Screening and Identification of Thermophilic Lactic Acid Bacteria Producing Antibacterial Substances Determined by OMP Analysis

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ABSTRACT

A total of forty-six lactic acid bacteria (LAB) strains were screened for production of antibacterial substances, out of which two strains, JAK-CS7-1 and JAK-M1-3, displayed broad inhibition spectra against Gram-positive and Gram-negative pathogenic bacteria. Based on physiological, biochemical and molecular methods, these two bioactive thermophilic LAB strains were identified as *Pediococcus acidilactici*. The amounts of lactic and acetic acids produced by these two LAB strains were determined by HPLC method and then used for preparing the organic acid model system performance (OMP). Thus, comparative evaluation of the antibacterial activities of the culture supernatants (CS) and the corresponding OMP, consisting of equivalent acids concentration and similar pH like CS, revealed that the OMP displayed lower activities than the CS. It was found that the antibacterial activities of the CS were greater than the OMP by 50% that could be attributed to the presence of additional antibacterial metabolites in the CS. The results of this study suggested that these two strains of *Pediococcus acidilactici* appear to contain other bioactive substances in addition to lactic and acetic acids.

Key words: antibacterial activity, lactic acid bacteria, *Pediococcus acidilactici*

INTRODUCTION

Lactic acid bacteria (LAB) are widely used as starter cultures for the fermentation of foods and beverages because of their contribution to flavor, texture and color development (De Vuyst and Vandamme, 1994). They also have the potential to inhibit the growth of pathogenic and spoilage microorganisms, thereby improving the hygienic quality and extending the shelf life of food products (Caplice and Fitzgerald, 1999). As a result, LAB have been the focus of intensive research, which principally aimed at development of suitable strains for use as food biopreservatives. Although reduction of pH, resulted from the fermentative conversion of carbohydrates to organic acids (lactic and acetic), is the primary effect exerted by these bacteria, they also produce other substances such as hydrogen peroxide, carbon dioxide, diacetyl, low molecular weight substances and bacteriocins; that are known to exhibit antagonistic activities against a wide range of pathogenic and spoilage microorganisms. The preservative effect of LAB during the manufacture and storage of fermented foods is therefore attributed to the combined action of these array of functional metabolites (Helander

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et al., 1997; Ouwehand, 1998).

Because of such unique metabolic characteristics, some LAB have also enjoyed considerable applications as inoculants for silage preparation. Effective silage fermentation require, among other factors, fast acid-producing LAB strains that essentially brought about the preservation of forage crops from spoilage microorganisms (Ennahar *et al.,* 2003). Thus, new LAB strains endowed with more broader applications for food and feed fermentations are still critically needed.

In the course of screening of LAB for novel antimicrobial substances, we have examined forty-six strains of LAB for antibacterial activities, from which two most effective thermophilic strains were identified.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

Forty-six strains of LAB, isolated from silage in Thailand, and the indicator strains used in this study were obtained from the culture collection of the Department of Biotechnology, Kasetsart University, Thailand. LAB strains were grown in MRS (Difco) broth at 45°C, under anaerobic or aerobic conditions and routinely maintained at 4°C on MRS agar slant culture. The indicator strains were cultivated in nutrient broth (Difco) at 37°C. *Eschericiha coli* DH5? was used as a host for cloning of PCR products and cultured in LB broth.

Screening of LAB strains for antibacterial activities

Cell-free culture supernatants (CS) of LAB strains were evaluated for antibacterial activities using the spot-on-lawn method (Hoover and Harlander, 1993). Nutrient agar (1.5% w/v) plates were overlaid with 5 ml of soft agar (0.7% w/v) that had been inoculated (0.2% v/v) with standardized culture of indicator strains (about 10^7 cfu/ml). An aliquot of 10 µl of CS samples

were spotted onto the surface of the freshly prepared indicator lawns. The plates were then incubated for 6 h at 37°C and subsequently examined for the formation of inhibition zones. The antibacterial activities were assayed by spotting serial dilutions of the CS and CS adjusted to pH 5.0 and 5.5 (to reduce the activity of organic acids) and expressed as arbitrary units (AU/ml). An arbitrary unit was defined as the reciprocal of the highest dilution producing a clear zone of growth inhibition of the indicator strain, which was calculated as (1000/10)D, where D is the dilution factor (Parente *et al.,* 1995).

Determination of morphology and motility

LAB strains were subjected to Gram-staining reaction and examined by phase contrast microscope to determine the cell morphology. The motility, catalase and spore tests were investigated by the method of Atlas (1997) and Forbes *et al.* (1998).

Determination of the growth on various temperatures, pH and NaCl concentrations

The growth of LAB strains at temperatures 10, 37, 45 and 50°C and at pH 4.5 and 9.6 were determined by inoculating one colony from one day culture into MRS medium. Salt tolerance was tested in MRS supplemented with 6.5 and 18 % NaCl. The abilities of growth under all these conditions were evaluated spectrophotometrically by measuring the turbidity at 600 nm, after 24 and 48 h incubation times, as previously described by Nitisinprasert *et al.* (2000).

Carbohydrate fermentation pattern

Carbohydrate fermentation patterns were determined, simultaneously at 37 and 45°C, with API 50CH Rapid fermentation strips (API, BioMérieux, France) in CHL medium as specified by the manufacturer.

PCR amplification of the 16S rRNA gene

Chromosomal DNA was isolated by a modification of the method of Anderson and Mckay (1983), in which the alkaline denaturation step was omitted. This DNA was used as a template for the polymerase chain reaction (PCR) that was conducted according to Newton and Graham (1997). Thus, the oligonucleotide primers 8UA (forward) and 1407B (reverse), previously designed by Nitisinprasert *et al.* (2000), were used to amplify about 1.4 kb fragment from the 16S rRNA gene. PCR was carried out in a volume of 10 µl containing 10xbuffer, 1.5mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (dNTP), 1 µM of each primer (KU-Vector DNA Synthesis Unit, Kasetsart University), 1 U of *Taq* polymersase (Promega, USA) and about 200 ng of template DNA. All amplification reactions were carried out in a DNA thermal cycle (PCR machine, Touchdown Hybaid) and the program was performed as follows: 1 cycle of 5 min denaturation at 94°C and further 35 cycles consisting of (i) 1 min denaturation at 94°C, (ii) 2 min primer annealing at 55°C and (iii) 2 min primer extension at 72°C. After the 35th cycle, the extension reaction was continued for another 15 min at 72°C to ensure the completion of the final extension step. PCR products were analyzed by electrophoresis on 1.5% agarose gel using TBE buffer.

Cloning and sequencing of the 16S rRNA gene

The PCR products were purified by using QIAEX II Gel Extraction Kit (QIAGEN Inc, USA) and then ligated into TA-cloning with pGEM-T easy vector (Promega, USA). The resulting ligation products were transformed into *E. coli* DH5 competent cells in accordance with the manufacturer protocol. Selection of the positive clones with *ca.* 1.4 kb PCR insert was based on the expressed blue/white phenotypes. White colonies were picked up and cultured in LB broth supplied with ampicillin (100 µg/ml) (Sigma), from which the recombinant plasmid DNA was isolated using the alkaline lysis procedure (Sambrook and Russell, 2001). Plasmid preparations were digested with *Eco*RI (Promega) under standard conditions in order to confirm the presence of insert corresponding to the size of the 16S rRNA gene. Double strand plasmid templates were prepared from positive clones and sequenced on both strands using T7 and SP6 sequencing primers on an ABI Prism DNA Sequencer model 3100, at the Bio Service Unit (BSU), in Thailand. Analysis of the sequence was performed with the ABI Prism Sequencing Analysis version 3.7. The resulting sequences were compared with the non-redundant nucleotide database at GenBank by using the BLAST program.

Determination of lactic and acetic acids content and their organic acid model system performance (OMP) preparation

The amounts of lactic and acetic acids produced during 12 - 48 h were determined by HPLC. The method previously described by Nitisinprasert *et al.* (2000) was used for the analysis of the organic acids. The corresponding organic acid model performance (OMP) consisting of equivalent lactic and acetic acid concentrations and similar pH like that of CS was prepared. The amounts of undissociated lactic and acetic acids were also calculated using the formula: Undissociated acid (mM) = (Total acid (mM))/(1 + 10^{pH-pKa}) (Ogawa *et al.*, 2001).

RESULTS

Screening of LAB strains for antibacterial activities

A total of 46 LAB strains were evaluated for antibacterial activities against *Escherichia coli* ATCC 8739 and *Salmonella* Typhimurium ATCC 13311, in conditions eliminating the effect of hydrogen peroxide by initial anaerobic cultivation of their cultures (Contreras *et al.,* 1997). This preliminary screening indicated that the CS of

most LAB strains, having reduced pH, displayed antibacterial activities against the indicator strains. However, some of these positive LAB strains after neutralization to pH 5.0 and 5.5 either lost activities or displayed inhibition activities against only one of the indicator strains. Whereas, the CS of two LAB strains, M1-3 and CS7-1, after being readjusted to pH 5.0 and 5.5 produced inhibition activities against the same two indicator strains, as shown in Table 1. Since LAB strains with broader inhibition spectra are preferred, only CS7-1 and M1-3 were selected for further studies.

The antibacterial activities of CS of these two selected strains have decreased by 50% after pH neutralization, which may be due to elimination of the effects from the lactic and acetic acids. This could also be attributed to the loss of synergistic activities of other antibacterial substance and these acids, upon increasing of pH.

Identification of two selected LAB strains

As shown in Table 2, both strains are Gram-positive, cocci in tetrads, catalase-negative and homofermentative. These strains were able to grow in MRS broth supplemented with NaCl concentration of 6.5 % and up to pH 9.6. Moreover, CS7-1 can grow in the range of 37-50°C while the maximum growth temperature for M1-3 was 37-45°C. Comparison of these properties with the differential characteristics of LAB described by Axelsson (1998) and Bergey's manual (Garvie, 1986) suggested that both CS7-1 and M1-3 could be assigned to the genus *Pediococcus.*

Both strains, CS7-1 and M1-3, showed identical carbohydrate fermentation patterns, at both 37 and 45°C and in particular they were able to metabolize pentoses and hexoses but unable to ferment most of the disaccharides (Table 3). The abilities of these two strains to ferment ribose and grow at higher pH clearly differentiate them from three of the pediococcal species such as *Pediococcus damnosus, P. dextrinicus and P. parvulus*, that are devoid of such properties

(Simpson and Taguchi, 1995). Accordingly, a preliminary identification made by the API database correlation indicated their similarities to *Pediococcus pentosaceus* and *Pediococcus acidilactici*. Furthermore, the differentiation schemes described by Simpson and Taguchi (1995) suggested that the ability to grow at 50°C and inability to ferment maltose are assigned to *P. acidilactici.* On the other hand, Stiles and Holzapfel (1997) indicated that *P. acidilactici* and *P. pentosaceus* are closely related species that may not be clearly differentiated by phenotypic characteristics. Therefore, the 16S rRNA sequences of CS7-1 and M1-3 were examined in order to confirm the conventional identification.

The PCR amplification of the 16S rRNA gene gave a product of about 1.4 kb for both LAB strains. This PCR product was cloned and the resulting recombinant plasmid, containing the 16S rRNA gene, was sequenced on both strands. Analysis of the DNA sequences by BLAST program revealed that CS7-1 showed highest level of similarity (99% identity) to *Pediococcus acidilactici* strains RO17 (Ennahar *et al.*, 2003) and LA3 (Cai *et al.,* 1999), whereas M1-3 showed highest level of similarity (99% identity) to *Pediococcus acidilactici* strains B1104 and DSM20284 (Heinz *et al.,* 2000). It was therefore concluded that our strains were *Pediococcus acidilactici* and designated as *Pediococcus acidilactici* JAK-CS7-1 and *Pediococcus acidilactici* JAK-M1-3.

Growth kinetics and production of antibacterial substances

The growth kinetics of strains CS7-1 and M1-3 were determined at 45°C and the results were presented in Figures 1 and 2. It can be seen in both strains that incubation times of 4-8 h and 8-12 h belong to the exponential and stationary growth phases, respectively. While after 12 h the cell counts started to decline and then entered the death phase. As shown in Figure 3, the CS of the

Table 1 Antibacterial activities of forty-six LAB strains, expressed as AU/ml.

LAB strain	pH[*]	Antibacterial activities against indicating strains					
		Escherichia coli ATCC 8739			*Salmonella* Typhimurium ATCC 13311		
		CS[**]	pH 5.0	pH 5.5	CS[**]	pH 5.0	pH 5.5
C1-1	6.30	0	0	0	0	0	0
C1-2	5.55	0	0	0	0	0	0
C1-3	6.01	0	0	0	0	0	0
C1-4	6.36	0	0	0	0	0	0
C1-5	5.49	0	0	0	0	0	0
C1-6	3.98	200	0	0	300	0	0
C1-7	4.44	200	0	0	200	0	0
C1-8	5.15	0	0	0	0	0	0
C1-9	4.35	100	0	0	100	0	0
C1-10	4.33	100	0	0	100	0	0
C1-11	4.05	300	0	0	200	0	0
M1-1	4.87	0	0	0	0	0	0
M1-2	4.77	0	0	0	0	0	0
M1-3	4.14	400	200	100	400	200	100
M1-4	4.49	100	0	0	0	0	0
M1-5	3.50	400	0	0	0	0	0
M1-6	3.37	300	0	0	0	0	0
M1-7	3.35	400	0	0	0	0	0
M1-10	3.18	300	0	0	0	0	0
M1-11	3.35	300	0	0	0	0	0
M1-12	3.19	300	0	0	0	0	0
W1-1	3.00	300	100	0	300	100	0
W1-2	2.96	300	100	0	300	100	0
W1-3	3.37	300	100	0	300	100	0
W1-4	3.54	200	0	0	200	0	0
W1-5	3.50	400	100	0	400	100	0
W1-6	3.39	400	0	0	300	0	0
W1-8	3.37	400	0	0	400	0	0
W1-9	3.24	300	0	0	300	0	0
W1-10	3.53	300	0	0	300	0	0
W1-11	3.48	400	0	0	400	0	0
CS6-1	3.74	300	0	0	300	0	0
CS6-2	3.60	300	0	0	300	0	0
CS6-3	3.57	300	0	0	300	0	0
CS6-4	3.63	300	0	0	300	0	0
CS6-5	3.55	300	0	0	300	0	0
CS6-6	3.55	300	0	0	300	0	0
CS7-1	4.11	400	200	100	400	200	100
CS7-2	3.53	400	100	0	300	0	0
CS7-3	3.61	300	100	0	200	0	0
CS7-4	3.61	300	100	0	200	0	0
CS7-5	3.68	300	100	0	200	0	0
CS7-6	3.66	300	100	0	200	0	0
CS8-1	3.63	200	0	0	200	0	0
CS8-2	3.55	200	0	0	200	0	0
CS8-3	3.74	200	0	0	200	0	0

[*] Final pH of the stationary growth phase culture; [**]non-neutralized cell-free culture supernatant

consecutive cultures displayed antibacterial activities, against both *E.coli* ATCC 8739 and *Salmonella* Typhimurium ATCC 13311, corresponding to the following values: 2 h culture showed 100 AU/ml, 4 h with 200 AU/ml, 6 – 8 h with 300 AU/ml and 10-24 h with 400 AU/ml. However, the CS of stationary growth phase cultures after pH neutralization to 5.0 and 5.5 exhibited antibacterial activities of 200 and 100 AU/ml, respectively. The decrease of antibacterial activities after pH neutralization may be ascribed to the reduction of the effects of lactic and acetic acids. The other more plausible justification could be the increased pH may not be equally favorable for all metabolites to effectively exert their antibacterial activities against the target strains.

Spectrum of inhibitory activity

The CS of the two strains displayed a remarkably broad inhibition spectrum over a wide range of Gram-positive and Gram-negative microorganisms. As can be seen from Table 4, Gram-negative indicator strains were more sensitive than the Gram-positive ones, which may be attributed to their difference in cell-wall composition. The inhibitory activities were more pronounced against strains of *E. coli* and *Salmonella* sp., of which strains E001-E012 and S001-S003 were isolated from chicken intestine and reported to be resistant to antibiotics (Nitisinprasert *et al.,* 2000). The CS pH 5 did not inhibit any of *Staphylococcus aureus* strains while the CS showed only moderate inhibitory activities. Except the first two *Staphylococcus aureus* strains, the other nine strains are known to cause mastitis diseases of cows. In contrast, CS7-1 and M1-3 did not exhibit any antibacterial activities against other LAB strains such as *Enterococcus faecalis* TISTR 927*, Lactobacillus plantarum* TISTR 541, *Leuconostoc mesenteroides* TISTR 473 and *Pediococcus acidilactici* TISTR 953. It is noteworthy that the neutralized CS of CS7-1 and

Table 2 Phenotypic characteristics of strains CS7-1 and M1-3.

Test	Characteristic	
	CS71-1	M1-3
1. Morphology	Cocci (in tetrads)	Cocci (in tetrads)
2. Gram stain	+	+
3. Motility	-	-
4. Spore	-	-
5. Catalase	-	-
6. Growth at 10°C	-	-
37°C	+	+
45°C	+	+
50°C	+	-
pH 4.5	+	+
pH 9.6	+	+
6.5% NaCl	+	+
18% NaCl	-	-
7. CO_2 gas from glucose	-	-

M1-3 were especially active against the Gram-negative pathogenic bacteria, which were reported to be resistant to some antimicrobials due to their protective outer membrane (Helander *et al.,* 1997).

The effect of antibacterial substances other than acids as determined by analysis of OMP

Analysis of the CS of CS7-1 and M1-3 by HPLC indicated that lactic and acetic acids were the only major acids produced by these two strains.

The production trend of these two acids during 12-48 h cultivation time was presented in Figure 4, from which the corresponding OMP were prepared.

The amounts of lactic and acetic acids produced by these strains at different cultivation times were used to compare their antibacterial activities. As can be seen from Figure 4, the amounts of total lactic acid and undissociated lactic acid (U-LA) of the CS7-1 and M1-3 were almost equivalent, whereas total acetic acid and

Table 3 Carbohydrate fermentation patterns of strains CS7-1 and M1-3.

Test	Strain		Test	Strain	
	CS7-1	M1-3		CS7-1	M1-3
1. Glycerol	-	-	25. Esculin	+	+
2. Erythritol	-	-	26. Salicin	+	+
3. D-Arabinose	-	-	27. Cellobiose	+	+
4. L-Arabinose	+	+	28. Maltose	-	-
5. Ribose	+	+	29. Sucrose	-	-
6. D-Xylose	+	+	30. Lactose	?	?
7. L-Xylose	-	-	31. Melibiose	-	-
8. Adonitol	-	-	32. Trehalose	+	+
9. β-Methyl-D-xyloside	-	-	33. Inulin	-	-
10. Galactose	+	+	34. Melezitose	-	-
11. D-Glucose	+	+	35. D-Raffinose	-	-
12. D-Fructose	+	+	36. Starch	-	-
13. D-Mannose	+	+	37. Glycogen	-	-
14. L-Sorbose	-	-	38. Xylitol	-	-
15. Rhamnose	+	+	39. β-Gentiobiose	+	+
16. Dulcitol	-	-	40. D-Turanose	-	-
17. Inositol	-	-	41. D-Lyxose	-	-
18. Mannitol	-	-	42. D-Tagatose	+	+
19. Sorbitol	-	-	43. D-Fucose	-	-
20. α-Methyl-D-mannoside	+	+	44. L-Fucose	-	-
21. α-Methyl-D-glucoside	-	-	45. D-Arabitol	-	-
22. N-Acetyl-glucosamine	+	+	46. L-Arabitol	-	-
23. Amygdalin	+	+	47. Gluconate	?	?
24. Arbutin	+	+	48. 2-Keto-gluconate	-	-
			49. 5-Keto-gluconate	-	-

+, acid production (positive); -, no acid produced; ?, weakly positive

undissociated acetic acid (U-AA) of M1-3 was higher than CS7-1, especially at 24 h. Thus, the antibacterial activities of CS pH 5 of M1-3 was higher towards indicator strains E001, E002, E004, E012 and S002 corresponding to 200 AU/ml, whereas CS pH 5 of CS7-1 did at 100 AU/ml against these indicator strains. It has been reported that acetic acid is the stronger inhibitor and has a

wide range of inhibitory activity (Ouwehand, 1998). Hence, with regard to the acid content, the greater antibacterial activity of strain M1-3 is justifiable.

The antibacterial activities of the CS and OMP were compared against *Escherichia coli* ATCC 8739 and *Salmonella* Typhimurium ATCC 13311. Both of these indicator strains were equally
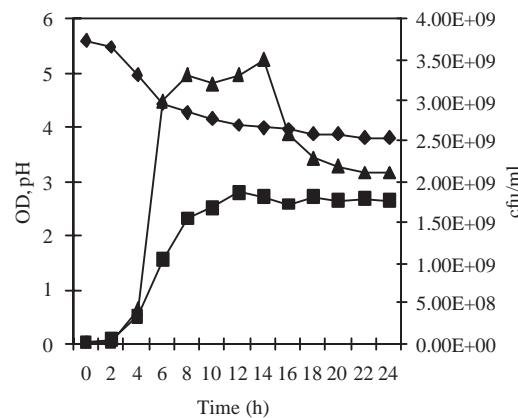


Figure 1 Growth kinetics of CS7-1
■ OD ◆ pH ▲ cfu/ml.
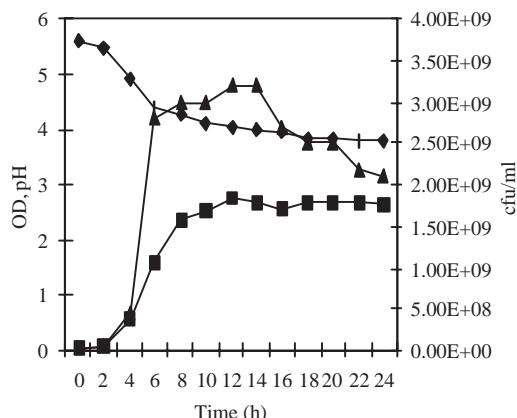
**Figure 2** Growth kinetics of M1-3
■ OD ◆ pH ▲ cfu/ml.
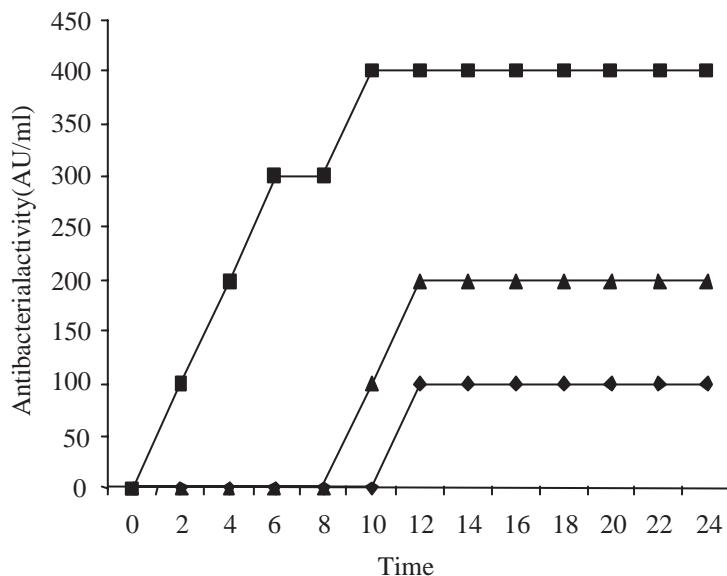
Figure 3 Relationships between antibacterial activities and incubation times for both CS7-1 and M1-3 ■ cs, ▲ pH5, ◆ pH5.5.

inhibited by both CS7-1 and M1-3. Thus, the result shown in Table 5, represented the antibacterial activities against both *Escherichia coli* ATCC 8739 and *Salmonella* Typhimurium ATCC 13311.

As can be seen from the above result, the CS exhibited antibacterial activities of 400 AU/ml, while the OMP showed only 200 AU/ml, against both indicator strains. Moreover, the CS

Table 4 Inhibition spectra of the CS of CS7-1 and M1-3, expressed as AU/ml.

Indicating strain	CS7-1			M1-3		
	CS*	pH 5.0	pH 5.5	CS*	pH 5.0	pH 5.5
Escherichia coli ATCC 11775	400	200	100	400	200	100
Escherichia coli E001	400	100	0	400	200	0
Escherichia coli E002	400	100	100	400	200	100
Escherichia coli E003	400	100	100	400	100	100
Escherichia coli E004	400	100	100	400	200	100
Escherichia coli E005	400	200	100	400	200	100
Escherichia coli E006	200	100	0	200	100	0
Escherichia coli E007	200	100	0	200	100	0
Escherichia coli E008	400	100	0	400	100	0
Escherichia coli E010	400	200	100	400	200	0
Escherichia coli E011	200	100	0	400	100	0
Escherichia coli E012	400	100	100	400	200	100
Salmonella sp. S001	200	0	0	200	0	0
Salmonella sp. S002	400	100	100	400	200	100
Salmonella sp. S003	400	200	100	400	200	100
Bacillus subtilis TISTR 025	200	100	0	200	100	0
Bacillus cereus (isolated from silage)	400	100	0	400	100	0
Staphylococcus aureus ATCC 13565	200	0	0	200	0	0
Staphylococcus aureus ATCC 6538-P	200	0	0	200	0	0
Staphylococcus aureus 32 D	200	0	0	200	0	0
Staphylococcus aureus 36C	100	0	0	100	0	0
Staphylococcus aureus 33A	200	0	0	200	0	0
Staphylococcus aureus 37C	100	0	0	100	0	0
Staphylococcus aureus mixed milk	200	0	0	200	0	0
Staphylococcus aureus 38A	100	0	0	100	0	0
Staphylococcus aureus mixed milk 67	200	0	0	200	0	0
Staphylococcus aureus 39D	200	0	0	200	0	0
Staphylococcus aureus 40D	200	0	0	200	0	0
Enterococcus faecalis TISTR 927	0	0	0	0	0	0
Lactobacillus plantarum TISTR 541	0	0	0	0	0	0
Leuconostoc mesenteroides TISTR 473	0	0	0	0	0	0
Pediococcus acidilactici TISTR 953	0	0	0	0	0	0

*Non-neutralized culture supernatant of CS7-1 and M1-3, having pH 4.11 and 4.14, respectively.

pH 5 showed 200 AU/ml; whereas the OMP, readjusted to pH 5, displayed only 100 AU/ml. Thus, the activities of the CS were greater than the corresponding OMP by 50%, before and after pH neutralization at all cultivation times and pHs. It was also found that the OMP at pH 5.5 did not show any activity while the CS at this pH exhibited antibacterial activities corresponding to 100 AU/ml. This comparative study suggested that the CS of both LAB strains may contain other antibacterial substances in addition to lactic and acetic acids.

DISCUSSION
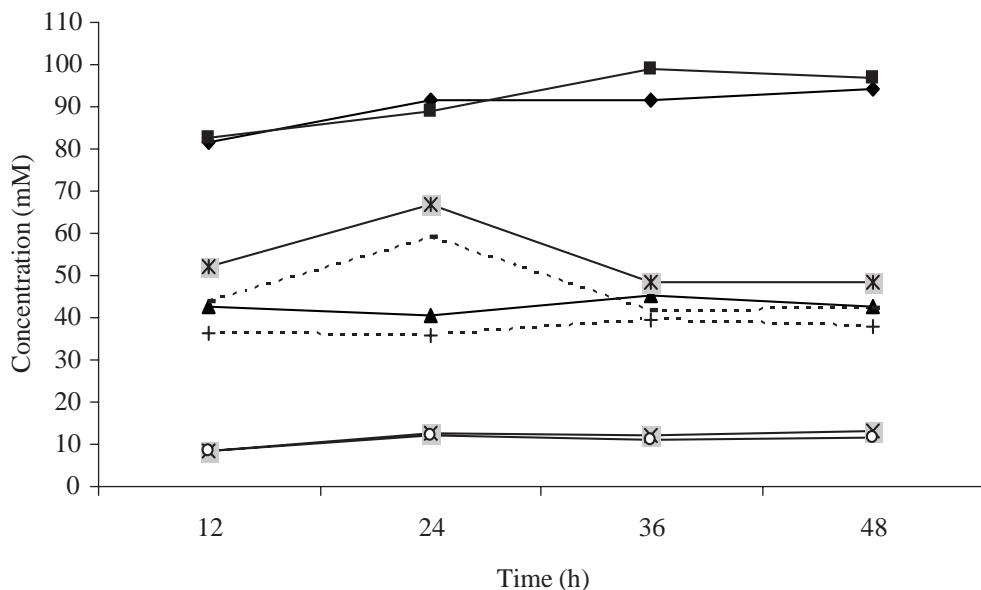
Figure 4 Amounts of total lactic and acetic acids, and their undissociated forms at different cultivation times

    ◆ Total LA of CS7-1   ■ Total LA of M1-3   ▲ Total AA of CS7-1
    ✳ Total AA of M1-3   ✕ U-LA of CS7-1   ○ U-LA of M1-3
    + U-AA of CS7-1   − U-AA of M1-3

Table 5 Antibacterial activities of CS and OMP, expressed as AU/ml.

Time (h)	CS7-1					M1-3				
	pH[*]	CS	CS pH 5	OMP	OMP pH 5	pH[*]	CS	CS pH 5	OMP	OMP pH 5
12	4.01	400	200	200	100	4.02	400	200	200	100
24	3.88	400	200	200	100	3.88	400	200	200	100
36	3.89	400	200	200	100	3.98	400	200	200	100
48	3.86	400	200	200	100	3.94	400	200	200	100

[*]pH of the cell free culture supernatant (CS), at which pH of the corresponding OMP was adjusted.

In this study two thermophilic LAB strains were identified as *Pediococcus acidilactici* JAK-CS7-1 and *Pediococcus acidilactici* JAK-M1-3 that exhibited wide spectrum of inhibitory activities against a range of indicator strains representing the species of food spoilage and pathogenic bacteria, of which Gram-negatives were more sensitive. It was found that LAB strain M1-3 exhibited greater inhibition activities against some *E. coli* strains which may be attributed to the higher amount of acids produced or the difference in the content of other inhibitory substances. The direct antimicrobial effects of lactic and acetic acids were reported to be resulted from the action of these acids on the bacterial cytoplasmic membrane which interferes with the maintenance of membrane potential and inhibits active transport (Caplice and Fitzgerald, 1999). It is assumed that the undissociated (neutral) form of these acids diffuse across the cell membrane because they are lipid-soluble. Thus, the undissociated molecule is considered to be the toxic form of a weak acid (Ouwehand, 1998; Ogawa *et al.,* 2001). Accordingly, strain M1-3 showed higher antibacterial activities because the amount of undissociated acetic acid was higher than CS7-1 at 24 h, the cultivation time used for investigating the inhibition spectrum.

In both strains the inhibition activities were more effective at lower pH. Reduction of the effects of lactic and acetic acids upon neutralization of the CS to pH 5 have resulted in the decrease of the activities by 50% from that of CS. This may also be due to the fact that most of the inhibitory substances produced by LAB are known to exert their activities at lower pH. It has been reported that many bacteriocins display greater antibacterial activities at lower pH values (pH 5 and below) than at physiological pH, because a higher amount of bacteriocin molecules are available at lower pH values. At lower pH values, the solubility is often increased, less aggregation of hydrophobic peptides occurs, and less bounding of bacteriocins to the

cell surface takes place. Also, hydrophilic bacteriocins may have an enhanced capacity to pass through hydrophilic regions of the cell surface of the sensitive target bacteria (Messens and De Vuyst, 2002).

Although the OMP contains equivalent amounts of acids and pH like that of CS, it displayed lower inhibition activities than CS. In fact, its activity before and after neutralization was reduced by 50% from the corresponding CS, indicating the presence of other inhibitory substances besides lactic and acetic acids. This may be more probable on account of the previous reports of some *Pediococcus acidilactici* strains to elaborate bacteriocins (Chikindas *et al.,* 1993; Cintas *et al.,* 1995; Elegado *et al.,* 1997).

Pediococci are often found living in association with plant materials, dairy and meat products. They were also reported as the dominant microbial population on forage crops and silage (Cai *et al.,* 1999). More recent information on the reclassification of *Pediococcus* species indicated that only five species belong to the genus: *Pediococcus acidilactici, Pediococcus damnosus, Pediococcus dextrinicus, Pediococcus parvulus* and *Pediococcus pentosaceus* (Barros *et al.,* 2001). Among these five recognized species *Pediococcus acidilactici* and *Pediococcus pentosaceus* have been more intensively investigated because of their enormous applications as starter cultures in the commercial fermentation of meats and vegetables (Stiles and Holzapfel, 1997).

The fact that our two strains, CS7-1 and M1-3, were isolated from silage may suggest that they have good potentials for future development of suitable inoculants for silage preparation. One of the factors that govern the success of an inoculant as a silage additive is the properties of the inoculant itself (Ennahar *et al.,* 2003). In this regard, a number of *Pediococcus acidilactici* strains have been reported to play beneficial effects as silage inoculants (Cai *et al.,* 1999). Thus, all these reported attractive features of *Pediococcus acidilactici*

strains essentially warrant further investigations to be conducted on our *Pediococcus acidilactici* JAK-CS7-1 and JAK-M1-3 strains. To this end, a study that aimed at characterizing the inhibitory substances other than organic acids is underway.

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