

Biochemical properties of *Streptococcus macedonicus* strains isolated from Greek Kasseri cheese

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KALANTZOPOULOS AND E. TSAKALIDOU. 2000. A total of 32 *Streptococcus macedonicus* strains, isolated from Greek Kasseri cheese, were screened for biochemical properties of technological importance in milk fermentation processing, such as acid production, proteolytic and lipolytic activity, citrate metabolism, exopolysaccharide production, antimicrobial activity and biogenic amines production. All strains were found to be moderate acidifiers in milk. Only four strains could hydrolyse milk casein, while 11 strains showed lipolytic activity against tributyrin. Using amino acid derivatives of 4-nitroaniline as substrates, the highest peptidase activities were determined against phenylalanine- and glycine-proline-4-nitroanilide. Using fatty acid derivatives of 4-nitrophenol, it was shown that all strains exhibited esterase activities up to caprylate, with highest values against butyrate and caproate. Only one showed activity up to palmitate; this was also the most active strain against tributyrin. Five of the 32 strains could metabolize citrate but none of them produced exopolysaccharides. Nine strains displayed antimicrobial activity towards *Clostridium tyrobutyricum*, while no antimicrobial activity was detected against *Listeria innocua* and *Propionibacterium freudenreichii* subsp. *shermanii*. Finally, none was able to decarboxylize ornithine, histidine or lysine, and only four strains produced tyramine from tyrosine.

INTRODUCTION

Lactic acid bacteria possess a large number of metabolic properties which are responsible for their successful use as starter cultures in the food and feed industry and as probiotics and dietary additives for nutritional and health purposes. They ferment lactose to lactic acid, which inhibits the growth of pathogenic and defect-producing bacteria; at the same time, this conversion is responsible for gel syneresis in cheese.

Lactic acid bacteria have limited abilities to synthesize amino acids, which are essential for their growth, and milk contains insufficient amounts of amino acids and low molecular mass peptides to sustain growth. However, they possess a complex proteolytic system capable of hydrolysing milk proteins to peptides and amino acids (Pritchard and Coolbear

1993). Although they are considered as weak proteolytic and lipolytic bacteria compared with other groups of microorganisms, it is generally accepted that the proteolytic and lipolytic systems of lactic acid bacteria contribute to the degradation of milk protein and fat and hence to the texture, taste and aroma of fermented products.

Compounds produced due to their secondary metabolism may have a special impact in certain applications, e.g. citrate metabolism for aroma compound production or exopolysaccharides for water retention and increasing viscosity, while decarboxylation of amino acids to biogenic amines is an undesirable feature. Finally, their bacteriostatic or bacteriocidal action against not only closely related bacteria, but also against food spoilers and pathogens, finds more and wider applications (Cogan and Hill 1993).

The nutritional value of foods fermented with lactic acid bacteria is considered to be higher than that of the corresponding raw materials due to, for example, predigestion of lactose and proteins, increased bioavailability of minerals,

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synthesis of vitamins and degradation of anti-nutritional components (Marteau and Rambaud 1993).

In order to design new starters for the production of fermented products of high and standardized quality, research on the selection and characterization of strains among all genera of lactic acid bacteria has been increasing over the last few years.

Recently, during a survey of the lactic acid bacterial flora of naturally fermented Greek Kasseri cheese, a group of 26 strains, phenotypically assigned to *Streptococcus thermophilus*, has been isolated. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis analysis of whole-cell proteins revealed that the group was quite different from *Strep. thermophilus* (Tsakalidou *et al.* 1994). Comparative 16S and 23S rRNA sequence analyses showed that the isolates represented a new species within the genus *Streptococcus*, which was named *Strep. macedonicus* (Tsakalidou *et al.* 1998).

In the present work, a total of 32 *Strep. macedonicus* strains, all isolated from naturally fermented Greek Kasseri cheese, were screened for biochemical properties of technological importance in milk fermentations. Strains were examined for acid production in milk, proteolytic and lipolytic activity, citrate metabolism, exopolysaccharide production, antimicrobial activity and biogenic amine production.

The aim of this study was to discover if these natural contaminants of Kasseri cheese possess properties which would allow them to be used either as starters or adjunct starters in cheese manufacture. Furthermore, we were interested in selecting strains possessing specific features, such as the production of bacteriocins and exopolysaccharides, which could be used and/or applied as future food additives.

MATERIALS AND METHODS

Bacterial strains

A total of 32 *Strep. macedonicus* strains were examined in the present study. They were isolated from Greek Kasseri cheese and belonged to the ACA-DC collection of the Laboratory of Dairy Research at the Agricultural University of Athens, Greece.

Acid production in milk

Strains were subcultured twice in skim milk (10% w/v), containing yeast extract (0.3% w/v), for 24 h at 37 °C (1% v/v inoculum). Final growth was performed in skim milk (10% w/v) for 6 h at 37 °C (1% v/v inoculum). Acid production was recorded as the final pH value.

Protease activity

Strains were subcultured twice in skim milk (10% w/v), containing yeast extract (0.3% w/v), for 24 h at 37 °C (1% v/v inoculum). Final growth was performed in skim milk (10% w/v) for 24 h at 37 °C (1% v/v inoculum). An equal volume of a 12% (w/v) trichloroacetic acid (TCA) solution was then added and the mixture vortexed and incubated for 10 min at room temperature. The peptides/amino acids in the supernatant fluid obtained after centrifugation (13 684 g for 5 min at 10 °C) were determined using the *o*-phthaldialdehyde method (Church *et al.* 1983). Results were expressed as leucine equivalents according to a standard curve, using leucine in a concentration range of 0–10 mmol l⁻¹.

Peptidase activity

Strains were subcultured twice in MRS broth (Oxoid, Basingstoke, UK) for 24 h at 37 °C (1% v/v inoculum). Final growth was performed in MRS broth for 24 h at 37 °C (1% v/v inoculum). Cells were collected by centrifugation (10 321 g, 10 min at 4 °C) and washed with a 0.9% (w/v) NaCl aqueous solution. They were then resuspended in 50 mmol l⁻¹ phosphate buffer, pH 7.0. Half of the cell suspension was used for the qualitative detection of peptidase activities. Cells in the remaining fraction were lysed using lysozyme (2 mg ml⁻¹, 2 h at 37 °C). The cell-free extract obtained after centrifugation (13 684 g, 5 min at 4 °C) was used for the quantitative determination of peptidase activities. The protein concentration was determined according to Lowry *et al.* (1951), using bovine serum albumin as a standard.

For the detection of peptidase activities, the following 10 substrates were used: alanine-, arginine-, glycine-, leucine-, lysine-, proline-, phenylalanine-, glycine-proline-, glycine-phenylalanine- and N-acetyl-alanine-4-nitroanilide. An intact cell suspension (25 µl) and amino acid derivatives of 4-nitroaniline (25 µl; 20 mmol l⁻¹ in methanol) were incubated in 200 µl 50 mmol l⁻¹ phosphate buffer, pH 7.0, at 30 °C for 60 min, and the intensity of the yellow colour against the blank measurement was visually recorded. In the case of positive reactions, the activity in the cell-free extract was photometrically determined at 410 nm by measuring the rate of substrate hydrolysis. A unit (U) of enzyme activity was defined as the amount of enzyme producing 1 µmol 4-nitroaniline min⁻¹ (ε₄₁₀ = 8800 mol⁻¹ l cm⁻¹). The specific activity was defined as the number of units mg⁻¹ protein.

Lipase activity

Strains were subcultured twice in skim milk (10% w/v), containing yeast extract (0.3% w/v), for 24 h at 37 °C (1% v/v inoculum). Final growth was performed in MRS broth for 24 h at 37 °C (1% v/v inoculum). A loopful of fresh culture

was inoculated on nutrient agar plates (pH 6.8) containing tributyrin (1% v/v) and arabic gum (1% w/v). The plates were incubated for 48 h at 37 °C and examined for halo formation around the colonies.

Esterase activity

For the detection of esterase activities 10 substrates were used: 4-nitrophenyl-acetate (C2), -propionate (C3), -butyrate (C4), -caproate (C6), -caprylate (C8), -caprate (C10), -laurate (C12), -myristate (C14), -palmitate (C16) and -stearate (C18). The preparation of the intact cell suspensions and the cell-free extracts, as well as the qualitative detection and the quantitative determination of esterases, was performed as in the case of the peptidase activities. The molecular extinction coefficient of 4-nitrophenol ($\epsilon_{410} = 7660 \text{ mol}^{-1} \text{ l cm}^{-1}$) was used for the calculation of the specific activity.

Citrate metabolism

Strains were subcultured twice in skim milk (10% w/v), containing yeast extract (0.3% w/v), for 24 h at 37 °C (1% v/v inoculum). Final growth was performed in skim milk (10% w/v) for 24 h at 37 °C (1% v/v inoculum). An equal volume of a 24% (w/v) TCA solution was then added and the mixture vortexed and incubated for 30 min at room temperature. The citrate in the supernatant fluid obtained after centrifugation (14 848 g, 5 min at 4 °C) was determined according to Marier and Boulet (1958).

Alternatively, strains were screened for citrate metabolism using Simmons Citrate Agar (SCA; Oxoid). A loopful of fresh culture was inoculated on SCA plates. Plates were incubated for 7 d at 37 °C and observed for bacterial growth and colour change.

Exopolysaccharide production

Strains were cultivated for 12 h in 11 enriched milk medium (10% w/v skim milk, 1% w/v peptone, 0.5% w/v yeast extract) at 42 °C (1% v/v inoculum). The isolation of exopolysaccharides from the fermented milk medium was performed in four steps as described elsewhere (De Vuyst *et al.* 1998).

Bacteriocin production

Strains were subcultured twice in MRS broth for 24 h at 37 °C (1% v/v inoculum). Final growth was performed in MRS broth for 24 h at 37 °C (1% v/v inoculum). The growth medium was then collected by centrifugation (10 321 g, 10 min at 4 °C). The pH of the cell-free supernatant fluid was

adjusted to 6.5 using 5 N NaOH and it was used for the bacteriocin screening.

Four indicator micro-organisms were used, *Listeria innocua* LMG 11387^T, *L. innocua* LMG 13568, *Propionibacterium freudenreichii* subsp. *shermanii* LMG 16424^T and *Clostridium tyrobutyricum* LMG 1285^T. These were kindly provided by the Laboratory of Microbiology Gent (University of Gent, Gent, Belgium). *Listeria innocua* strains were incubated in BHI broth (Oxoid) or brain heart infusion (BHI) agar at 30 °C. *Propionibacterium freudenreichii* subsp. *shermanii* LMG 16424^T was incubated in YEL broth (1% w/v tryptone, 1% w/v yeast extract, 2.1% v/v sodium lactate, 0.025% w/v K₂HPO₄, 0.005% w/v MnSO₄) or on YEL agar (strictly anaerobically) at 30 °C. *Clostridium tyrobutyricum* LMG 1285^T was incubated in RCM broth or agar (Oxoid), strictly anaerobically, at 30 °C.

Two methods were used for the bacteriocin production screening, the well diffusion assay (WDA) and the soft agar assay (SAA; De Vuyst *et al.* 1996).

For the WDA, growth medium (containing 1.5% w/v agar) was inoculated with the respective indicator strain (1% v/v fresh inoculum). Holes were opened in the solid growth medium and 40 µl of the cell-free supernatant fluid were added. The plates were incubated under the appropriate conditions for each indicator strain and observed for the formation of inhibition zones (halos) after 24 h.

For the SAA, growth medium (containing 1.5% w/v agar) appropriate for each indicator strain was poured in Petri dishes. A quantity of 3.5 ml of the same growth medium (containing 0.7% w/v agar) inoculated with the respective indicator strain was added to the plates. Supernatant fluid (10 µl) was spotted onto the surface of the plates. The plates were incubated for 24 h under the appropriate conditions for each indicator strain and examined for halo formation.

Biogenic amine production

Strains were subcultured twice in MRS broth (Oxoid) for 24 h at 37 °C (1% v/v inoculum). A loopful of fresh culture was inoculated on plates containing tryptone (0.5% w/v), yeast extract (0.5% w/v), NaCl (0.5% w/v), glucose (0.1% w/v), Tween 80 (0.05% v/v), MgSO₄·7H₂O (0.02% w/v), CaCO₃ (0.01% w/v), bromocresol purple (0.006% w/v), MnSO₄·4H₂O (0.005% w/v), FeSO₄·7H₂O (0.004% w/v), agar (1.5% w/v) and a precursor amino acid (2% w/v, ornithine, histidine, lysine, tyrosine) at pH 5.0 (Joosten and Northolt 1989). The plates were incubated at 30 °C for 7 d and observed for purple colour formation in the case of ornithine, histidine and lysine and for clarification in the case of tyrosine.

RESULTS

Acid production in milk

All *Strep. macedonicus* strains examined were found to be rather moderate acid producers as, after 6 h of growth in milk, the pH value only ranged from 5.6 to 6.1 (Table 1).

Proteolytic activity

According to the results obtained in the present study using the *o*-phthalaldehyde method, all *Strep. macedonicus* strains examined exhibited low extracellular proteolytic activity. Only four strains, namely *Strep. macedonicus* ACA-DC 205, 209, 211 and 266, were able to hydrolyse milk casein. The free amino groups determined were expressed in leucine equivalents and ranged between 0.07 and 0.80 mmol l⁻¹ (Table 1).

No extracellular proteolytic activity could be detected for all other strains. Synthetic substrates were also used for the detection of peptidase activities in cell-free extracts of the *Strep. macedonicus* strains. Although no activity was detected against proline-, glycine-phenylalanine- or N-acetyl-L-alanine-4-nitroanilide, the majority of the strains exhibited a rather broad specificity; the highest activities were observed against phenylalanine-4-nitroanilide (Table 2). Several strains were found to be active against glycine- (three strains), phenylalanine- (seven strains) and arginine-4-nitroanilide (19 strains), with an activity ranging from 0.01 to 1.32 U mg⁻¹. All strains except one exhibited peptidase activity against alanine-, and/or lysine- and/or leucine-4-nitroanilide, with values ranging from 0.01 to 0.14 U mg⁻¹. Finally, 21 of the 32

Table 1 Acid production and proteolytic and lipolytic activity of *Streptococcus macedonicus* strains expressed as pH, leucine equivalents (mmol l⁻¹) and halo formation, respectively

Strain ACA-DC	pH (6 h in milk)	Proteolysis (leucine, mmol l ⁻¹)	Lipolysis (halo formation)
186	6.1	—	+++
187	5.7	—	—
188	6.1	—	—
190	5.6	—	—
191	6.0	—	+
192	6.1	—	—
193	5.9	—	+
194	6.0	—	—
197	5.7	—	+
198	6.0	—	—
199	6.0	—	—
200	6.1	—	+
203	5.8	—	+
205	5.9	0.30	—
206 ^T	6.0	—	—
207	5.9	—	—
208	5.7	—	—
209	5.7	0.45	+
210	5.6	—	+
211	6.0	0.80	+
243	5.6	—	—
244	6.0	—	—
245	6.0	—	—
246	5.7	—	—
247	5.6	—	—
248	5.8	—	—
249	6.0	—	—
250	5.9	—	—
251	6.0	—	—
255	5.9	—	+
256	5.7	—	—
266	5.7	0.07	+

Table 2 Peptidase activities ($\text{U mg}^{-1} \times 100$) of *Streptococcus macedonicus* strains, measured against derivatives of 4-nitroaniline with various amino acids

Strain ACA-DC	gly	phe	arg	leu	lys	gly-pro	ala
186	1	0	1	0	2	0	1
187	0	0	3	2	3	13	3
188	0	8	1	0	0	1	1
190	0	0	4	7	7	27	8
191	0	0	6	4	6	27	7
192	0	0	8	4	6	25	6
193	0	0	0	0	0	3	2
194	0	0	0	4	2	10	2
197	0	0	0	0	0	1	2
198	0	0	0	0	0	2	1
199	0	132	7	9	11	14	10
200	0	0	10	12	16	13	14
203	0	0	0	0	0	11	0
205	1	0	0	0	2	0	1
206 ^T	0	0	2	0	1	10	2
207	0	0	0	1	2	15	2
208	0	0	0	0	1	1	1
209	0	29	0	0	1	1	1
210	0	0	0	1	1	2	1
211	1	25	1	1	0	0	1
243	0	0	1	2	3	11	3
244	0	0	6	3	6	14	7
245	0	87	4	4	8	23	7
246	0	77	0	0	1	1	1
247	0	0	0	0	2	1	2
248	0	0	2	2	2	11	2
249	0	0	6	8	10	14	9
250	0	0	3	2	2	7	2
251	0	63	4	5	9	23	6
255	0	0	6	8	10	20	8
256	0	0	0	1	1	8	1
266	0	0	4	4	7	26	6

strains tested exhibited X-prolyl-dipeptidyl aminopeptidase activity, ranging from 0.01 to 0.27 U mg^{-1} , as they were found to be active against glycine-proline-4-nitroanilide.

Lipolytic activity

In the present study, using the agar assay with tributyrin as substrate, only *Strep. macedonicus* strain ACA-DC 186 was found to be highly lipolytic (Table 1). Another 10 strains, namely ACA-DC 191, 193, 197, 200, 203, 209, 210, 211, 255 and 266, exhibited lower lipolytic activity. In contrast, when synthetic substrates were used, esterase activities were detected for all strains (Table 3). All strains were active against 4-nitrophenyl-butyrate (C4), -caproate (C6) and -caprylate (C8), exhibiting specific activities in a range from 0.01 to 0.90

U mg^{-1} . Several strains were able to hydrolyse 4-nitrophenyl-acetate (C2; 12 strains) and 4-nitrophenyl-propionate (C3; seven strains), with specific activities ranging from 0.01 to 0.63 U mg^{-1} , while seven strains were active against 4-nitrophenyl-caprate (C10), with lower activities (0.01–0.19 U mg^{-1}). Only strain ACA-DC 186, also highly active against tributyrin, could hydrolyse 4-nitrophenyl-laurate (C12) and palmitate (C16), while no activity was detected against 4-nitrophenyl-myristate (C14) and stearate (C18).

Citrate metabolism

Five of the *Strep. macedonicus* strains tested, namely ACA-DC 186, 188, 205, 209 and 211, could grow on SCA; only strains ACA-DC 188, 205 and 209 caused a colour change of

Table 3 Esterase activities ($\text{U mg}^{-1} \times 100$) of *Streptococcus macedonicus* strains, measured against derivatives of 4-nitrophenol with various fatty acids

Strain ACA-DC	C2	C3	C4	C6	C8	C10	C12	C16
186	19	55	90	60	24	6	6	3
187	6	0	4	10	4	3	0	0
188	10	16	33	24	12	3	0	0
190	0	0	7	24	1	0	0	0
191	2	7	24	11	7	0	0	0
192	0	0	11	15	7	10	0	0
193	0	0	31	46	0	0	0	0
194	0	0	14	22	7	0	0	0
197	3	0	27	36	46	0	0	0
198	0	0	22	18	12	0	0	0
199	6	3	36	23	13	0	0	0
200	1	0	10	4	3	0	0	0
203	0	0	24	30	28	0	0	0
205	9	42	61	46	9	6	0	0
206 ^T	0	0	35	22	27	0	0	0
207	0	0	23	11	29	0	0	0
208	0	0	11	18	45	0	0	0
209	2	18	39	27	5	1	0	0
210	0	0	20	32	41	0	0	0
211	23	63	66	56	18	19	0	0
243	2	0	30	11	1	0	0	0
244	0	0	16	15	25	0	0	0
245	1	0	12	10	18	0	0	0
246	0	0	28	26	20	0	0	0
247	0	0	16	18	0	0	0	0
248	0	0	19	25	3	0	0	0
249	0	0	13	13	5	0	0	0
250	0	0	26	25	40	0	0	0
251	0	0	17	9	21	0	0	0
255	0	0	22	15	20	0	0	0
256	0	0	21	31	12	0	0	0
266	0	0	8	15	7	0	0	0

the growth medium. Using the photometric assay for citrate determination (initial concentration in milk $1909 \mu\text{g ml}^{-1}$) the same set of strains, except ACA-DC 211, was found to catabolize citrate (Table 4).

Exopolysaccharide production

According to the results obtained in this study, none of the *Strep. macedonicus* strains produced exopolysaccharides in enriched milk medium.

Bacteriocin production

None of the *Strep. macedonicus* strains tested showed antimicrobial activity against both *L. innocua* and *P. freudenreichii*

subsp. *shermanii* strains used as indicator micro-organisms. However, nine strains were active against the *Cl. tyrobutyricum* strain used; the highest activity was obtained with strain ACA-DC 198 (Table 4).

Biogenic amine production

None of the *Strep. macedonicus* strains tested could decarboxylize ornithine, lysine or histidine, and only four strains produced tyramine from tyrosine (Table 4).

DISCUSSION

A rapid pH decrease during the initial steps of cheese preparation is of crucial importance in cheese manufacture. The

Table 4 Citrate metabolism, bacteriocin production against *Clostridium tyrobutyricum* and tyramine production of the *Streptococcus macedonicus* strains

Strain ACA-DC	Citrate metabolized ($\mu\text{g ml}^{-1}$)	Citrate screening on SCA production (growth/colour change)	Bacteriocin production	Tyramine production
186	1204	+/-	-	+
188	1024	+ /blue	+	+
190	0	-/-	+	-
191	0	-/-	+	-
192	0	-/-	+	-
193	0	-/-	+	-
198	0	-/-	+++	-
205	289	+ /blue	-	+
209	932	+ /blue	+	+
210	0	-/-	+	-
211	0	+/-	+	+

SCA, Simmons citrate agar.

Strep. macedonicus strains examined in this study were found to be rather moderate acid producers and could, therefore, be used as starters preferably in combination with other lactic acid bacteria strains that would produce lactic acid from lactose more rapidly.

The degradation of milk casein plays an important role in the development of texture in cheese. In addition, certain peptides contribute to the formation of flavour, whereas other, undesirable bitter-tasting peptides can lead to off-flavours. According to the results obtained in the present study, *Strep. macedonicus* strains exhibited low extracellular proteolytic activity. Using the same method, the results were similar to those obtained in our laboratory for other thermophilic cocci, which were in turn still much lower than those obtained for thermophilic lactobacilli or lactococci (unpublished data). Thus, it could be concluded that the contribution of the *Strep. macedonicus* strains to the initial degradation of milk casein during cheese manufacture would be rather limited.

However, it is well established that intracellular peptidases of cheese starters can be released after cell lysis in the curd during ripening (Gasson 1996). These peptidases are considered to play an important role in proteolysis during cheese preparation. For this reason, synthetic substrates were used for the detection of peptidase activities in cell-free extracts of the *Strep. macedonicus* strains. The majority of the strains exhibited a rather broad specificity and most of them exhibited X-prolyl-dipeptidyl aminopeptidase activity. Since milk casein is rich in proline, this activity could be important in view of using these strains in milk fermentations.

Milk fat hydrolysis during cheese manufacture is due to the endogenous milk lipase, the lipolytic enzymes of starter

and non-starter bacteria, lipases from psychrotrophic bacteria and, depending on the cheese variety, exogenous enzyme preparations. Fatty acids produced can be further converted to methylketones and thioesters, which are also implicated as cheese flavour compounds. Lactic acid bacteria are generally considered to be only weakly lipolytic, as compared with other groups of micro-organisms (El Soda *et al.* 1995). However, due to the low taste threshold of some fatty acids, a large number of weakly lipolytic bacteria may play an important role in products which are stored for a long period, such as ripened cheeses (Crow *et al.* 1993). In the present study, using an agar assay with tributyrin as substrate, only one *Strep. macedonicus* strain was found to be highly lipolytic. On the contrary, when synthetic substrates were used, esterase activities were detected for all strains. In this respect, *Strep. macedonicus* strains were similar to enterococci (Tsakalidou 1997) and other streptococci (Formisano *et al.* 1974). It must be noted, however, that the values obtained for the esterase activities were generally higher than those determined for the peptidase activities. This could be an indication that the *Strep. macedonicus* strains would have more impact on lipolysis than on proteolysis during cheese ripening.

Although the concentration of citrate in milk is low, its metabolism is important in determining the texture and flavour of cheese. The carbon dioxide produced is responsible for eye formation in Dutch-type cheeses, while diacetyl, acetoin, butanediol, acetaldehyde and acetate have very distinct aroma properties and contribute significantly to the flavour of cheese. Most of the knowledge about the metabolic pathways involved in citrate metabolism has been derived from *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*. However,

these pathways seemed to be a common trait for other lactic acid bacteria (Hugenholtz 1993). Five of the *Strep. macedonicus* strains tested were found to catabolize citrate. According to the results obtained using the photometric method, it is evident that some of the *Strep. macedonicus* strains possessed at least an uptake mechanism and perhaps citrate catabolism enzymes. The fact that these strains could also grow on SCA agar with citrate as sole carbon source indicates that they can also use citrate as energy source. However, further research is necessary in order to determine the exact pathway and the final products. Only when these data are available, can we draw conclusions about the role of citrate on the growth of *Strep. macedonicus* and its production of aroma compounds.

Many lactic acid bacteria produce exopolysaccharides. These are either common homopolysaccharides such as α -D-glucans (dextrans, mutans and alternan), β -D-glucans and fructans (e.g. levan), or heteropolysaccharides produced by both mesophilic and thermophilic lactic acid bacteria (De Vuyst and Degeest 1999). The latter group of exopolysaccharides (EPS) has received renewed interest since they play an important role in the rheology, texture, body and mouthfeel of fermented milk drinks. Most of the EPS-producing lactic acid bacteria studied in more detail were isolated from dairy products, e.g. Scandinavian ropy fermented milk products, various yogurts and fermented milks, and milky and sugary kefir grains. Cheese and fermented meat and vegetables also served as a source of EPS-producing lactic acid bacteria strains. According to the results obtained in this study, none of the *Strep. macedonicus* strains produced exopolysaccharides in enriched milk medium.

Bacteriocin production is one of the most important characteristics of lactic acid bacteria, as they exhibit a bacteriostatic or bacteriocidal action against other Gram-positive bacteria (De Vuyst and Vandamme 1994). They display varied spectra of inhibition; those which inhibit pathogens and spoilage bacteria are potentially useful. Although to date only nisin has been used commercially, it is of great importance to know the capacity of lactic acid bacteria strains to produce bacteriocins. The potential of *Strep. macedonicus* strains to inhibit *Cl. tyrobutyricum* growth is important, as these sporulating bacteria can provide a late swelling during the ripening of cheese. This may be ascribed to a bacteriocin molecule.

Many lactic acid bacteria possess amino acid decarboxylases and may thus produce biogenic amines (Joosten and Northolt 1989; Tham *et al.* 1990). Histamine poisoning has been attributed to the consumption of different sea fish species, but it may also occur after the consumption of cheese or other types of fermented foods. Tyramine has been proved to be a cause of adverse reactions, involving headache, hypersensitive crisis and interactions with antidepressive drugs, which were observed after the consumption of ripening cheese. An increase in amine concentration during cheese ripening under conditions of enhanced proteolysis in the pre-

sence of starter and spoilage lactobacilli has been observed (Leuschner *et al.* 1998).

Although none of the *Strep. macedonicus* strains tested could decarboxylize ornithine, lysine or histidine, four strains produced tyramine from tyrosine. In the case of tyramine, quantitative determination is necessary to draw conclusions about the potential pathogenicity of these strains.

In conclusion, with respect to their acidifying and proteolytic activity, *Strep. macedonicus* strains could hardly be used as sole starters in cheese making. However, as adjunct starters they could contribute to the hydrolysis of milk fat and the degradation of milk citrate, but also to the secondary hydrolysis of milk casein due to their peptidolytic activities. Moreover, the screening work performed in this study revealed strains with technologically interesting properties, such as those possessing antimicrobial activity against *Cl. tyrobutyricum*. In this case, the description of a more integral inhibitory spectrum of the producer strains, the characterization of the antimicrobial molecules and the optimization of their production deserve further investigation.

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