Journal of Industrial Microbiology, 1 (1986) 209–217
Elsevier
SIM 00028

A rapid method for differentiation of dairy lactic acid bacteria by enzyme systems

B.H. Lee[a,b,*], S. Haché[b] and R.E. Simard[b]

[a]*Food Research Center, Agriculture Canada, St-Hyacinthe, Québec J2S 4Z4*, and [b]*Département de sciences et technologie des aliments, Université Laval, Ste-Foy, Québec G1K 7P4, Canada*

Received 30 June 1985
Revised 25 April 1986
Accepted 6 May 1986

Key words: Lactic acid bacteria; Lactobacilli; *Streptococcus;* Homofermentative bacteria; Heterofermentative bacteria; *Leuconostoc*; Dairy microbiology

SUMMARY

A rapid and simple technique utilizing the APIZYM enzymatic patterns complemented with arginine dihydrolase and citratase was developed for species differentiation of 40 lactic acid bacteria relevant to the dairy industry. *Streptococcus* species in general produced no β-galactosidase, except for *Streptococcus thermophilus*. *Lactobacillus* species showed strong aminopeptidases and galactosidases but contained no arginine dihydrolase and citratase. Among the group N-streptococci, *Streptococcus diacetylactis* produced citratase, whereas *Streptococcus cremoris* differed by the production of butyrate esterase. *Streptococcus faecalis* was readily distinguishable from *Streptococcus lactis* by butyrate esterase activity that was the basis of the differential agar developed. Heterofermentative lactobacilli differed from homofermentative lactobacilli in possessing arginine dihydrolase and citratase but by not producing leucine-aminopeptidase.

INTRODUCTION

Cheeses and fermented milk products contain a defined starter flora, associated flora and an undefined highly variable adventitious flora. Mesophilic starters (*Streptococcus lactis, Streptococcus cremoris*) and thermophilic starters (*Streptococcus thermophilus, Lactobacillus bulgaricus, Lactobacillus helveticus* or *Lactobacillus lactis*) are normally used in a variety of cheeses and fermented dairy products. In cottage cheese, associated starters (*Streptococcus diacetylactis, Leuconostoc cremoris*) are used, and *Streptococcus diacetylactis* and/or *Leuconostoc* species which produce CO_2 are also added to provide a desired open texture for mold ripened cheese or dutch cheese.

Unlike starter and associated flora, adventitious flora are accidentally acquired through the survival of thermoduric microorganisms from pasteurized milk or postpasteurization contamination in the plant. Gas defects in blowing of cheese in blocks and in retail packs were caused by high levels of growth of citrate-fermenting heterofermentative streptococci, lactobacilli and *Leuconostoc* species

* To whom correspondence should be addressed.

[16]. The separation of the group N-streptococci (*S. lactis, S. diacetylactis, S. cremoris*) from *Leuconostoc* species and *S. thermophilus,* and further differentiation between *S. lactis* and *S. diacetylactis* from the group D streptococci (*S. faecalis*) is difficult and requires many tests [4,9,11].

The fecal streptococcus, *S. faecalis* occurs in cheese as high as 10^4–10^7 per gram [2] and there is no simple and rapid procedure for distinguishing *S. lactis* from *S. faecalis,* though the separation of these mixtures was recently tried by simple affinity chromatography on an ion exchange resin [15].

The feasibility of the APIZYM (micro-enzyme) system has been demonstrated in the identification of various pathogenic bacteria [3,8], *S. thermophilus* in yogurt [1] and lactobacilli in meats [12].

Since it is possible to determine minute amounts of specific enzymes accurately and rapidly, a quick APIZYM test (supplementing other enzymes) has been applied to several lactic acid bacteria relevant to the dairy industry, particularly with emphasis on the differentiation of homo- and heterofermentative lactobacilli, and *S. lactis* from *S. faecalis.*

MATERIALS AND METHODS

Bacteria and maintenance. Strains of lactic acid bacteria (mostly of dairy origin), commercial starters and members of the coliform group are listed in Table 1. Lyophilized culture were reconstituted in 5 ml of Lactobacilli MRS (Difco) or Brain Heart Infusion (Difco) broth followed by incubation at 30°C for 48 h. *L. bulgaricus* was incubated at 45°C. All cultures were activated in 5 ml of Lactobacilli MRS broth through two transfers before each experiment. Stock cultures were maintained at −30°C in 20% (v/v) skim milk (Difco) solution diluted equally with growth medium.

APIZYM procedure. Bacterial growth (24 h) from the surface of an MRS agar plate was removed with a loop and suspended in 5 ml of MRS (Difco) broth with a turbidity of a McFarland No. 6 standard. Two drops of the resulting suspensions were inoculated with a pasteur pipet into each microcupule of the gallery containing 19 dehydrated chromogenic enzyme substrates. Each suspension was assayed in duplicate. After incubation of the strips of 4 h at 37°C, reactions were visualized after the addition of the detector reagents supplied with the system (API Laboratory Products Ltd., St-Laurent, Québec).

Determination of arginine dihydrolase, citratase and growth temperature. Arginine dihydrolase activity was initially studied by three methods: arginine dihydrolase test in Thornley's semisolid medium [21], ammonia production from arginine broth, and Minitek arginine disk (BBL). Except for some variation with cultures of *S. cremoris*, the three methods gave similar results.

Citratase activity was also studied by three methods: citratase assay [6], citrate agar [10] and Minitek citrase disk impregnated with 0.01% phenol red prepared by the procedure of Gilliland and Speck [5] but the citrate disk method was finally chosen for this test.

Growth at 15, 39 or 45°C was determined by streaking the culture on MRS agar and incubating it in an anaerobic jar (Gas-pak system, BBL) for 48 h.

Differential agar medium for separating S. lactis and S. faecalis strains. The differential medium contained 0.5% Bacto-peptone, 0.3% Bacto-yeast extract (Difco), 1.0% 2-naphthyl butyrate (Sigma) and 1.5% agar. The pH of the medium was adjusted to 6.5, and the medium was sterilized by autoclaving it at 121°C for 15 min.

A streak- or spread-plating method was used for growth of *S. lactis* (six strains) and *S. faecalis* (six strains) on solid medium. Plates were incubated in anaerobic jars (BBL) for 48 h at 30°C and then flooded with the reagents supplied with th APIZYM system.

RESULTS AND DISCUSSION

The results of the APIZYM technique, designed for the detection of 19 constitutive enzymes, showed that *S. lactis* differed from *S. diacetylactis* in failing to produce citratase and arginine dihydrolase as expected (Table 2). All results are the

Table 1

Strains and sources of lactic acid bacteria used

Strains	Identification No.	Obtained (from)
1. *Streptococcus lactis*	ML3	Agriculture Canada[a]
2. *lactis*	14871	ATCC[b]
3. *diacetylactis*	10166	Agriculture Canada
4. *diacetylactis*	11007	ATCC
5. *cremoris*	ML1	Agriculture Canada
6. *cremoris*	9596	ATCC
7. *faecalis*	7080	ATCC
8. *faecalis*	A69	Agriculture Canada
9. *faecalis*	19433	ATCC
10. *thermophilus*	1001	Express dairies[c]
11. *Lactobacillus bulgaricus*	1373	NCDO[d]
12. *acidophilus*	4356	ATCC
13. *acidophilus*	1002	Agriculture Canada
14. *helveticus*	10797	ATCC
15. *lactis*	1437	NCDO
16. *lactis*	12315	ATCC
17. *casei*	242	NCDO
18. *casei-rhamnosus*	7469	ATCC
19. *plantarum*	14917	ATCC
20. *brevis*	47	LSPQ[e]
21. *fermentum*	AN2-255A	LSPQ
22. *Leuconostoc cremoris*	19254	ATCC
23. *cremoris*	1003	Visby lab.[f]
24. *dextramicum*	19255	ATCC
25. *mesenteroides*	23386	ATCC
26. *lactis*	19256	ATCC
27. Commercial starter	4318L	Agropur[g]
28. Commercial starter	371K	Kraft[h]
29. Commercial starter	374K	Kraft
30. Commercial starter	H72	Hansen[i]
31. Commercial starter	H96	Hansen
32. Commercal starter	MAR-1	Miles[j]
33. *E. coli*	11775	ATCC
34. *K. oxytoca*	AN2-474	LSPQ
35. *Streptococcus lactis*	19435	ATCC
36. *lactis*	11454	ATCC
37. *lactis*	14872	ATCC
38. *lactis*	14873	ATCC
39. *faecalis*	19433	ATCC
40. *faecalis*	8043	ATCC
41. *faecalis*	882	ATCC
42. *faecalis*	6057	ATCC

[a] Agriculture Canada Food Research Institute, Ottawa.
[b] The American Type Culture Collection, Rockville, MD.
[c] Express Dairies, Holland, U.K..
[d] National Collection Dairy Organisms (NCDO), Reading, U.K.
[e] Laboratoire de santé publique du Québec, Ste-Anne-de-Bellevue, Québec.
[f] Enolacto Ltd., Middlesex, U.K.
[g] Agropur coopérative agro-alimentaire, Grandby, Québec.
[h] Kraft Inc., Glenview, IL.
[i] Chr. Hansen's Lab., Inc., Milwaukee, WI.
[j] Marschall Division, Madison, WI.

Table 2

Differentiation of lactic acid bacteria relevant to the dairy industry by enzyme systems

Species	Alkaline phosphatase	Acid phosphatase	Esterase (C_4)	Esterase-lipase (C_8)	Leucine-amino-peptidase	Valine-amino-peptidase	Cysteine-amino-peptidase	Phosphoamidase	α-Galactosidase	β-Galactosidase	β-Glucuronidase	α-Glucosidase	β-Glucosidase	β-Glucosaminidase	Arginine dihydrolase	Citratase
1. *S. lactis*	(1)[a]	2		2[b]	1			1						(1)	V[a]	–
2. *lactis*	(1)	2		2	1			1							–	–
3. *diacetylactis*		1		1	1			1							+	+
4. *diacetylactis*	(2)	2		1	1			2							+	+
5. *cremoris*		2	1	1	(1)			1					(1)		V	–
6. *cremoris*		1	2	2	1			(1)					3	1	V	–
7. *faecalis*	1	1	2	3	2			1					3	4	+	+
8. *faecalis*	(1)	(1)	2	2	1			1					2		+	–
9. *faecalis*	1	2	2	2	3			1					(1)	(1)	+	–
10. *thermophilus*				1	2			(1)		4					–	–
11. *Lactobacillus bulgaricus*		(1)		(1)	3			1		3					–	–
12. *acidophilus*		(1)		1	1			1		4		(1)	(2)		–	–
13. *acidophilus*	(1)			1	2			1		3			(3)	(2)	–	–
14. *helveticus*		2			3			(1)	5						–	–
15. *lactis*					2	2		(1)		2		(1)	3	2	–	V
16. *lactis*					3	(1)		1	(3)			(2)	2	3	–	V
17. *casei*	(1)	(1)	1	3	4	4	(1)	1		2			2		–	–
18. *casei-rhamnosus*	(1)	(1)	3	3	4	4	(1)	1	(1)	2			2		–	–
19. *plantarum*				1	1	(1)		(1)		1		(1)	2	(1)	–	–
20. *brevis*		2		2					3	4	2	2			+	+
21. *fermentum*		2	2	3					3	2		2			+	+

[a] ( ) and V, variable reaction obtained from triplicate experiments, each run in duplicate.

[b] 1–4 (intermediate activity: 5–30 nM); 5 (maximum activity: 40 nM).

[c] +, positive reaction or growth; –, negative reaction or no growth.

averaged values obtained from three separate experiments, each run in duplicate. *S. cremoris* could be differentiated from *S. lactis* by β-glucosidase and butyrate esterase but it differed from *S. faecalis* by not producing alkaline phosphatase. *S. thermophilus* was characterized by strong production of β-galactosidase. This is in agreement with the results of Bouillanne and Desmajeaud [1] who showed similar activities from 53 different strains of *S. thermophilus* by APIZYM tests. It confirms that *S. thermophilus* metabolizes lactose mainly by β-galactosidase, whereas group N streptococci involve a PEP-phosphotransferase (PTS) system and subsequent cleavage by β-phosphogalactosidase. However, it has been disputed as to whether the lactose transport system in *S. thermophilus* involved a PET-PTS system [7,20].

Thermobacteria (*L. bulgaricus, L. acidophilus, L. helveticus, L. lactis*) and streptobacteria (*L. casei, L. casei* sub *sp. rhamnosus, L. plantarum*) could clearly be separated from heterofermentative lactobacilli (betabacteria: *L. brevis, L. fermentum*) on the basis of arginine dihydrolase and citratase (Table 2). As yet, too few lactobacilli have been examined to enable identification of characteristic patterns. Despite several difficulties found in the differentiation of *L. helveticus, L. jugurt* and *L. bulgaricus* [14], *L. helveticus,* however, had a strong α-galactosidase. *L. lactis* also did not produce acid- or alkaline-phosphatase or esterases. *L. casei* and *L. casei* subsp. *rhamnosus* had many activities on different substrates. *L. plantarum* had only weak activities of caprylate esterase-lipase, leucine-amino-peptidase, β-galactosidase and β-glucosidase.

Betabacteria (*L. brevis, L. fermentum*) did not produce leucine-aminopeptidase or phosphoamidase, compared with the homofermentative lactobacilli. *L. brevis* was characterized by β-glucuronidase which was not detected in any of the lactobacilli tested. There was no characteristic enzyme which permitted good differentiation of two yogurt lactics: *S. thermopilus* and *L. bulgaricus* but their morphologies are easily differentiated microscopically.

Leuconostoc species (*L. cremoris, L. dextranicum, L. lactis, L. mesenteroides*) that constitute the aroma and flavor producers in milk products (Table 3) are not easily distinguished from streptococci (Table 2). Among *Leuconostoc* species studied, however, *L. cremoris* was characterized by the production of butyrate esterase but slight variations were found between different *L. cremoris* strains where occasional aberrant reactions on α- anb β-glucosidases were noted in *L. cremoris* (known as *L. citrovorum*). Reactions of *L. dextranicum* and *L. lactis* were similar to those of *S. diacetylactis. L. mesenteroides* had many variable reactions and did not produce arginine dihydrolase, despite the fact that this species ususally hydrolyzes arginine. *L. cremoris* and *L. lactis* consistently produced arginine dihydrolase in this study but these strains did not produce arginine dihydrolase in other studies [19].

The cheesemaker has no way of knowing the strain composition of various types of starters. When multiple or mixed commercial starters are allowed to developed in MRS broth overnight at 25°C, the starter culture strains having reactions similar to *S. lactis* become dominant. In most starter cultures for cheese, both *S. lactis* and *S. cremoris* contribute 90–95% of the bacteria present but of the two, *S. lactis* tends to be more active in fermentation [18]. Consequently, to identify undefined mixures of commercial starters, one may dilute the lyophilized cultures in milk and grow them directly on MRS agar plates before studying taxonomic and enzymatic characteristics. Members of the coliform group such as strains of *Escherichia coli* and *Klebsiella oxytoca* were also tested for the detection of their specific enzymatic activities to assess the feasibility of using the APIZYM system in discriminating contaminants from the lactic starters. Without knowing gram-negative and catalase-positive characteristics, the enzymatic patterns alone were distinct those from of other lactic acid bacteria. *E. coli* and *K. oxytoca* had strong β-galactosidase and acid phosphatase activities but *K. oxytoca* could be separated from others on the basis of myristate lipase activity.

In the case of cheeses or fermented milk products where only a few species of *S. lactis* and *S. faecalis* are concerned, a simplified differentiation

Table 3

Differentiation of lactic acid bacteria from coliform group by enzyme systems

Species	Alkaline phosphatase	Acid phosphatase		Esterase lipase (C_8)	Lipase (C_{14})	Leucine-amino-peptidase	Phosphoamidase	β-Galactosidase	α-Glucosidase	β-Glucosidase	β-Glucosaminidase	Arginine dihydrolase	Citratase
22. *Leuconostoc cremoris*	(1)[a]	1	(1)	1		(1)	1[b]					+	+
23. *cremoris*	1	(2)	2	2		1	1		(4)	(1)		+	V[a]
24. *dextranicum*		2		1		1	1					+	+
25. *mesenteroides*		(1)		(1)		(1)	1	1	(3)	(3)	2	–	V
26. *lactis*		2		1		(1)	(1)					+	V
27. 4318 L		2		1		1	(1)					–	–
28. 371 K	(1)	3		1		(1)	(1)	(2)				–	–
29. H 72	(1)	3		1		1	(1)					–	V
30. H 96		3		(1)		1	(1)					–	–
31. 374 K		2		1		(1)	(1)					–	–
32. MAR-1		3		(1)		1	(1)					V	–
33. *E. coli*	2	4				2	(1)	4				–	+
34. *K. oxytoca*		5			5			5				–	+

[a] ( ) and V, variable reaction obtained from triplicate experiments which were run in duplicate.

[b] 1–4 (intermediate activity: 5–30 nM); 5 (maximum activity: 40 nM).

[c] +, positive reaction or growth; –, negative growth or no growth.

Table 4

Differentiation of *Streptococcus lactis* from *Streptococcus faecalis* strains by enzyme systems and growth temperature

Species	Alkaline phosphatase	Acid phosphatase	Esterase (C_4)	Esterase-lipase (C_8)	Leucine-amino-peptidase	Chymotrypsin	Phosphoamidase	α-Glucosidase	β-Glucosidase	β-Glucosaminidase	Arginine dihydrolase	Citratase	Growth at 15°C	Growth at 39°C	Growth at 45°C
1. *Streptococcus lactis*	(1)[a]	2		2	1		1			(1)	+[b]	−	+	+	−
2. *lactis*	(1)	2		2	1		1				−	−	+	−	−
35. *lactis*		1		1	2		1	1			+	−	+	+	−
36. *lactis*		2		1	2		1		1		+	V	+	+	−
37. *lactis*		2		1	2		2				−	−	+	−	−
38. *lactis*		2		1	2		1				−	−	+	−	−
7. *faecalis*	1	1	2	3	2	1	1		3	4	+	+	+	+	+
8. *faecalis*		(1)	2	2	1		1		2		+	−	+	+	+
9. *faecalis*	1	2	2	2	3		1		(1)	(1)	+	−	+	+	+
39. *faecalis*			2	3	1	2	1	3			+	−	+	+	+
40. *faecalis*		1	1	2	1		(1)				+	+	+	+	+
41. *faecalis*			2	3	1		2				+	−	+	+	+
42. *faecalis*		(1)	1	2	2		1				+	−	+	+	+

[a] ( ) and V, variable reaction obtained from triplicate experiments which were run in duplicate.

[b] +, positive reaction or growth; −, negative growth or no growth.

system was established (Table 4); *S. lactis* differed from *S. faecalis* in failing to produce butyrate esterase. *S. faecalis* was also characterized by a stronger activity of caprylate esterase-lipase than *S. lactis*. Among different strains of *S. lactis* that we have studied, three strains did not resemble known species of *S. lactis* which usually produce arginine dihydrolase. Two strains of *S. faecalis* differed from the remaining *S. faecalis* strains in producing chymotrypsin. *S. faecalis* strains also differed from *S. lactis* by their thermophilic nature which is in agreement with the results for known species in the literature.

In view of the many different species involved in this study, clear-cut results may not be attainable. However, simple and fast enzyme tests for differentiation of dairy lactics are very useful and offer a reasonable substitute for the more cumbersome and time-consuming conventional methods. Arginine and citrate utilizations were the basis of differentiating homofermentative from heterofermentative lactobacilli (*L. brevis, L. fermentum*) but these characteristics did not form the basis of differentiation among streptococci and *Leuconostoc* species studied. Since many lactic streptococci strains exhibit variability on arginine and citrate, probably by plasmid involvement [13], this trait may not be a stable characteristic.

We have demonstrated interesting and consistent differences in the butyrate esterase activity between *S. lactis* and *S. faecalis*. Thus, a differential agar medium containing β-naphthyl butyrate was developed as a rapid means for distinguishing two strains in dairy products. *S. faecalis* produced a violet color on this medium after the addition of the color reagents because of their ability to produce β-naphthol from naphthyl butyrate, while *S. lactis* did not. This differential agar will offer many advantages to the dairy industry and starter culture manufacturers for rapid and economical differentiation or selection of potential starter strains, and for investigations of compatability and dominance of mixed strain starters [17].

ACKNOWLEDGEMENT

Contribution No. 35 of Agriculture Canada Food Research Center, St-Hyacinthe, Québec, Canada.

REFERENCES

1 Bouillanne, C. and M.H. Desmajeaud. 1980. Etude de quelques caractères de souches de *S. thermophilis* utilisées en fabrication de yoghourt et proposition d'une méthode de classement. Le Lait, 60: 458–473.

2 Clark, W.S. and G.W. Reinbold. 1966. Enterococci in young cheddar cheese. J. Dairy Sci. 49: 1214–1218.

3 Cohen, B., M.J. Hudson and M.J. Hill. 1980. Comparison of the API20A. APIZYM and conventional methods for the identification of anaerobic bacteria. J. Med. Microbiol. 13, 8.

4 Diebel, R.H. and H.W. Seeley. 1978. Family, Streptococcaceae. In: Bergey's Manual of Determinative Bacteriology, (Buchanan, R.E. and N.E. Gibbons, eds.), 8th Edn., Williams and Wilkins Co., Baltimore.

5 Gilliland, S.E. and M.L. Speck. 1977. Use of the Minitek system for characterizing lactobacilli. Appl. Environ. Microbiol. 33: 1289–1292.

6 Harvey, R.J. and E.B. Collins. 1963. The citratase of *Streptococcus diacetylactis*. J. Biol. Chem. 238: 2648–2653.

7 Hemme, D., D. Wahl and M. Nardi. 1980. Variations de l'équipement enzymatique de *Streptococcus thermophilus*. Le Lait, 60: 111–129.

8 Humble, M.W., A. King and I. Phillips. 1977. APIZYM: a simple rapid system for the detection of bacterial enzymes. J. Clin. Pathol. 30: 275–277.

9 Huggins, A.R. 1984. Progress in dairy starter culture technology. Food Technol. 38(6): 41–50.

10 Kempler, G.M. and L.L. McKay. 1980. Improved medium for detecting of citrate-fermenting *Streptococcus lactis* subsp. *diacetylactis*. Appl. Environ. Microbiol. 39: 926–927.

11 Law, B.A. and M.E. Sharpe. 1978. Streptococci in the dairy industry. Streptococci, (Skinner, F.A. and L.B. Quesnel, eds.), pp. 263–278, Academic Press, London.

12 Lee, B.H. and R.E. Simard. 1984. Three systems for biochemical characterizations of lactobacilli associated with meat spoilage. J. Food Prot. 47: 937–942.

13 McKay, L.L. 1983. Functional properties of plasmids in lactic streptococci. Antonie Van Leeuwenhoek J. Microbiol. Serol., 49: 259–274.

14 Matteuzzi, D. 1972. Lactic acid production in milk and DNA homology relationships in the species *Lactobacillus jugurt*. Can. J. Microbiol. 18: 1893–1895.

15 Paquin, C. and J. Goulet. 1984. Séparation d'un mélange de streptocoques par adsorption sélective. Can. Inst. Food Sci. Technol. J. 17(3): 33 (abstract).
16 Prentice, G.A. and J.V. Brown. 1983. The microbiology of cheddar cheese manufacture. Dairy Ind. Intern. 48: 23–26.
17 Reddy, M.S., E.R. Vedamuthu, C.J. Washam and G.W. Reinbold. 1969. Differential agar medium for separating *Streptococcus lactis* and *Streptococcus cremoris*. Appl. Microbiol. 18: 755–759.
18 Robinson, R.K. 1983. Starter cultures for milk and meat processing. In: Biotechnology, Vol. 3 (Rehm, H.J. and G. Reed, eds.), pp. 192–202, Verlag chemie, Weinheim.
19 Roissert, H.B. 1983. Que sont les bactéries lactiques? La technique laitière. No. 979, 41–47.
20 Somkuti, G.A. and D.H. Steinberg. 1979. Adaptability of *Streptococcus thermophilus* to lactose, glucose and galactose. J. Food Prot. 42: 885–887.
21 Thornley, M.J. 1960. The differentiation of *Pseudomonas* from other gram-negative bacteria on the basis of arginine metabolism. J. Appl. Bacteriol. 23: 37–52.