tolerant properties.
4. Strain CS 1-8, isolated from TMR silage and tentatively assigned to *Pediococcus* sp., exhibited good growth at the early stage of fermentation but the accumulation of lactate was about half of that by strain SP 1-3 after 21-day incubation.
5. Napiergrass silage inoculated with strain SP 1-3 or strain CS 1-8 was of low pH, and contained large amounts of lactate and small amounts of acetate. The counts of CFB and yeast in the silage were at a low level.
6. From these results, selected strains SP 1-3 and CS 1-8 were evaluated as suitable strains for silage fermentation inoculant use in tropical regions.

References

1. Flores, D. F. (1991) Biotechnology and the improvement of silage (tropical and temperate) rumen digestion, a mini-review. *Appl. Microbiol. Biotechnol.*, **35**, 277–282.
2. Uchimura, T. & Okada, S. (1992) Nyusankin jikken manual [Manuals for experiments of lactic acid bacteria]. Asakura Shoten, Tokyo, pp.162 [In Japanese].
3. Lindgren, S. E., Axelsson, L. T. & McFeeter, R. F. (1990) Anaerobic L-lactate degradation by *Lactobacillus plantarum*. *FEMS Microbiol. Lett.*, **66**, 209–214.
4. Muhlbach, P. R. F. (1999) Additives to improve the silage making process with tropical forages. Silage making in the tropics with particular emphasis on small holders. *In* FAO plant production and protection paper 161, 151–164.
5. Ohmomo, S. et al. (1995) Screening of lactic acid bacteria for silage-making in tropical regions. *JARQ*, **29**, 251–256.
6. Ohmomo, S. et al. (2000) Purification and some characteristics of enterocin ON-157, a bacteriocin produced by *Enterococcus faecium* NIAI 157. *J. Appl. Microbiol*., **88**, 81–89.
7. Ohmomo, S. et al. (2002) Silage and microbial performance, old story but new problems. *JARQ*, **36**, 59–71.
8. Ohmomo, S., Nitisinprasert, S. & Hiranpradit, S. (2002) Silage-making and recent trend of dairy farming in Thailand. *JARQ*, **36**, 227–234.
9. Ohmomo, S. et al. (2004) Modification of the pouch method to evaluate the ability of lactic acid bacteria strains for making good quality silage in Thailand. *JARQ*, **38**, 119–124.
10. Tanaka, O. & Ohmomo, S. (1994) A repeatable model system for silage fermentation in culture tubes. *Biosci. Biotechnol. Biochem*., **58**, 1412–1415.
11. Tanaka, O. & Ohmomo, S. (1998) Lactic acid productivity of the selected strains of the genus *Lactobacillus* in laboratory-scale silage. *Grassl. Sci.*, **43**, 374–379.
12. Technical Cooperation Division of Agriculture Development Cooperation Department (1998) Final evaluation report on the dairy farming development project in the central region of the Kingdom of Thailand. Japan International Cooperation Agency, Tokyo, pp.136.
13. Tjandraatmadja, M., Norton, B. W. & MacRae, I. C. (1990) A numerical taxonomic study of lactic acid bacteria from tropical silages. *J. Appl. Bacteriol*., **68**, 543–553.