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The performance of *Streptococcus macedonicus* ACA-DC 198 as starter culture in Kasseri cheese production

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Abstract

Streptococous macedonicus ACA-DC 198 was used both as an adjunct (Cheese B) and as sole starter (Cheese C) in Kasseri cheese production. Control cheese (Cheese A) was prepared using a commercial starter culture. In all cheeses, numbers of all microbial groups examined initially increased, but declined after Baski scalding and remained practically stable up to day 90. The presence of *S. macedonicus* was confirmed until the end of ripening (Cheeses B and C); this was also the case for the bacteriocin produced by *S. macedonicus* ACA-DC 198. *X*-propyl-dipeptidyl-aminopeptidase activity was detected in all cheeses after day 15. The physicochemical characteristics of the mature Cheese C were in agreement with those defined by the Greek legislation. The sensory properties of all mature cheeses corresponded with the ones characterizing the traditional Kasseri cheese. It was concluded that *S. macedonicus* ACA-DC 198 can be used as an adjunct in Kasseri cheese production.

Keywords: Kasseri cheese; *Streptococcus macedonicus*; Microbiological; Physicochemical; Sensory; Bacteriocin; *X*-propyl-dipeptidyl-aminopeptidase

1. Introduction

Food fermentation has been used for centuries as a method to preserve perishable food products, such as milk, meat, fish, cereals, vegetables, and fruits. Besides yeasts and moulds, lactic acid bacteria are widely used in the production of fermented foods (Leroy & De Vuyst, 2004). In fact, lactic acid bacteria constitute worldwide the majority in volume and value of the commercial starter cultures, with the largest amount being applied in the dairy industry (Hansen, 2002).

Among the various functions of lactic acid bacteria in food fermentations, one that has gained increasing attention in recent years is the bioprotective role (Leroy & De Vuyst, 2004). Besides organic acids and other low molecular mass inhibitory compounds, such as diacetyl or hydrogen peroxide, many lactic acid bacteria produce ribosomally synthesized peptides or proteins, called bacteriocins, which exert an antimicrobial effect against not only closely related bacteria, but often also against spoilage and pathogenic bacteria (De Vuyst & Vandamme, 1994). These bacteria offer great potential in biopreservation of foods. Today, biopreservation, in contrast to the chemical (artificial) preservation of foods, reflects the consumers' demands for safe and minimally processed foods of adequate shelf life and convenience, and the global need for increasing the supply of healthy and safe foods.

During a survey of the lactic acid bacteria microbiota in naturally fermented Greek Kasseri cheese, a group of strains, phenotypically assigned to *Streptococcus thermophilus*, was isolated. SDS-PAGE analysis of whole-cell proteins and comparative 16S and 23S rRNA sequence analyses indicated that the isolates represented a new species within the genus *Streptococcus*, which was named *Streptococcus macedonicus* (Tsakalidou et al., 1994, 1998).

S. macedonicus strains were shown to posses generally low milk acidification, proteolytic, and citrate-catabolizing

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activity, and moderate lipolytic activity (Georgalaki, Sarantinopoulos, Ferreira, De Vuyst, Kalantzopoulos, & Tsakalidou, 2000). *X*-prolyl-dipeptidyl-aminopeptidase (PepX) was shown to be the dominant peptidase activity (Georgalaki, Papadelli, Anastasiou, Kalantzopoulos, & Tsakalidou, 2002). Several strains produced an antimicrobial compound active against *Clostridium tyrobutyricum* and other microorganisms (Georgalaki et al., 2002). Milk casein is rich in proline and thus PepX is assumed to play an important role in casein degradation during cheese ripening (Kunji, Mierau, Hagting, Poolman, & Konings, 1996; Mierau, Kunji, Venema, & Kok, 1997). *C. tyrobutyricum* is considered responsible for the so-called "late blowing", which is a serious defect, especially in hard and semi-hard cheeses, such as Kasseri, Emmental, Gruyère, Grana, Edam and Gouda, where *Clostridium* growth is favored by the rather high pH of these cheeses (Steffen, Eberhard, Bosset, & Rüegg, 1993; Walstra, Noomen, & Geurts, 1993).

The aim of the present work was to examine how *S. macedonicus* ACA-DC 198 performs during Kasseri cheese production, when used either as adjunct or sole starter. More specifically, the research assesses survival under the stress conditions prevailing during Kasseri cheese production, evaluates the production and stability of bacteriocin under these conditions, and, finally, evaluates the impact of the strain on the technological characteristics of Kasseri cheese.

2. Materials and methods

2.1. Bacterial strains and media

S. macedonicus ACA-DC 198 as well as a commercial, freeze-dried, mixed starter culture (LYOBAC X 10 UF, Alce S.R.L., Novara, Italy), containing *S. thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lb. casei*, were used as starters in Kasseri cheese production. *S. macedonicus* ACA-DC 198 was isolated from naturally fermented Kasseri cheese and belongs to the ACA-DC culture collection of the Laboratory of Dairy Research (Department of Food Science and Technology, Agricultural University of Athens, Greece). Before cheese-making experiments, the strain was subcultured twice in skim milk (10%, w/v; Oxoid, Basingstoke, Hampshire, UK), containing yeast extract (0.3%, w/v; Oxoid) at 37 °C for 24 h (1%, v/v, inoculum). Final subculturing was performed in pasteurized sheep's milk under the same conditions.

2.2. Cheese making and sampling

Kasseri cheese, a "pasta-filata" type cheese, was prepared using either the commercial starter culture solely (Cheese A), or both the commercial starter and *S. macedonicus* ACA-DC 198 (Cheese B), or *S. macedonicus* ACA-DC 198 as sole starter (Cheese C). The trials consisted of three repetitions of the entire set of cheeses (A–C), which were performed in three successive weeks within the same lactation period.

The procedure for Kasseri cheese making was performed as follows. Sheep's milk (100 kg) was pasteurized at 68 °C for 10 min and then cooled to 37 °C. The commercial starter (freeze-dried) was added at a concentration of 5 g per 100 kg milk, while *S. macedonicus* ACA-DC 198 was added as a 1 %(v/v) inoculum. Rennet (3 g per 100 kg milk; 950 IMCU; Vlachopoula E.P.E., Athens, Greece) and $CaCl_2$ (10 g per 100 kg milk; JT Baker, Deventer, The Netherlands) were then added to allow coagulation (35 min). The curd was cut into pieces of 0.5 cm size, heated up to 45 °C within 10 min and held at this temperature for 10 min under stirring. The whey was removed and the drained cheese curd (Baski 1) was left at 21 °C for 48 h (Cheeses A and B) or 5 days (Cheese C) in order to allow the pH to reach 5.2. The ripened cheese curd (Baski 2) was cut into small thin slices, which were scalded in water (75–80°C) and kneaded into a homogenous, elastic mass (Kasseri 0). Salt (2%, w/w, for Cheeses A and B; 1%, w/w, for Cheese C) was added during moulding.

After moulding, Cheeses A and B were left at 18 °C for 1 week (Kasseri 7). Then vacuum-packaged and ripened at 4 °C for 11 weeks (Kasseri 15, Kasseri 30, Kasseri 60 and Kasseri 90). Cheese C, after moulding was held at 21 °C, and in the first week turned twice and dry-salted (total salt added 0.26%, w/w). After 1 week the cheese was transferred to 16 °C and left there for further 7 weeks (Kasseri 15, Kasseri 30 and Kasseri 60). Then, the cheese was vacuum-packaged and left for 4 weeks at 4 °C (Kasseri 90).

2.3. Microbiological analyses

The following groups of microorganisms were enumerated through plating: (i) thermophilic lactobacilli on MRS agar (pH 5.4; Biokar Diagnostics, Beauvais, France) at 42 °C for 72 h, anaerobically (double agar layer); (ii) NSLAB on Rogosa agar (Biokar Diagnostics) at 30 °C for 3–5 days, anaerobically (double agar layer); (iii) mesophilic cocci on M17 agar (Biokar Diagnostics) at 22 °C for 48 h; (iv) thermophilic cocci on M17 agar (Biokar Diagnostics) at 42 °C for 48 h; (v) enterococci on KAA agar (Merck, Darmstadt, Germany) at 37 °C for 24 h; (vi) micrococci on Mannitol Salt Agar (Biokar Diagnostics) supplemented with cycloheximide (100 $\mu g\,mL^{-1}$; Sigma-Aldrich) at 30 °C for 48 h; and (vii) coliforms on Violet Red Bile Agar (Biokar Diagnostics) at 37 °C for 24 h, anaerobically (double agar layer). M17 agar, supplemented with 25 $\mu g\,mL^{-1}$ streptomycin (Biokar Diagnostics), was used as selective medium for *S. macedonicus* enumeration after 48 h of incubation at 37 °C. *S. macedonicus* colonies were analyzed by species-specific PCR according to Papadelli, Manolopoulou, Kalantzopoulos, and Tsakalidou (2003). The total microbiota of the pasteurized milk was enumerated on PCA (Biokar Diagnostics) at 30 °C for 72 h.

2.4. Physicochemical analyses

The pH of the curd and cheese was measured using a glass electrode (pH-Meter 632, Metrohm Herisau, Switzerland), which was calibrated using standard solutions of pH 4.0, 7.0 and 9.0. Moisture content was measured by heating the samples at 105 °C to constant weight. The salt and ash content were determined according to IDF Standard 17A (1972) and IDF Standard 27 (1964), respectively, and the salt content was expressed as salt in moisture (SIM). Fat content was determined according to the Gerber-van Gulik method (Ardö & Polychroniadou, 1999) and expressed as fat in dry matter (FDM). Total nitrogen (TN) and water-soluble nitrogen (WSN) were determined using the Kjeldahl method (Ardö & Polychroniadou, 1999). Proteolytic index (PI) was expressed as the WSN % of the TN. All determinations were performed in triplicate.

Casein fractions in cheese samples were separated by urea–polyacrylamide gel electrophoresis according to Andrews (1983). A suspension of 240 mg cheese in 10 mL stacking gel buffer, containing 6 M urea (Sigma-Aldrich, St. Louis, MO, USA), 0.1 M β-mercaptoethanol and 0.4 mL tracking dye solution, was held at 40 °C for 15 min, and then centrifuged at $3000 \times g$ for 15 min at 4 °C. The solidified fat layer was discarded and 10 μL of the aqueous supernatant were used for electrophoresis. Electrophoresis was carried out on a vertical slab unit (LKB vertical electrophoresis unit 2001, Bromma, Sweden) in slabs 140×160 mm with thickness 1.5 mm. Gels were stained overnight according to Blakesley & Boezi (1977) and destained using distilled water. Casein fractions of cheese samples were identified by comparison with an isoelectric ovine casein standard prepared by acidification of defatted ovine milk at pH 4.4 using 1M HCl (Moatsou, Samolada, Katsabeki, & Anifantakis, 2004).

Analysis of WSN extracts was performed using an automated HPLC system (Waters, Milford, MA, USA), consisting of one pump (Waters 600), a diode array UV/Vis detector (Waters 996), a helium degasser and a Rheodyne injector (Model 7125, Rheodyne Inc., Cotati, CA, USA). The data acquisition and processing were performed by the Millenium v. 2.15 software (1994, Waters Corp.). A reverse-phase C_{18} Nucleosil wide pore column (5 μm, 30 nm, 300×4.0 mm, Bischoff, Germany) with a guard column (30×4.0 mm) was used. The chromatographic conditions were: solvent A, 1 mL L^{-1} trifluoroacetic acid (TFA; Merck, Darmstadt, Germany) in water; solvent B, a mixture of 600 mL L^{-1} acetonitrile (gradient grade for LC; Merck), 399 mL L^{-1} water and 1 mL L^{-1} TFA at a flow rate of 0.75 mL min^{-1}. The samples were eluted at room temperature, first with 100% solvent A for 10 min, then with a gradient of 0-80% solvent B over 80 min and finally with 100% solvent B for 10 min. The absorbance of the eluate was monitored at 214 nm. Solvents and samples were filtered through 0.45 μm Nylon 66 and cellulose acetate filters, respectively (Lida Manufacturing Corp., Kenosha, WI, USA).

2.5. Detection of X-prolyl-dipeptidyl-aminopeptidase (PepX)

A 10 g aliquot of grated cheese sample was homogenized in a Stomacher blender (Stomacher 400, Seward Medical Ltd., London, UK) for 5 min with 10 mL of 50 mM Tris-HCl buffer, pH 7.0. The suspension was centrifuged ($12\,000 \times g$ for 30 min at 4 °C) and the supernatant obtained was filtered through Acrodisc® GHP membrane filters of 0.22 μm pore size (Pall, NY, USA). Cheese extract (125 μL) and substrate (125 μL; arginyl-prolyl-β-naphthylaminde 0.656 mM, and Fast Garnet 0.656 mM, in 50 mM phosphate buffer, pH 7.0) were incubated in microtiter plates at 37 °C for 10 min (Gatti, Fornasari, Mucchetti, Addeo, & Neviani, 1999). Peptidase activity was assayed at 580 nm (Sunrise photometer, Tecan GmbH, Grödig, Austria) against a blank containing 125 μL of the extraction buffer instead of the cheese extract (Bouquien, Corrieu, & Desmazeaud, 1988). Determination of the PepX activity was performed in all three cheese samples (A–C) in triplicate.

2.6. Detection of macedocin

The well-diffusion assay was used for the determination of macedocin activity in cheese samples (Tagg & McGiven, 1971), using *Lactococcus lactis* LMG 6890 T as indicator strain. Briefly, 15 mL of M17 agar (Biokar Diagnostics), containing 0.1% (v/v) of a fresh culture of the sensitive strain, were poured into a Petri dish, and wells of 5 mm diameter were made in the solidified medium. The wells were filled with 50 μL of cheese or whey extracts, and determination of inhibition zones was done after 12 h of incubation at 30 °C.

Cheese and whey extracts were prepared as follows. Grated cheese sample (10 g) was homogenized in a Stomacher blender for 5 min with 40 mL of 2% (w/v) sodium citrate solution. The suspension was adjusted to pH 6.5 with 5 M NaOH and then 10 mL were mixed with 50 mL of a methanol/chloroform mixture (2:1, v/v). Three phases (water, intermediate and organic) were separated by leaving the samples at room temperature for 24 h. Water and organic solvents were removed using a Flash Evaporator (Büchi 461, Büchi Labotechnik A.G., Flawil, Switzerland). For the bacteriocin assay, the liquid residue of the organic phase was used as such, while 500 and 300 μL of water were added to the water phase residue and the intermediate phase, respectively. For the whey samples, 10 mL were mixed directly with the methanol/chloroform mixture, and then the same procedure as above was followed.

Alternatively, grated cheese and whey samples were heated for 10 min at 80 °C and put on the agar medium inoculated with the sensitive strain before it was solidified or into the wells made after solidification, respectively (Foulquié Moreno, Rea, Cogan, & De Vuyst, 2003). Incubation of the Petri dishes took place as above.

Detection of macedocin activity was performed in all three cheese samples (A–C) in triplicate.

2.7. Sensory evaluation

After 90 days of ripening (full-ripened cheese), cheeses were subjected into a blind organoleptic evaluation by a 15-member non-professional taste panel (all members of the Laboratory of Dairy Research) familiar with Kasseri cheese. The panel graded the color [scale 0–15; bad (0–5), medium (6–10) good (11–15)], the texture [scale 0–30; bad (0–14), medium (15–19), good (20–24), very good (25–30)] and the taste and aroma [scale 0–55; bad (0–15), medium (16–37), good (38–47), very good (48–55)].

2.8. Statistical analysis

The data analysis was performed with SYSTAT statistics package v. 8.0 (SPSS Inc., 1998, Chicago, IL, USA). One-way analysis of variance (ANOVA) was used a) to test the differences (at $P<0.05$) among Cheeses A–C, concerning pH and the microbiological analyses throughout ripening, as well as for the sensory evaluation of the final product, and b) to test the differences (at $P<0.05$) in the chemical analyses of Cheese C during ripening.

3. Results and discussion

3.1. Microbiological analyses

The mean ($\pm$SD) total microbiota of the raw milk was 6.71 ± 0.49 log cfu mL^{-1}, while after pasteurisation it decreased to 3.37 ± 0.41 log cfu mL^{-1}. The counts for the microbial groups examined in the present study during the ripening of the Kasseri cheese are shown in Table 1.

Counts of *S. macedonicus* in Cheeses B and C increased during Baski ripening (from Baski 1 to 2), but declined after Baski scalding (Kasseri 0). Significantly higher counts were determined in Cheese C in Kasseri 7 and 15. This might be due to the different ripening temperatures applied directly after Baski scalding. In fact, this trend in recovery directly after Baski scalding was observed for all microbial groups in Cheese C (apart from coliforms), but it was more pronounced and statistically significant in the case of *S. macedonicus*, enterococci and micrococci. The presence of *S. macedonicus* in Cheeses B and C was confirmed by species-specific PCR applied on colonies isolated from the selective medium used for the enumeration of *S. macedonicus*. On the contrary, in the case of Cheese A, no PCR product was obtained from colonies isolated from the same medium.

Similar evolution was observed for the thermophilic lactobacilli, NSLAB, thermophilic cocci and mesophilic cocci. Counts at day 90 were similar to those reported by Kaminaridis, Paraschopoulos, and Beri (1999) and by Moatsou, Kandarakis, Moschopoulou, Anifantakis, and Alichanidis (2001) for Kasseri cheese prepared with either raw or pasteurized milk. No significant differences among the three cheeses were observed, apart from Kasseri 0, where counts of thermophilic lactobacilli, NSLAB and thermophilic cocci in Cheese C were significantly lower than in Cheeses A and B.

Interestingly, significant differences between Cheese C and Cheeses A and B were observed in the case of enterococci throughout ripening, apart from Baski 1. After Baski scalding, counts in Cheeses A and B decreased and by the end of ripening they were almost diminished. This might be due to an antimicrobial compound produced by the commercial starter culture, which inhibited the growth of enterococci in these cheeses. On the contrary, in Cheese C, enterococci recovered almost fully 1 week after Baski scalding and remained stable at levels close to those reported for commercial samples of Kasseri cheese from the Greek market (Kalogridou-Vassiliadou & Manolkidis, 1984).

Despite the decrease observed for micrococci counts after Baski scalding, these increased within the first 2 weeks and afterwards they either remained stable (Cheeses A and B), or started decreasing again (Cheese C). Significantly higher counts were determined for Cheese C in Baski 2, and Kasseri 7, 15 and 30. This might be related to the longer Baski ripening time and the higher temperatures applied directly after Baski scalding.

In all cheeses, counts of coliforms initially increased but diminished between Kasseri 7 and 15, as previously reported by Kaminaridis et al. (1999) and Moatsou et al. (2001). Significantly higher counts were determined in Cheese C in Baski 2, probably due to the longer ripening of the Baski in Cheese C.

3.2. Physicochemical analyses

The mean ($\pm$SD) composition of the milk (pH 6.64 ± 0.09) used was as follows: fat, 6.07 ± 0.42%; protein, 5.27 ± 0.20%; lactose, 5.00 ± 0.22%; solids-non-fat, 11.15 ± 0.28%; total solids, 17.22 ± 0.68%. Changes in pH, moisture, ash, SIM, FDM and PI are shown in Table 2. Apart from pH, determinations were performed only for Cheese C.

In Cheeses A and B, the pH of Baski 1 decreased within 2 days to 5.03 and 4.97 (Baski 2), respectively. After Baski ripening, Cheese C (5 days) reached a pH of 5.27, which was significantly higher than the pH of Baski 2 in Cheeses A and B. This is attributed to the fact that *S. macedonicus* is a slow milk acidifier (Georgalaki et al., 2000). The final pH (Kasseri 90) increased in all three cheeses, reaching a final value of 5.67, close to those recorded in samples of Kasseri cheese on the Greek market (Anifantakis, 1991) or experimental cheeses (Kaminaridis, Siavaras, & Potetsianaki 1995; Kaminarides et al., 1999). Throughout ripening, no significant differences were observed among the cheeses, apart from Kasseri 30 and 60, where significantly higher values were observed in Cheese C.

In Cheese C, the moisture initially decreased during Baski ripening. This significant loss of moisture can be

Table 1
Mean counts (±SD; log cfu g^{-1}) of the microbial groups examined during Kasseri cheese ripening

	B1	B2	K0	K7	K15	K30	K60	K90
Cheese A[a]								
Thermophilic lactobacilli[b]	6.73±0.75	8.09±0.57	7.38±0.26	6.56±1.01	7.09±0.61	6.57±0.85	7.04±0.59	7.11±0.47
NSLAB[c]	6.78±0.46	8.09±0.32	7.46±0.29	7.34±0.53	7.13±0.82	6.77±0.60	7.23±0.38	7.42±0.49
Mesophilic cocci[d]	7.06±0.71	8.05±0.66	5.59±1.25	5.62±0.83	5.24±0.95	5.18±1.23	5.32±0.52	6.06±0.18
Thermophilic cocci[e]	7.84±0.35	8.50±0.89	7.75±0.54	6.82±0.70	6.46±1.10	5.87±0.39	6.17±0.70	6.52±0.61
Enterococci[f]	2.40±0.83	4.09±0.76	1.00±0.87	0.57±0.98	0.57±0.98	0.33±0.58	0.23±0.40	0.00
Micrococci[g]	2.49±0.44	3.26±0.42	2.35±0.75	3.40±0.60	4.07±0.39	3.72±0.33	3.07±0.80	3.11±0.24
Coliforms[h]	1.83±0.51	4.27±0.24	0.95±0.92	0.57±0.51	0.00	0.00	0.00	0.00
S. macedonicus[i]	ND	ND	ND	ND	ND	ND	ND	ND
Cheese B								
Thermophilic lactobacilli[j]	6.73±0.56	7.85±0.80	7.19±0.42	6.04±0.49	6.35±1.14	6.16±0.70	6.11±0.48	6.09±1.14
NSLAB[k]	7.19±0.70	8.27±0.65	7.61±0.39	7.03±0.69	6.80±0.78	6.62±0.94	6.94±0.69	6.59±0.39
Mesophilic cocci[l]	7.40±1.20	8.45±0.72	7.23±0.38	5.29±0.65	5.55±0.71	5.77±1.08	6.12±1.29	6.11±0.79
Thermophilic cocci[m]	7.69±0.35	8.66±1.02	7.88±0.34	5.72±0.88	5.86±1.11	5.30±0.75	5.26±0.08	5.59±0.74
Enterococci[n]	2.35±0.54	3.46±0.49	2.07±0.93	0.47±0.81	0.47±0.40	0.23±0.40	0.57±0.51	0.23±0.40
Micrococci[o]	2.37±0.65	2.87±0.23	2.42±0.26	3.58±0.65	3.96±0.67	3.21±0.40	3.47±0.71	2.88±0.32
Coliforms[p]	2.39±0.91	3.77±0.95	1.13±0.99	0.00	0.00	0.00	0.00	0.00
S. macedonicus[q]	6.62±0.18	7.31±0.29	6.03±0.57	2.79±0.45	3.17±0.50	3.50±0.92	3.90±0.26	3.12±0.72
Cheese C								
Thermophilic lactobacilli[r]	7.40±0.40	8.31±0.30	6.11±0.52	7.00±0.48	6.47±0.83	6.65±0.98	6.13±0.66	6.24±0.91
NSLAB[s]	7.44±0.38	8.83±0.21	6.72±0.20	7.36±0.43	6.75±0.86	6.93±0.82	6.32±0.81	6.26±0.92
Mesophilic cocci[t]	5.75±0.72	8.54±0.57	5.84±0.59	6.64±0.99	6.97±1.11	6.05±1.23	5.42±0.49	5.44±1.13
Thermophilic cocci[u]	7.47±0.46	8.86±0.12	6.80±027	7.18±0.56	5.58±0.41	6.44±0.72	5.51±0.61	5.98±0.78
Enterococci[v]	1.89±0.76	5.84±1.10	4.09±0.81	6.03±0.52	5.53±0.65	5.71±0.88	4.37±0.79	5.11±1.42
Micrococci[w]	3.89±0.83	4.71±0.05	3.01±0.28	5.21±0.76	6.34±0.48	5.40±0.46	4.03±0.71	3.42±0.57
Coliforms[x]	3.42±1.36	8.00±0.28	2.73±0.41	1.61±0.82	0.00	0.00	0.00	0.00
S. macedonicus[y]	6.61±1.54	8.33±0.80	5.34±1.22	6.24±0.91	6.03±0.47	4.88±1.77	3.97±1.96	3.85±1.67

[a]Cheese A: prepared with the commercial starter culture solely. Cheese B: prepared with the combination of the commercial starter culture and *S. macedonicus* ACA-DC 198. Cheese C: prepared with *S. macedonicus* ACA-DC 198 as sole starter culture. B1: unripened Baski; B2: ripened Baski; K0: unripened Kasseri; K7: Kasseri day 7; K15: Kasseri day 15; K30: Kasseri day 30; K60: Kasseri day 60; K90: Kasseri day 90. ND: not detected

[b]Significant differences between Cheese C and Cheeses A and B in K0 ($P = 0.0194$).

[c]Significant differences between Cheese C and Cheeses A and B in K0 ($P = 0.0259$).

[d]No significant differences among Cheeses A, B and C.

[e]Significant differences between Cheese C and Cheeses A and B in K0 ($P = 0.0314$).

[f]Significant differences between Cheese C and Cheeses A and B in B2 ($P = 0.0296$), K0 ($P = 0.0129$), K7 ($P = 0.0002$), K15 ($P = 0.0002$), K30 ($P = 0.0001$), K60 ($P = 0.0002$) and K90 ($P = 0.0005$).

[g]Significant differences between Cheese C and Cheeses A and B in B2 ($P = 0.0004$), K7 ($P = 0.0308$), K15 ($P = 0.0023$) and K30 ($P = 0.0013$).

[h]Significant differences between Cheese C and Cheeses A and B in B2 ($P = 0.0002$)

[i]Significant differences between Cheese B and Cheese C in K7 ($P = 0.0171$) and K15 ($P = 0.0020$).

[j]Significant differences between Cheese C and Cheeses A and B in K0 ($P = 0.0194$).

[k]Significant differences between Cheese C and Cheeses A and B in K0 ($P = 0.0259$).

[l]No significant differences among Cheeses A, B and C.

[m]Significant differences between Cheese C and Cheeses A and B in K0 ($P = 0.0314$).

[n]Significant differences between Cheese C and Cheeses A and B in B2 ($P = 0.0296$), K0 ($P = 0.0129$), K7 ($P = 0.0002$), K15 ($P = 0.0002$), K30 (P = 0.0001), K60 (P = 0.0002) and K90 ($P = 0.0005$).

[o]Significant differences between Cheese C and Cheeses A and B in B2 ($P = 0.0004$), K7 ($P = 0.0308$), K15 ($P = 0.0023$) and K30 ($P = 0.0013$).

[p]Significant differences between Cheese C and Cheeses A and B in B2 ($P = 0.0002$).

[q]Significant differences between Cheese B and Cheese C in K7 ($P = 0.0171$) and K15 ($P = 0.0020$).

[r]Significant differences between Cheese C and Cheeses A and B in K0 ($P = 0.0194$).

[s]Significant differences between Cheese C and Cheeses A and B in K0 ($P = 0.0259$).

[t]No significant differences among Cheeses A, B and C.

[u]Significant differences between Cheese C and Cheeses A and B in K0 ($P = 0.0314$).

[v]Significant differences between Cheese C and Cheeses A and B in B2 ($P = 0.0296$), K0 ($P = 0.0129$), K7 ($P = 0.0002$), K15 ($P = 0.0002$), K30 ($P = 0.0001$), K60 ($P = 0.0002$) and K90 ($P = 0.0005$).

[w]Significant differences between Cheese C and Cheeses A and B in B2 ($P = 0.0004$), K7 ($P = 0.0308$), K15 ($P = 0.0023$) and K30 ($P = 0.0013$).

[x]Significant differences between Cheese C and Cheeses A and B in B2 ($P = 0.0002$).

[y]Significant differences between Cheese B and Cheese C in K7 ($P = 0.0171$) and K15 ($P = 0.0020$).

Table 2
Mean values (±SD) for pH, moisture, ash, salt in moisture (SIM), fat in dry matter (FDM), proteolytic index (PI) in Kasseri cheese samples[a] during ripening

Sample	pH[b]			Moisture (%)	Ash (%)	SIM (%)	FDM (%)	PI (%)
	A	B	C	C	C	C	C	C
B1	6.04 ± 0.08^{a}	5.94 ± 0.07^{a}	6.36 ± 0.08^{a}	54.28 ± 1.64^{a}	2.21 ± 0.02^{a}	0.24 ± 0.05^{a}	53.75 ± 3.23^{a}	5.23 ± 1.38^{a}
B2	5.03 ± 0.06^{b}	4.97 ± 0.07^{b}	5.27 ± 0.02^{b}	42.01 ± 1.34^{b}	2.86 ± 0.12^{b}	0.37 ± 0.11^{a}	54.35 ± 2.17^{a}	6.90 ± 1.14^{b}
K0	5.08 ± 0.09^{b}	$5.08\pm0.12^{b,c}$	5.15 ± 0.14^{b}	44.25 ± 1.23^{b}	3.74 ± 0.22^{c}	2.63 ± 0.53^{b}	51.74 ± 2.84^{a}	5.10 ± 1.14^{a}
K7	5.26 ± 0.06^{b}	$5.30\pm0.13^{c,d}$	5.43 ± 0.06^{c}	35.42 ± 1.62^{c}	4.24 ± 0.20^{d}	3.97 ± 0.34^{c}	52.85 ± 2.01^{a}	6.98 ± 0.89^{b}
K15	$5.35\pm0.14^{b,c}$	5.31 ± 0.12^{d}	5.47 ± 0.05^{c}	34.09 ± 3.67^{c}	4.63 ± 0.28^{e}	4.37 ± 0.62^{d}	51.01 ± 2.46^{a}	8.04 ± 1.44^{c}
K30	5.30 ± 0.13^{b}	$5.27\pm0.04^{c,d}$	$5.55\pm0.05^{c,d}$	32.84 ± 2.57^{c}	4.50 ± 0.30^{e}	5.15 ± 0.96^{d}	53.46 ± 2.66^{a}	8.89 ± 1.31^{d}
K60	5.32 ± 0.12^{b}	$5.26\pm0.04^{c,d}$	$5.55\pm0.06^{c,d}$	31.28 ± 3.53^{c}	4.45 ± 0.29^{e}	4.56 ± 0.88^{d}	53.10 ± 0.69^{a}	10.99 ± 1.75^{d}
K90	$5.67\pm0.19^{a,c}$	5.67 ± 0.03^{a}	5.67 ± 0.07^{d}	32.14 ± 2.94^{c}	4.61 ± 0.13^{e}	5.39 ± 0.92^{d}	51.97 ± 1.36^{a}	9.34 ± 1.70^{d}

[a]Cheese A: prepared with the commercial starter culture solely; Cheese B: prepared with the combination of the commercial starter culture and *S. macedonicus* ACA-DC 198; Cheese C: prepared with *S. macedonicus* ACA-DC 198 as sole starter culture. B1: unripened Baski; B2: ripened Baski; K0: unripened Kasseri; K7: Kasseri day 7; K15: Kasseri day 15; K30: Kasseri day 30; K60: Kasseri day 60; K90: Kasseri day 90. Different superscripts within each column show statistically significant differences ($P<0.05$).

[b]Significant differences between Cheese C and Cheeses A and B in K0 ($P = 0.0012$), K30 (0.0136) and K60 (0.0103).

attributed to the longer than usual Baski ripening time. Moreover, a severe loss of moisture was observed during the first week of Kasseri ripening (from Kasseri 0 to Kasseri 7). This is probably due to the higher than usual temperature applied (21 °C versus ⩽18 °C). Additionally, cheese was left unpackaged in the 21 °C ripening room. Then the moisture in Cheese C slightly decreased; however these changes were not statistically significant. The final value obtained (~33.15%,) is in accordance to the respective legislation (moisture ⩽45%; Greek Codex Alimentarius, 2003), but considerably lower than the one commonly observed (Anifantakis, 1991; Kaminaridis et al., 1995).

The ash content of Cheese C increased until Kasseri 15 and then remained stable until the end of ripening at levels close to those previously reported (Kaminaridis et al., 1995). Similar to ash, the SIM content increased until Kasseri 15 reaching a plateau of ~4.87%. On the other hand, the FDM content remained stable throughout production and ripening, with a value of ~52.78%, which is in accordance to the respective legislation (FDM ⩾ 40%; Greek Codex Alimentarius, 2003). The considerably lower SIM content observed in the present study compared with previous ones (Anifantakis, 1991; Kaminaridis et al., 1999) is probably due the less salt used in the case of Cheese C, namely 1% (w/w) during moulding and 0.26% (w/w) during dry-salting of Kasseri 0.

During Baski ripening a significant increase of the PI was observed, while after Baski scalding the PI decreased. This might be due to the loss of peptides during Baski scalding and kneading, as previously reported by Moatsou et al., (2001). Afterwards (Kasseri 7), the PI increased again until Kasseri 30 and it then remained practically stable (~9.74%) throughout ripening. These values were in agreement with those reported for Kasseri cheese prepared from pasteurized milk (Kaminaridis et al., 1995, 1999), although higher PI values have been reported for raw milk Kasseri cheese (Moatsou et al., 2001).

Urea-PAGE electrophoretic patterns of casein fractions in Cheese C revealed that the degradation of casein started during Baski ripening and continued during aging, thus confirming the results obtained for the PI (Fig. 1). Degradation was more pronounced in the case of α_{s1}-casein. The initial casein degradation during Baski ripening may be attributed to the residual rennet, which is retained in the cheese curd (Fox, 1989) although it should be noted that β-casein is only slightly sensitive to the action of the rennet (Mulvihill & Fox, 1978). During Baski scalding, however, the residual rennet in the cheese curd is inactivated (Fox, 1989), while the same procedure is expected to enhance the action of milk's indigenous proteinase, plasmin (Van den Berg & Exterkate, 1993). Thus, casein degradation after Baski scalding should be attributed to the action of plasmin (Van den Berg & Exterkate, 1993) and/or of microbial enzymes (Fox & McSweeney, 1996), and particularly to the specificity of the proteolytic system of *S. macedonicus* ACA-DC 198, which was used as starter culture in this study.

These results were in accordance with those obtained when arginyl-prolyl-β-naphthylamide was used to detect activity of PepX. In all three cheeses, activity (expressed at $\Delta E/\Delta t$, min^{-1}) was detected in Baski 1 and 2 (0.019 ± 0.003 and 0.024 ± 0.003, respectively), as well as in Kasseri 15 (0.017 ± 0.002), Kasseri 30 (0.016 ± 0.003), Kasseri 60 (0.014 ± 0.002) and Kasseri 90 (0.010 ± 0.002), while no activity was detected in Kasseri 0 and 7. No significant differences were observed among the three cheeses. The activity detected in the initial stages of the production (Baski 1 and 2) as well as the absence of activity in Kasseri 0 and 7 may be attributed to the residual rennet in the cheese curd and its consequent inactivation during Baski

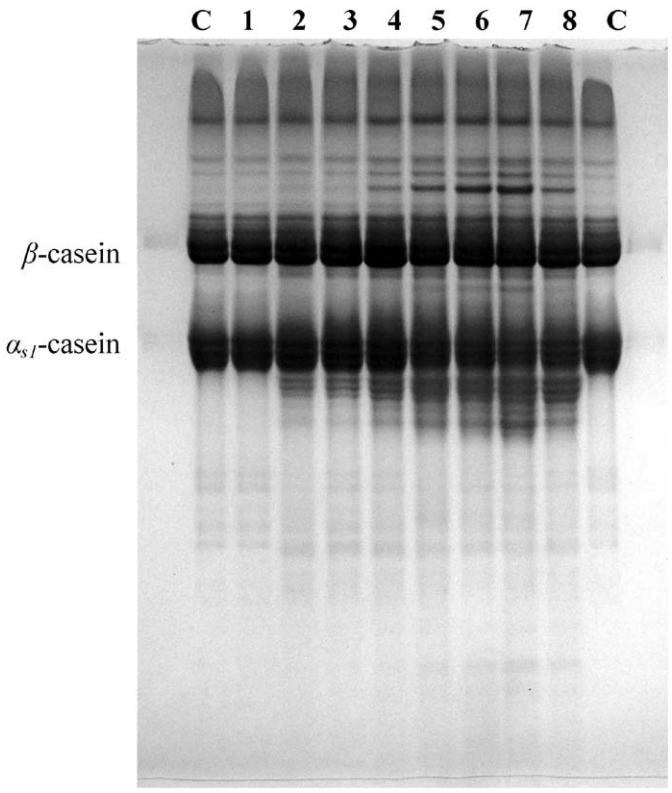

Fig. 1. Urea-PAGE electrophoretic patterns of casein fractions during ripening in Cheese C (prepared with *S. macedonicus* ACA-DC 198 as sole starter culture) during ripening. C: control (isoelectric ovine casein) 1: B1 (unripened Baski); 2: B2 (ripened Baski); 3: K0 (unripened Kasseri); 4: K7 (Kasseri day 7); 5: K15 (Kasseri day 15); 6: K30 (Kasseri day 30); 7: K60 (Kasseri day 60); 8: K90 (Kasseri day 90).

scalding, respectively. The absence of activity, however, in Kasseri 0 and 7 excludes the presence of plasmin in cheese. In this context, the activity detected in Kasseri 15 and thereafter until the end of ripening may be credited to microbial enzymes, among them intracellular peptidases released after cell lysis during cheese ripening. It should be noted here that *S. macedonicus* ACA-DC 198 is known to possess rather low proteolytic activity towards casein. However, among its intracellular peptidases, PepX is the most active (Georgalaki et al., 2000). The release of microbial intracellular peptidases in cheese and their role in cheese ripening has been discussed in the literature before (Chapot-Chartier, Deniel, Rousseau, Vassal, & Gripon, 1994; El-Kholy, El-Soda, Ezzat, & El-Shafei, 1998; Gatti et al., 1999; Wilkinson, Guinee, O'Callaghan, & Fox, 1994).

Changes in the RP-HPLC profile of the WSN fractions in Cheese C were observed throughout ripening (Fig. 2). According to the literature, peptides that elute first from a RP-HPLC column are mainly hydrophilic with a molecular mass < 3000 Da (Belitz & Kaiser, 1993; Kaiser, Belitz, & Fritsch, 1992), while hydrophobic peptides are eluted in the rear region. Large-size peptides are generally eluted later than those with low molecular mass, although differences may exist in the retention time of medium- and large-size peptides with the same amino acid composition but slightly different sequences, due to conformational effects (Lee & Warthesen, 1996; Polo, de Gonzalez Llano, & Ramos, 1992). Results shown in Fig. 2 confirmed the considerable proteolysis, which occurred during Baski ripening, and were obvious from both the urea-PAGE (Fig. 1) and the determination of the PI (Table 2). They further verified the loss of nitrogeneous fractions during scalding, which was recorded in the determination of the PI and was more severe for the peptides eluted between 40 and 70 min. Practically, the phenomenon of proteolysis restarted after Baski scalding. Thereafter, changes observed in the most hydrophilic area (10–40 min) were mainly quantitative, while transformations in the more hydrophobic areas 40–70 min and 70–100 min were qualitative as well. The RP-HPLC profile of ripened Kasseri (Kasseri 90) was considerably different from the respective profile of the ripened Baski (Baski 2).

3.3. Detection of macedocin

Using either intact cheese samples or cheese extracts to detect macedocin activity, it was clear that this was present only in Cheeses B and C, where *S. macedonicus* ACA-DC 198 was used as a starter culture (Fig. 3; data shown for Cheese C). Intact cheese samples produced more clear inhibition zones compared with the cheese extracts. This might be due to the distribution of macedocin in the three different phases (water, intermediate and organic) obtained through the extraction. Indeed, all three phases gave inhibition haloes, with the intermediate one giving slightly clearer zones. This might be an indication that macedocin is equally distributed in the solid and water phases of cheese. No activity was detected in whey samples from either initial cheese curd drainage or obtained during Baski scalding. This indicates that the bacteriocin was retained in the cheese curd. Macedocin was produced in the initial stages of cheese making and remained constant throughout ripening, thus confirming the stability of the molecule under the conditions prevailing in Kasseri cheese production (Georgalaki et al., 2002).

The ability of cheese starters to produce bacteriocin in situ has been previously reported by either detecting the bacteriocin activity in cheese or in challenge cheese making experiments (Foulquié Moreno et al., 2003; Nuñez, Rodríguez, García, Gaya, & Medina, 1997; Sulzer & Busse, 1991). However, problems concerning low production levels and instability of bacteriocins in certain food environments still need to be addressed (Leroy & De Vuyst, 2004). It is generally accepted that the use of bacteriocin-producing cultures in food is of considerable advantage over using purified bacteriocin preparations. The latter application requires extensive and costly purification schemes, toxicology tests, and may suggest a non-natural image of additives, for instance with respect to the applied concentrations.

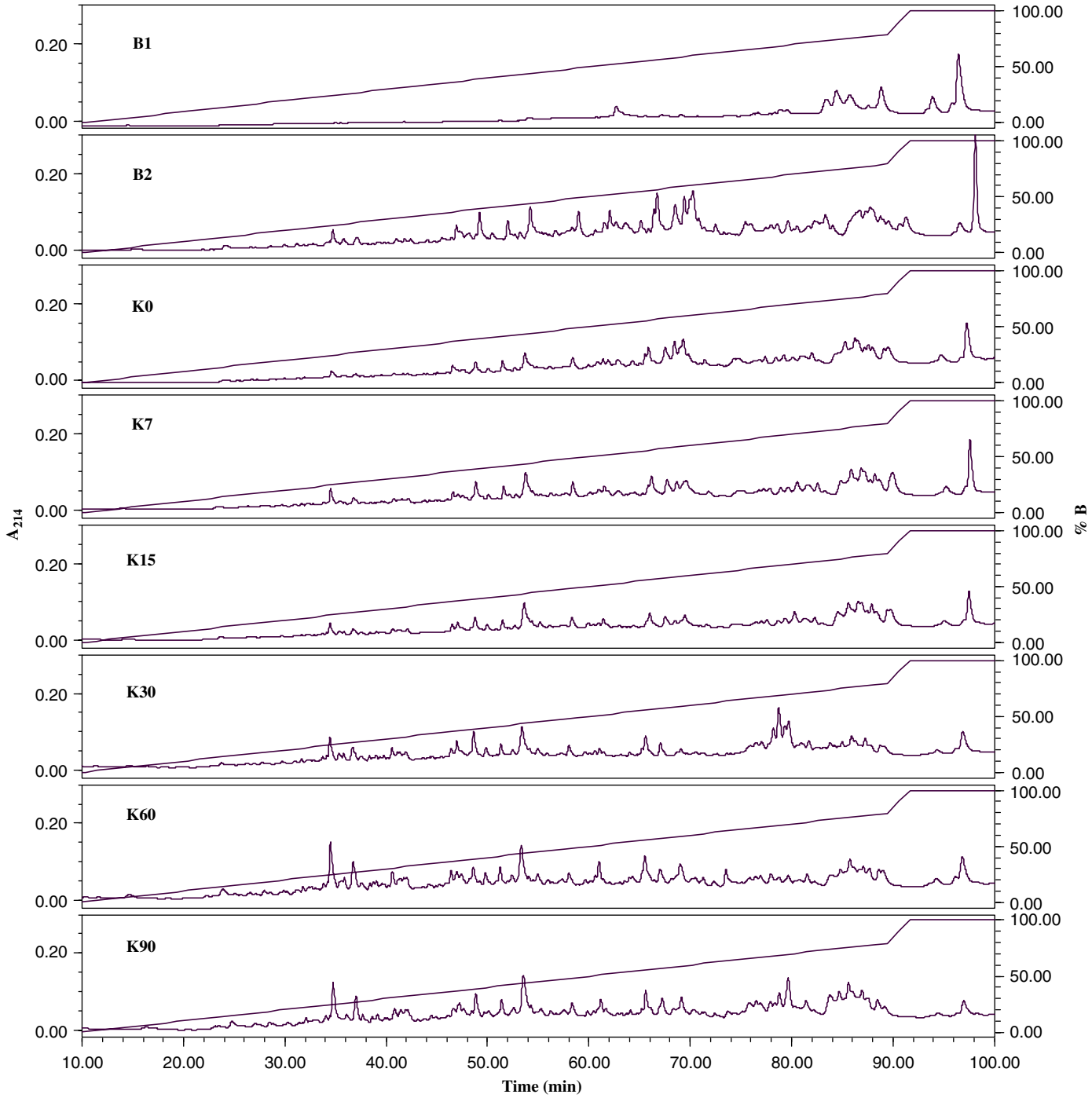

Fig. 2. Reverse-phase (RP)-HPLC profiles of the water soluble nitrogen (WSN) extracts in Cheese C (prepared with *S. macedonicus* ACA-DC 198 as sole starter culture) during ripening. Elution was performed at room temperature with 100% solvent A for 10 min, then with a gradient of 0–80% solvent B over 80 min and finally with 100% solvent B for 10 min.The absorbance of the eluate was monitored at 214 nm. B1: unripened Baski; B2: ripened Baski; K0: unripened Kasseri; K7: Kasseri (day 7); K15: Kasseri (day 15); K30: Kasseri (day 30), K60: Kasseri (day 60); K90: Kasseri (day 90).

3.4. Sensory evaluation

The sensory assessment of the full-ripened Kasseri cheese (90 days) is presented in Table 3. All cheeses were characterized as hard. Kasseri prepared with *S. macedonicus* ACA-DC 198 as sole starter culture (Cheese C) possessed a significantly better color than cheeses A and B. However, cheese prepared with the combination of the commercial starter and *S. macedonicus* (Cheese B) was significantly superior compared to cheeses A and C with respect to texture and taste/aroma, and thus to the total sensory score.

4. Conclusions

In the present study *S. macedonicus* ACA-DC 198 was used both as adjunct and as sole starter culture in Kasseri

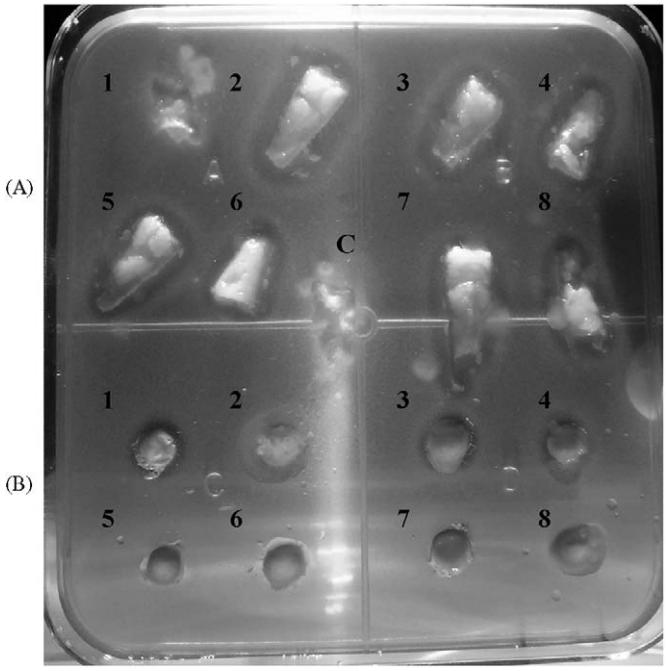

Fig. 3. Macedocin detection in cheese C (with *S. macedonicus* ACA-DC 198 as sole starter culture) during ripening. (A) Cheese samples heated at 80 °C for 10 min; (B) cheese extracts — intermediate phase. 1: B1 (unripened Baski); 2: B2 (ripened Baski); 3: K0 (unripened Kasseri); 4: K7 (Kasseri day 7); 5: K15 (Kasseri day 15); 6: K30 (Kasseri day 30); 7: K60 (Kasseri day 60); 8: K90 (Kasseri day 90). In the center the negative control (Cheese A; prepared with the commercial starter culture solely) is displayed.

Table 3
Sensory evaluation of full-ripened Kasseri cheese (90 days)[a]

Cheese	Color (0–15)	Texture (0–30)	Taste-Aroma (0–55)	Total (0–100)
A	7.21 ± 0.58^{a}	17.98 ± 1.66^{a}	35.12 ± 3.77^{a}	60.31 ± 5.78^{a}
B	6.59 ± 0.67^{a}	21.66 ± 0.91^{b}	40.79 ± 1.22^{b}	69.04 ± 2.32^{b}
C	9.31 ± 0.63^{b}	18.35 ± 0.63^{a}	30.91 ± 2.87^{a}	58.57 ± 2.61^{a}

[a]Values are means (±SD) for three different trials and 15 panelists. Cheese A: prepared with the commercial starter culture solely; Cheese B: prepared with the combination of the commercial starter culture and *S. macedonicus* ACA-DC 198; Cheese C: prepared with *S. macedonicus* ACA-DC 198 as sole starter culture. Different superscripts within each column show statistically significant differences ($P < 0.05$).

cheese making. Based on the overall evaluation of the results obtained from the microbiological, physicochemical and sensorial analyses, it is concluded that *S. macedonicus* ACA-DC 198 can be used as an adjunct in Kasseri cheese production. However, its application as sole starter demands modifications in Kasseri cheese-making technology in order to ensure higher organoleptic quality. On the other hand, the production of macedocin in the initial stages of cheese making and its stability until the end of ripening give confidence for the potential use of *S. macedonicus* ACA-DC 198 as a protective culture for the production of semi-hard and hard cheeses.

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