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NOTE

Novel characteristic for distinguishing *Lactococcus lactis* subsp. *lactis* from subsp. *cremoris*

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***Lactococcus lactis* strains were examined for their ability to produce γ-aminobutyric acid (GABA). Results showed that strains of *L. lactis* subsp. *lactis* were able to produce this acid, whereas *L. lactis* subsp. *cremoris* were not. GABA production thus represents another effective characteristic for distinguishing *L. lactis* subsp. *lactis* from *L. lactis* subsp. *cremoris*.**

Keywords: *Lactococcus lactis*, glutamate decarboxylase, γ-aminobutyric acid, lactic acid bacteria

The species *Lactococcus lactis* is subdivided into *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. These taxa were formerly classified as *Streptococcus lactis*, *Streptococcus cremoris* and '*Streptococcus diacetylactis*'. '*S. diacetylactis*', a producer of diacetyl from citrate, is now recognized as *L. lactis* subsp. *lactis* biovar diacetylactis (Mundt, 1986). Although conspecificity of the three taxa is suggested by the high sequence similarity of their chromosomal DNA (Jarvis & Jarvis, 1981), three subspecies have been recognized because they contribute important properties to fermented dairy products.

L. lactis subsp. *cremoris* is generally preferred in cheese starters because it imparts flavour to the cheese. Orla-Jensen (1919) differentiated *L. lactis* subsp. *cremoris* from *L. lactis* subsp. *lactis* based on characteristics that included the tendency to form longer chains, lower maximum growth temperature and reduced fermentative power. Other differences between the two species have also been observed (Ayers *et al.*, 1924; Hills, 1940; Niven *et al.*, 1942; Yawger & Sherman, 1937); a key difference is the deimination of arginine by *L. lactis* subsp. *lactis* but not by *L. lactis* subsp. *cremoris*.

Glutamate decarboxylase (GAD; EC 4.1.1.15), which catalyses the decarboxylation of glutamate, produces γ-aminobutyric acid (GABA) in bacteria (Gale, 1946), higher plants (Okunuki, 1937; Schales *et al.*, 1946) and animals (Blindermann *et al.*, 1978; Roberts & Frankel, 1951). Gale (1946) proposed that bacterial GAD maintains physiological pH under acidic conditions.

Abbreviations: GAD, glutamate decarboxylase; GABA, γ-aminobutyric acid.

This study has shown that *L. lactis* subsp. *lactis* strains are able to produce GABA. In addition to the ability to hydrolyse arginine, GABA production represents another characteristic that may be used for distinguishing *L. lactis* subsp. *lactis* from *L. lactis* subsp. *cremoris*.

Bacterial strains and growth conditions

The strains in Table 1 were isolated from commercial cheese starters (Nomura *et al.*, 1998). *L. lactis* subsp. *lactis* ATCC 19435^T (T = type strain), *L. lactis* subsp. *cremoris* ATCC 19257^T and *L. lactis* subsp. *lactis* biovar diacetylactis ATCC 13675^T were obtained from the American Type Culture Collection, Manassas, VA, USA. The other strains in Table 3 were from laboratory collections. The bacteria were maintained in sterile litmus milk and subcultured once a week. Actively growing cultures were obtained by transferring 1% inoculum to autoclaved reconstituted skimmed milk (8% milk solids) and incubating at 30 °C for 16 h. TYGG medium contained 0·5% tryptone (Difco), 0·5% yeast extract (Difco), 1% glucose and 10 mM L-monosodium glutamate (pH 7·0). Lactic acid bacteria were identified by a previously described method (Nomura *et al.*, 1998). Arginine hydrolysis was determined with API 20 Strep (bioMérieux).

Determination of GABA production by lactic acid bacteria

Actively growing culture (1 ml) was transferred to 100 ml skim milk supplemented with L-monosodium glutamate (20 mM) and incubated at 30 °C for 4 d. A 5 ml portion of this culture was then mixed with 1 ml 30%(w/v) trichloroacetic acid. After centrifugation

Table 1. GABA production by lactic acid bacteria isolated from cheese starters

Values represent the mean of duplicate determinations.

Strain	pH	GABA (μM)	Classification*
Group I			
01-1	4·32	0·0	cremoris
01-2	4·30	0·0	cremoris
01-3	4·29	0·0	cremoris
01-8	4·37	0·0	cremoris
01-9	4·67	0·0	cremoris
01-11	4·65	0·0	cremoris
02-8	4·24	0·0	cremoris
40-1	4·28	0·0	cremoris
40-2	4·75	0·0	cremoris
40-3	4·28	0·0	cremoris
53-5	4·43	0·0	cremoris
53-6	4·66	0·0	cremoris
53-8	4·71	0·0	cremoris
53-9	4·25	0·0	cremoris
95-1	4·48	0·0	cremoris
95-2	4·30	0·0	cremoris
95-3	4·30	0·0	cremoris
Group II			
01-10	4·31	0·0	cremoris
53-2	4·71	0·0	cremoris
53-4	4·69	0·0	cremoris
Group III			
01-4	4·54	237·6	diacetylactis
01-5	4·39	26·2	diacetylactis
01-6	4·67	164·9	diacetylactis
01-7	4·68	262·8	diacetylactis
02-1	4·57	94·1	diacetylactis
02-2	4·76	53·3	diacetylactis
02-3	4·66	99·9	diacetylactis
02-4	4·65	72·7	diacetylactis
02-5	4·52	41·7	diacetylactis
02-6	4·48	54·3	diacetylactis
02-7	4·58	28·1	diacetylactis
53-1	4·74	240·5	diacetylactis
53-3	4·81	108·6	diacetylactis
53-7	4·68	57·2	diacetylactis
HAZ-1	4·36	211·4	diacetylactis
HAZ-2	4·27	190·1	diacetylactis
HAZ-3	4·26	204·6	diacetylactis

* Data for classification are presented in Table 2. cremoris, *L. lactis* subsp. *cremoris*; diacetylactis, *L. lactis* subsp. *lactis* biovar diacetylactis.

for 20 min at 1800 ***g***, the soluble fraction was analysed using a Hitachi L-8500 amino acid analyser.

GAD activity assay

Bacterial inoculum (1 ml) was transferred to 100 ml TYGG medium and incubated at 30 °C for 16 h. A culture sample (1 ml) was then transferred to 100 ml fresh TYGG medium and incubated at 30 °C for 14 h. Cells were harvested by centrifugation at 1800 ***g*** for 20 min at 4 °C, washed once with PBS (0·15 M NaCl, 10 mM sodium phosphate; pH 7·3), and suspended in 5 ml distilled water. The cell suspension was frozen at − 50 °C and lyophilized. For the enzyme assay, the cell powder was suspended in 1 ml 50 mM sodium acetate buffer (pH 4·7). GAD activity was assessed by a previously described method (Nomura *et al.*, 1998). The reaction mixture contained 1 ml 50 mM sodium acetate buffer (pH 4·7), 2 mM L-glutamate, 0·1 mM pyridoxal phosphate, and aliquots of the cell suspension. The reaction was carried out for 20 h at 30 °C. GABA production was measured with an amino acid analyser. One katal of GAD was defined as the amount of enzyme required to produce 1 mol GABA s^{-1} at 30 °C and pH 4·7.

GABA production by bacteria from cheese starters

Of 37 strains of lactic acid bacteria isolated from cheese starters, 17 strains produced GABA (Table 1).

The isolates were classified based on selected physiological characteristics. Two groups that could not produce GABA were noted; they differed only in their capacity to hydrolyse aesculin (groups I and II in Table 2). These isolates were non-motile, catalase-negative, Gram-positive cocci that occurred in pairs or short chains. Characteristics such as production of L-lactate from glucose, carbohydrate fermentation patterns, the absence of ammonia production from arginine, and the absence of carbon dioxide and diacetyl production from citrate suggested that these bacteria were strains of *L. lactis* subsp. *cremoris* (Table 2). All the GABA-producing strains were phenotypically identical and possessed the characteristics of *L. lactis* subsp. *lactis* biovar diacetylactis, e.g. ammonia production from arginine, carbon dioxide and diacetyl formation from citrate, and fermentation of ribose and trehalose (group III in Table 2).

GABA production by *L. lactis*

Sixteen strains of *L. lactis* were examined for GABA production (Table 3). GABA was generated in all cultures of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *lactis* biovar diacetylactis strains, but not in any cultures of strains of *L. lactis* subsp. *cremoris*. GABA production appeared to occur concurrently with the ability of the strains to hydrolyse arginine.

GAD activity was measured (Table 3); activity correlated with GABA production. GAD activity was present in all strains of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *lactis* biovar diacetylactis, but not in *L. lactis* subsp. *cremoris* strains that failed to produce GABA. The lack of GABA production appears to be linked to the absence of GAD activity. GABA production is thus a novel and additional feature for distinguishing *L. lactis* subsp. *lactis* from *L. lactis* subsp. *cremoris*.

Table 2. Properties of lactic acid bacteria isolated from cheese starters

Property	Group I	Group II	Group III	ATCC 13675^T*	ATCC 19257^T*
L-Lactate formed	+	+	+	+	+
Hydrolysis of:					
Arginine	−	−	+	+	−
Aesculin	−	+	+	+	−
Acid from:					
D-Ribose	−	−	+	+	−
L-Arabinose	−	−	−	−	−
D-Mannitol	−	−	−	−	−
D-Sorbitol	−	−	−	−	−
Lactose	+	+	+	+	+
D-Trehalose	−	−	+	+	−
Inulin	−	−	−	−	−
D-Raffinose	−	−	−	−	−
Starch	−	−	−	−	−
Utilization of citrate:					
Gas production	−	−	+	+	−
Diacetyl	−	−	+	+	−
GABA production	−	−	+	+	−

*ATCC 13675^T, *L. lactis* subsp. *lactis* biovar diacetylactis ATCC 13675^T. ATCC 19257^T, *L. lactis* subsp. *cremoris* ATCC 19257^T.

Table 3. GABA production activity of *Lactococcus lactis*

Values represent the mean of duplicate determinations. ND, Not determined.

Strain	pH	GABA (μM)	GAD activity [μkat (kg dry wt bacteria)$^{-1}$]	Arginine hydrolysis
L. lactis subsp. *lactis*				
ATCC 19435^T	4·45	210·4	5·1	+
527	4·17	439·3	24·0	+
565	4·22	529·5	6·5	+
712	4·14	111·5	31·7	+
7C-5	4·41	8·7	28·6	+
SlN	4·50	368·5	58·0	+
L. lactis subsp. *lactis* biovar diacetylactis				
ATCC 13675^T	4·12	226·9	5·7	+
CVT3	4·56	106·7	58·9	+
DRC-1	4·17	249·2	5·9	+
N-7	4·50	255·0	41·5	+
L. lactis subsp. *cremoris*				
ATCC 19257^T	4·18	0·0	0·0	−
924	4·30	0·0	0·0	−
F-16	4·30	0·0	0·0	−
H-61	4·21	0·0	0·0	−
HP	4·22	0·0	0·0	−
ML	4·31	0·0	0·0	−
Blank	6·50	0·0	ND	ND

L. lactis subsp. *cremoris* is generally distinguished from *L. lactis* subsp. *lactis* based on the following phenotypic criteria: *L. lactis* subsp. *cremoris* strains cannot grow at 40 °C, in 4% NaCl or at pH 9·2, and they cannot deiminate arginine. Crow & Thomas (1982) showed that the inability to produce ammonia

from arginine is due to the absence of arginine deiminase activity. Agar media now exist (Reddy *et al.*, 1969; Thomas, 1973; Turner *et al.*, 1963) that can be used to differentiate *L. lactis* subsp. *cremoris* from *L. lactis* subsp. *lactis* based on the capacity to hydrolyse arginine. The data in Table 2 further show that the ability to ferment ribose and trehalose can also differentiate the two strains. *L. lactis* strains may be classed as two distinct phylogenetic groups by Southern hybridization (Godon *et al.*, 1992), and by analysis of 16S rRNA (Salama *et al.*, 1991) and lactate dehydrogenase gene (Swindell *et al.*, 1994) sequences.

Recently, *Lactobacillus hordniae*, which is an aetiological agent of Pierce's disease of grapevines, has been transferred into *Lactococcus lactis* subsp. *hordniae* based on nucleic acid hybridization studies (Schleifer *et al.*, 1985). Growth of this bacterium is poor in milk, while strains of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* grow rapidly. As GAD activity of this organism has not been examined, it would be useful to know if this activity is also a feature of this subspecies.

The present study revealed that *L. lactis* subsp. *lactis* consistently produced GABA and that *L. lactis* subsp. *cremoris* did not. The lack of GABA production in *L. lactis* subsp. *cremoris* can be attributed to the absence of GAD activity (Table 3). Reasons for the absence of GAD in *L. lactis* subsp. *cremoris* have not been determined. The data presented here show that GABA production is another useful criterion for differentiating *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis*.

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