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Short Communication

Diacetyl of lactic acid bacteria from milk and fermented foods in Thailand

Amnat Pakdeeto,[1] Nuanphan Naranong,[1] and Somboon Tanasupawat[2,*]

[1] *Department of Applied Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand*

[2] *Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand*

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Diacetyl (biacetyl; 2,3-butanedione; dimethyl diketone; 2,3-diketobutane) is a major flavor compound essential in many dairy products such as butter, cream, and some cheeses. In addition to butter and other dairy products, it is found in red and white wines, brandy, roasted coffee, ensilage, and many other fermented foods (Jay, 1982). Its production was shown to depend on the strains used as starter cultures and the conditions of fermentation such as pH, oxygen, and temperature (Bassit et al., 1993; Gasson, 1983; Monnet et al., 1994). Moreover, it is a metabolic end product that is synthesized from pyruvate aerobically as well as anaerobically (Condon, 1987) that is actually produced by citrate fermenting lactic acid bacteria (Hugenholtz, 1993). The strains of lactic acid bacteria in the genera *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Pediococcus*, and *Lactococcus* (*Streptococcus*) can produce diacetyl as well as other organisms (Jay, 1982; Kaneko et al., 1990, 1991). In food, diacetyl is important not only because it is responsible for the desirable flavor in many foods, but also because it has antimicrobial properties (Ray, 1992). Since there are a diversity of lactic acid bacteria found in various fermented food products in Thailand (Tanasupawat and Komagata, 1995) there were no reports on diacetyl or acetoin. The aim of this work was to screen and identify the diacetyl-producing lactic acid bacteria, including the study of effects on growth and pH for the diacetyl and acetoin production.

Lactic acid bacteria were isolated from 5 samples of pasteurized milk and 21 of fermented foods obtained at the markets, and from 6 of raw cow's milk at the Dairy Farming Promotion Organization of Thailand. GYPB-0.3% $CaCO_3$ or MRS-0.3% $CaCO_3$ agar plate (De Man et al., 1960; Tanasupawat et al., 1998) was used for isolation. All the isolates obtained from various sources were screened for diacetyl/acetoin production in 15 ml MMRS broth (Phalip et al., 1994) by using the colorimetric method, as described by Mattessich and Cooper (1989). The selected strains that showed high diacetyl/acetoin production were further studied for their ability to produce diacetyl in MMRS medium. Cells grown in MRS broth were harvested at the end of the exponential growth phase, and the absorbance of cultures at 575 nm (Boumerdassi et al., 1997) was measured with a spectrophotometer (UV-160: Shimadzu, Kyoto, Japan) for preparing the inoculum. A 1% (v/v) inoculum was transferred into 400 ml MMRS broth in a 1,000 ml Erlenmeyer flask and incu-

* Address reprint requests to: Dr. Somboon Tanasupawat, Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand.

E-mail: tsomboon@chula.ac.th

bated at 30°C with shaking (200 rpm) and static condition (Kaneko et al., 1991). The fermentation broth was sampled every 6 h for observing the growth (O.D. at 575 nm) by spectrophotometer and for measuring the pH by Beckman pH-meter. Diacetyl and acetoin were determined by gas-liquid chromatography (Chrompack CP9000) as described by Thornhill and Cogan (1984).

Determination of morphological, cultural, physiological, and biochemical characteristics and the isomers of lactic acid were all from previous papers (Okada et al., 1978; Tanasupawat et al., 1998). Diaminopimelic acid (DAP) in the peptidoglycan was determined by the method of Komagata and Suzuki (1987). DNAs were isolated and purified from previous papers (Saito and Miura, 1963; Yamada and Komagata, 1970). Photobiotin labeling DNA-DNA hybridization was carried out in 2× SSC (saline trisodium citrate) and 50% formamide solution at 40 or 45°C for 12 h (Ezaki et al., 1989). DNA-DNA similarity was determined by using the colorimetric method as reported by Tanasupawat et al. (2000).

A total of 137 strains isolated from 5 samples of pasteurized milk were 5 rods and 44 cocci, 6 of raw cow's milk were 14 rods and 26 cocci and 21 of fermented foods were 38 rods and 10 cocci, respectively. All were gram positive, nonmotile, and nonsporing. Colonies on MRS agar plates were circular, low convex with entire margin, and nonpigmented. They did not produce catalase or reduce nitrate. Most of the rod-shaped strains were found in fermented foods (Tanasupawat et al., 1993, 1995, 1998) while the coccal strains were distributed in pasteurized milk and raw cow's milk (Devriese et al., 1991). The results showed that 115 isolates which could produce diacetyl/acetoin ranged from 0.01 to 6.49 mM. Homofermentative rod-shaped strains produced 0.01 to 6.49 mM and the homofermentative coccal strains 0.04 to 5.09 mM as reported in *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* CNRZ 125 (Phalip et al., 1994). Furthermore, the heterofermentative rod-shaped and coccal strains produced diacetyl/acetoin 0.12 to 3.62 mM and 2.62 to 4.41 mM, respectively. The selected strains SR8-1,

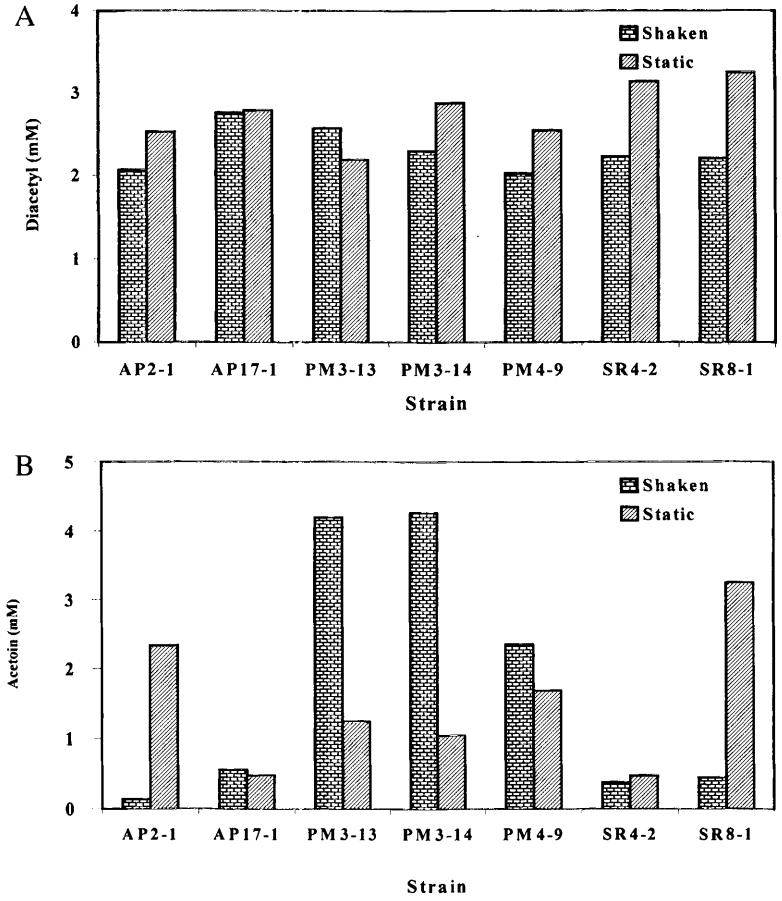

Fig. 1. (A) Diacetyl production of selected strains under shaken and static conditions at 30°C for 24 h. (B) Acetoin production of selected strains under shaken and static conditions at 30°C for 24 h.

Table 1. Characteristics of strains.

Characteristics	AP17-1	[a]SR4-2	[a]SR8-1	[b]*L. pentosus* NRCT 1069^T	[b]*L. plantarum* NRCT 1067^T	AP2-1	[c]*W. confusa* NRIC 0207^T	[c]*L. mesenteroides* NRIC 1541^T	PM3-13	PM3-14	PM4-9	[d]*E. faecium* NRIC 1145^T	[d]*E. faecalis* NRIC 1142^T
Cell form	Rods					Cocci							
Cell size (μm)	0.8–1.0×1.5–5.0					5.0–1.0							
Cell arrangement	Singly, in pairs or chains												
Gas from glucose	−	−	−	−	−	+	+	+	−	−	−	−	−
Arginine hydrolysis	−	−	−	−	−	+	+	−	+	+	+	+	+
Slime formation	−	−	−	−	−	+	+	+	−	−	−	−	−
Growth at 45°C	−	−	−	−	−	−	−	−	+	+	+	+	+
pH 4.0	+	+	+	+	+	+	+	+	−	−	−	ND	ND
pH 9.6	w	+	+	+	−	−	−	−	+	+	+	ND	ND
Isomer of lactic acid	DL	DL	DL	DL	DL	DL	DL	D	L	L	L	L	L
Peptidoglycan type: *meso*-DAP	+	+	+	+	+	−	−	−	−	−	−	−	−
Acid from:													
L-Arabinose	+	+	+	+	+	−	−	+	+	+	+	+	−
Gluconate	+	+	+	+	+	+	+	−	+	+	+	+	−
Glycerol	+	+	+	+	−	w	ND	ND	w	w	−	−	+
Lactose	+	+	+	+	+	−	−	+	+	+	+	+	+
D-Mannitol	+	+	+	+	+	−	−	+	w	w	+	+	+
D-Melibiose	+	+	+	+	+	−	−	+	w	w	w	−	−
D-Melezitose	w	w	w	−	+	−	−	ND	−	−	−	−	+
Raffinose	w	w	w	−	+	−	−	+	−	−	w	−	−
L-Rhamnose	w	+	w	+	−	−	−	−	w	w	w	−	−
D-Ribose	+	+	+	+	+	+	+	−	+	+	+	ND	ND
Salicin	+	+	+	+	+	−	−	+	+	+	+	+	+
D-Sorbitol	+	+	+	+	+	−	−	−	−	−	−	−	+
Sucrose	+	+	+	+	+	+	+	+	w	w	+	+	−
D-Trehalose	+	+	+	+	+	−	−	+	w	w	w	−	+
D-Xylose	+	+	+	+	−	+	+	+	w	−	−	−	−

+, positive; w, weak; −, negative reaction; ND, no data.

All produced acid from D-cellobiose, esculin, D-fructose, D-galactose and D-glucose. NRIC, NODAI Research Institute Culture Collection, Tokyo, Japan.

[a] Data from Tanasupawat et al. (2002).

[b] Data from Tanasupawat et al. (1992a, 1993, 2000).

[c] Data from Collins et al. (1993); Hammes et al. (1992); Kandler and Weiss (1986); Schillinger et al. (1989); Tanasupawat et al. (1993, 2000).

[d] Data from Facklam and Collins (1989); Tanasupawat et al. (1992b).

SR4-2, PM3-14, AP17-1, PM4-9, AP2-1, PM3-13 were cultivated in 400 ml MMRS broth for 24 h. As determined by gas-liquid chromatography, their diacetyl productions were 3.25, 3.14, 2.88, 2.79, 2.55, 2.53 and 2.19 mM under static conditions, and were 2.21, 2.23, 2.30, 2.76, 2.03, 2.07 and 2.57 mM under shaken conditions, respectively (Fig. 1A). On the other hand, their acetoin productions were 3.26, 0.48, 1.05, 0.48, 1.69, 2.34 and 1.25 mM under static conditions, and were 0.45, 0.38, 4.27, 0.56, 2.36, 0.14 and 4.20 mM under shaken conditions, respectively (Fig. 1B).

On the basis of phenotypic characteristics, isomer of lactic acid and peptidoglycan type of cell wall (Table 1), the selected strains AP17-1, SR4-2 and SR8-1 were included in the genus *Lactobacillus* (Hammes et al., 1992; Kandler and Weiss, 1986). A strain AP2-1 was in *Weissella* (Collins et al., 1993; Schillinger et al., 1989) which was different from genus *Leuconostoc* (Collins et al., 1993) and the strains PM3-13, PM3-14 and PM4-9 were in *Enterococcus* (Devriese et al., 1991; Tanasupawat et al., 1992b). From the DNA-DNA similarity results as shown in Table 2, the strains AP17-1, SR4-2 and SR8-1 showed high DNA similarity (93.3–111.6%) with *Lactobacillus pentosus* NRIC 1069^T but showed low DNA similarity with *Lactobacillus plantarum* NRIC 1067^T. They were identified as *Lactobacillus pentosus* (Tanasupawat et al., 1992a, 1998, 2000; Wayne et al., 1987). The strain AP2-1 showed high DNA similarity (100.5%) with *Weissella confusa* NRIC 0207^T. It was identified as *Weissella confusa* (Collins et al., 1993; Hammes et al., 1992; Kandler and Weiss, 1986; Schillinger et al., 1989; Tanasupawat et al., 1993, 2000; Wayne et al., 1987). The strains PM3-13, PM3-14 and PM4-9 showed high DNA similarity (72.1–99.6%) with *Enterococcus faecium* NRIC 1145^T but showed low DNA similarity (2.7–17.1%) with *Enterococcus faecalis* TISTR 379^T. They were identified as *Enterococcus faecium* (Facklam and Collins, 1989; Tanasupawat et al., 1992b; Wayne et al., 1987).

Table 2. DNA-DNA similarity of strains.

Strains	% Similarity with labeled strains				
	NRIC 1069^T	NRIC 1067^T	NRIC 0207^T	NRIC 1145^T	TISTR 379^T
AP17-1	111.6	19.6			
SR4-2[a]	93.3	30.9			
SR8-1[a]	100.9	53.6			
L. pentosus NRIC 1069^T	100.0	53.3			
L. plantarum NRIC 1067^T	38.9	100.0			
AP2-1			100.5		
W. confusa NRIC 0207^T			100.0		
PM3-13				83.8	5.5
PM3-14				72.1	2.7
PM4-9				99.6	17.1
E. faecium NRIC 1145^T				100.0	3.4
E. faecalis TISTR 379^T				19.7	100.0

[a] Data from Tanasupawat et al. (2002).

TISTR, Thailand Institute of Scientific and Technological Research, Bangkok, Thailand.

The determination of growth, pH, diacetyl and acetoin of 3 selected strains in 400 ml MMRS broth revealed that diacetyl production was found at the exponential phase (Figs. 2, A and B, 3, A and B, 4, A and B). All selected strains could produce the maximum of diacetyl concentration at 18 to 36 h of fermentation time under stationary phase as reported by Ray (1992). *L. pentosus* SR4-2 could produce 2.97 mM diacetyl under shaken conditions and 3.35 mM under static conditions while the pH decreased to 5.81 and 5.74 after 30 h incubation, respectvely (Fig. 2, A and B). In addition, *L. pentosus* SR8-1 produced 2.35 mM diacetyl under shaken conditions and 3.25 mM under static conditions while the pH decreased to 5.84 and 6.48 after 36 and 24 h incubation, respectively and diacetyl production of *L. pentosus* AP17-1 did not differ between conditions (data not shown). *W. confusa* AP2-1 produced 2.84 mM diacetyl under shaken conditions and 3.16 mM under static conditions while the pH increased to 7.27 and 7.51 after 36 and 30 h incubation, respectively (Fig. 3, A and B). *E. faecium* PM3-14 produced 2.30 mM diacetyl under shaken conditions while the pH increased to 7.96 after 24 h incubation and 3.27 mM under static conditions while the pH decreased to 6.41 after 18 h incubation, respectively (Fig. 4, A and B). *E. faecium* PM3-13 produced 2.57 mM diacetyl under shaken conditions while the pH increased to 7.94 after 24 h incubation and 2.98 mM under static conditions while the pH increased to 7.0 after 36 h incubation. *E. faecium* PM4-9 produced 2.46 mM diacetyl

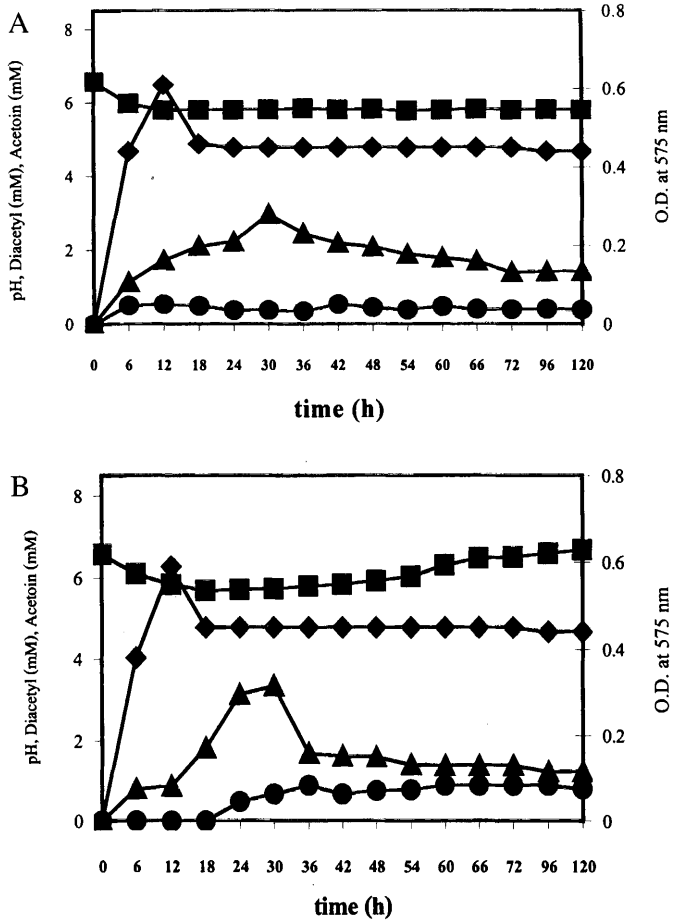

Fig. 2. (A) Relation among growth, pH, diacetyl (mM) and acetoin (mM) production of *Lactobacillus pentosus* SR4-2 under shaken conditions at 30°C for 120 h. (B) Relation among growth, pH, diacetyl (mM) and acetoin (mM) production of *Lactobacillus pentosus* SR4-2 under static conditions at 30°C for 120 h.

◆ growth, ■ pH, ▲ diacetyl, ● acetoin.

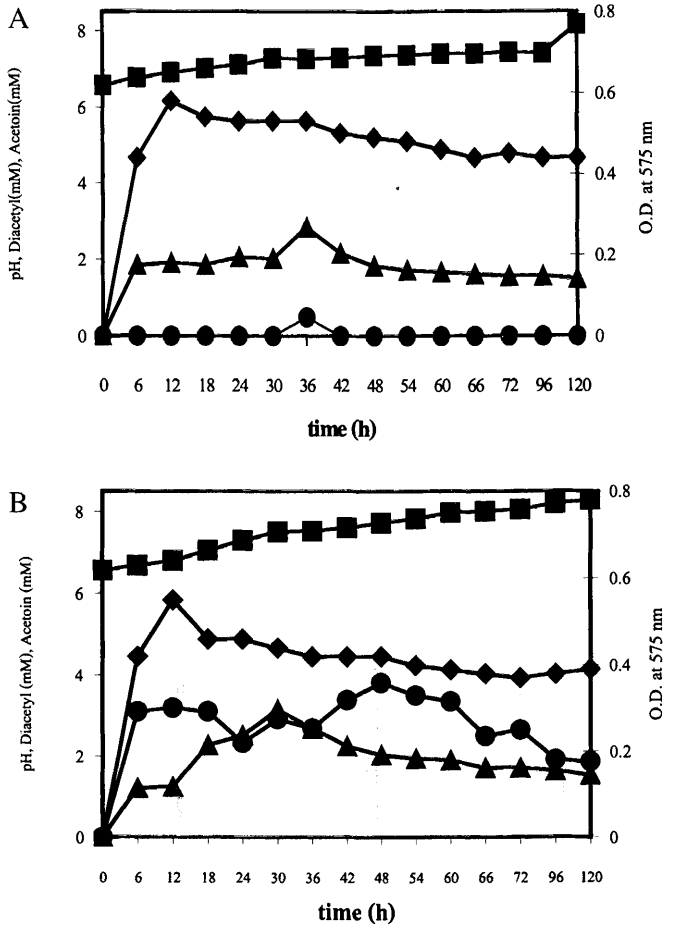

Fig. 3. (A) Relation among growth, pH, diacetyl (mM) and acetoin (mM) production of *Weissellas confusa* AP2-1 under shaken conditions at 30°C for 120 h. (B) Relation among growth, pH, diacetyl (mM) and acetoin (mM) production of *Weissella confusa* AP 2-1 under static conditions at 30°C for 120 h.

◆ growth, ■ pH, ▲ diacetyl, ● acetoin.

under shaken conditions while the pH increased to 7.55 after 18 h incubation and 3.04 mM under static conditions while the pH increased to 6.96 after 30 h incubation, respectively (data not shown).

On the other hand, SR4-2, AP2-1 and PM3-14 strains could produce 0.54, 0.49 and 4.59 mM acetoin under shaken conditions after 12, 36 and 120 h incubation while their productions were 0.89, 3.82 and 1.88 mM under static conditions after 66, 48 and 60 h incubation, respectively (Figs. 2, A and B, 3, A and B, 4, A and B). In addition, the strains SR8-1, AP17-1, PM3-13 and PM4-9 could produce 0.67, 0.75, 4.20 and 2.36 mM acetoin under shaken conditions after 48, 30, 24 and 24 h incubation, while their productions were 6.36, 1.18, 2.03 and 1.84 mM under static conditions after 96, 42, 60 and 36 h incubation, respectively (data not shown). As mentioned above, most of homofermentative strains could produce more diacetyl than the heterofermentative strain did as reported previously (Christensen and Pederson, 1958). Moreover, all selected strains could produce high diacetyl concentration in the medium containing citrate under static conditions, which was different from previous reports (Boumerdassi et al., 1996; Kaneko et al., 1990, 1991). However, most lactic acid bacteria produced a high amount of diacetyl in the medium without citrate under aerobic conditions (Kaneko et al., 1990, 1991). Therefore, the diacetyl metabolism and enzyme activities cultivated in the medium with citrate of these strains should be further studied.

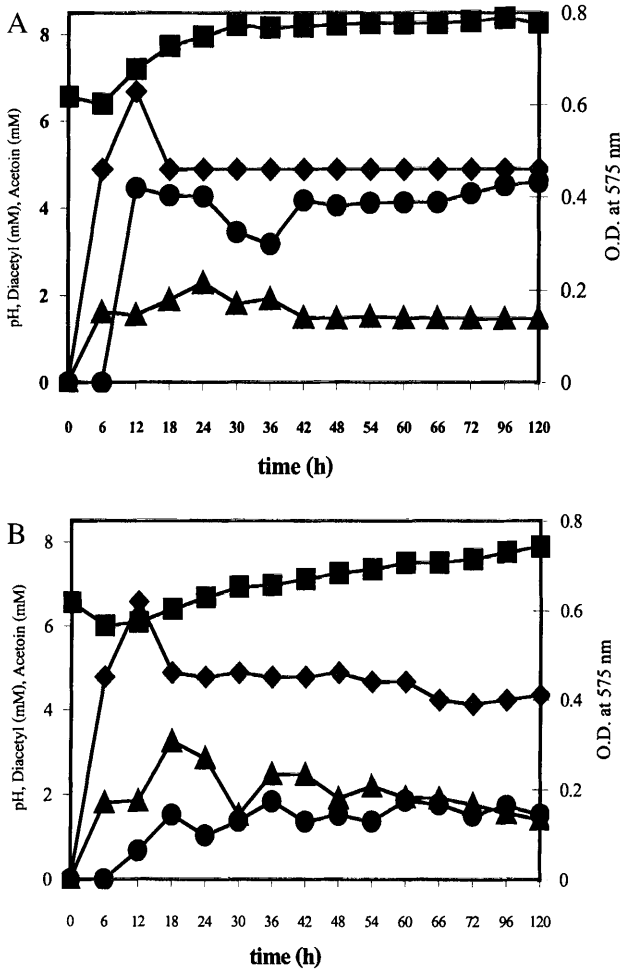

Fig. 4. (A) Relation among growth, pH, diacetyl (mM) and acetoin (mM) production of *Enterococcus faecium* PM3-14 under shaken conditions at 30°C for 120 h. (B) Relation among growth, pH, diacetyl (mM) and acetoin (mM) production of *Enterococcus faecium* PM3-14 under static conditions at 30°C for 120 h.

◆ growth, ■ pH, ▲ diacetyl, ● acetoin.

In fermented milks, the aroma and flavor are basically due to the production of nonvolatile and volatile acids and carbonyl compounds by starter cultures. Diketones, 2,3-butanedione and 2,3-pentanedione belong to the key aroma compounds and 2,3-butanedione can be reduced to 2,3-butanediol through acetoin (Beshkova et al., 2003). In Thailand, *L. pentosus* SR4-2 and SR8-1 were isolated from soy sauce mash (Tanasupawat et al., 2002), *L. pentosus* AP17-1 from fermented fish (*pla-ra*), *W. confusa* AP2-1 from pork sausage (*mu-yor*), and *E. faecium* PM3-13, PM3-14 and PM4-9 were isolated from pasteurized milk. Their diacetyl production as flavor will be a role other than that of lactic acid fermentation in foods.

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