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Detection of *Streptococcus macedonicus* in Greek cheeses

Marina Georgalaki [a], Eugenia Manolopoulou [a], Rania Anastasiou [a], Marina Papadelli [a,b], Effie Tsakalidou [a,*]

[a] Laboratory of Dairy Research, Department of Food Science and Technology, Agricultural University of Athens, Iera Odos 75, 118 55 Athens, Greece
[b] Department of Agricultural Products' Technology, Technological Education Institute of Kalamata, Antikalamos, 24 100 Kalamata, Greece

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ABSTRACT

The aim of the present work was the detection and enumeration of *Streptococcus macedonicus* in Greek traditional cheeses. A total of 68 traditional cheese samples have been examined. Enumeration was performed by plating on the *S. macedonicus* differential medium Bromothymol Blue *Streptococcus macedonicus* (BBSM). Detection of *S. macedonicus* was confirmed by species-specific polymerase chain reaction (PCR), using as template either cheese DNA or DNA from colonies isolated from the BBSM medium. Counts of *S. macedonicus* on BBSM medium ranged from less than 1 to 6.84 log cfu g^{-1}. *S. macedonicus* has been detected in 15 samples out of the 68 tested, corresponding to 13 out of the 20 different cheese varieties examined, originating from various geographical areas in Greece. This indicates that *S. macedonicus* is widespread in Greek cheeses. However, its presence could not be correlated either with the cheese variety or the cheese manufacturing procedures.

1. Introduction

During a survey of the lactic acid bacteria microbiota of naturally fermented Greek PDO Kasseri cheese, the species *Streptococcus macedonicus*, member of the LAB group, was isolated (De Vuyst & Tsakalidou, 2008; Tsakalidou et al., 1998). This thermophilic and homofermentative dairy streptococcus belongs to the *Streptococcus bovis/Streptococcus equinus* complex and possesses a food-grade and non-pathogenic status (Poyart et al., 2002). *Streptococcus waius* described by Flint et al. (1999) for isolates from stainless steel dairy apparatus exposed to skim milk is meanwhile considered as synonymous to *S. macedonicus* (Manachini et al., 2002; Papadelli et al., 2003; Poyart et al., 2002). Interestingly, although Schlegel et al. (2003) proposed to split *Streptococcus gallolyticus* into three subspecies, namely *S. gallolyticus* subsp. *gallolyticus*, *S. gallolyticus* subsp. *pasteurianus* and *S. gallolyticus* subsp. *macedonicus*, the Subcommittee of the International Committee on Systematics of Prokaryotes, which deals with the taxonomy of staphylococci and streptococci, recommended that *S. gallolyticus* should remain a separate species (Subcommittee of the International Committee on Systematics of Prokaryotes, 2003).

The phenotypic description of *S. macedonicus* allows its clear discrimination from its closest phylogenetic neighbour, *S. bovis*, principally by its failure to produce a blackening reaction in medium containing aesculin, and from other dairy streptococci, in particular *Streptococcus* thermophilus, with respect to the utilization of maltose and cellobiose by *S. macedonicus* strains (Manachini et al., 2002; Tsakalidou et al., 1998). The species *S. macedonicus* is nutritionally fastidious (e.g. multiple amino acid auxotrophies), requiring the addition of appropriate nitrogen sources (e.g. yeast extract) to grow in MRS medium and to perform well in milk media (Georgalaki et al., 2002; Georgalaki et al., 2000; Van den Berghe et al., 2006). Growth is sustained between 25 and 45 °C and between pH 5.0 and 8.5 (Van den Berghe et al., 2006).

Strains of *S. macedonicus* are weak milk acidifiers and posses generally low proteolytic and citrate-catabolizing activity, but moderate lipolytic activity. Interestingly, *X*-prolyl-dipeptidyl aminopeptidase is their dominant peptidase activity (Georgalaki et al., 2000; Lombardi et al., 2004) while several strains produce exopolysaccharides (Vincent et al., 2001), or exhibit antimicrobial activity (Georgalaki et al., 2000; Zoumpopoulou et al., 2008). The strain *S. macedonicus* ACA-DC 198 produces a food-grade lantibiotic, named macedocin, with anti-clostridial activity (Georgalaki et al., 2002). These physiological and technological properties make *S. macedonicus* a multi-functional candidate co-culture for dairy food applications, as suggested by Lombardi et al. (2004) and confirmed by fermentations simulating Kasseri cheese technology (Poirazi et al., 2007) as well as experimental Kasseri cheese making trials (Anastasiou et al., 2007).

Since its description by Tsakalidou et al. (1998), *S. macedonicus* has been detected in various European cheeses (Andrighetto et al., 2002; Aponte et al., 2008; Baruzzi et al., 2002; Callon et al., 2004; Lombardi et al., 2004; Pacini et al., 2006; Poznanski et al., 2004) and in one Mexican fermented maize beverage (Díaz-Ruiz et al., 2003). However, no further data exist in the literature about the

* Corresponding author. Tel.: +30 210 529 4661; fax: +30 210 529 4672.
E-mail address: et@aua.gr (E. Tsakalidou).

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occurrence of *S. macedonicus* in Greek cheeses other than Kasseri cheese. Thus, the aim of the present study was to detect and enumerate *S. macedonicus* in Greek cheeses and try to correlate its occurrence with the cheese variety, cheese type, as well as the geographical distribution of cheeses all over Greece.

2. Materials and methods

2.1. Media and strains

For the selective enumeration of *S. macedonicus* the SM medium (*Streptococcus macedonicus* medium) described by Pacini et al. (2006), slightly modified (BBSM medium, Bromothymol Blue *Streptococcus macedonicus*), was used. More precisely, the BBSM medium consisted of 35 g L^{-1} CASF (Count Agar Sugar free, Oxoid, Basingstoke, UK), 2.5 g L^{-1} yeast extract (Biokar Diagnostics, Beauvais, France), 0.5 g L^{-1} potassium ascorbate (Himedia Laboratories, Mumbai, India), 19 g L^{-1} glycerolphosphate (Sigma-Aldrich, Steinheim, Germany) and 0.015 g L^{-1} bromothymol blue (Hopkin & Williams Ltd, London, UK), instead of the bromocresol purple used in the original SM medium. After sterilization (121 °C, 1 atm, 15 min), 5 g L^{-1} of filter-sterilized raffinose (Serva, Heidelberg, Germany) were added.

A total of 19 LAB strains were used to evaluate the selectivity of the BBSM medium. Eight *S. macedonicus* strains, namely ACA-DC 190, 193, 198, 205, 206^T, 243, 246 and 247, along with 11 strains belonging to other LAB species, namely *S. bovis* LMG 8518^T, *S. gallolyticus* LMG 16802^T, *Streptococcus pasteurianus* LMG 22273^T, *S. thermophilus* LMG 6896^T, ACA-DC 4 and ACA-DC 6, *Enterococcus durans* LMG 10746^T, *Enterococcus faecalis* LMG 7937^T, *Enterococcus faecium* LMG 11423^T, and *Lactobacillus rhamnosus* ACA-DC 3430 and LMG 6400^T (ACA-DC: Agricultural College of Athens-Dairy Collection, Athens, Greece, and LMG: Collection of the Laboratorium voor Microbiologie, Gent, Belgium). All strains were streaked on the BBSM medium and incubated at either 42 or 44 °C, under aerobic or anaerobic conditions (anaerobic incubator, 8% CO_2) for 48 h.

2.2. Cheese samples

A total of 68 cheese samples representing 20 Greek cheese varieties have been examined. Cheese samples were obtained either from super markets or family producers. They belonged to five cheese types grouped according to the manufacturing conditions, namely, (i) white brine cheeses (15 samples): Feta, Kalathaki, Sfela and Touloumotyri, (ii) soft cheeses (four samples): Katiki and Kopanisti, (iii) semihard cheeses (14 samples): Kasseri and Mastelo, (iv) hard cheeses (30 samples): Arseniko, Formaella, Graviera, Kefalograviera, Kefalotyri, Ladotyri, Metsovone, San Giorgio, and San Michali, and (v) whey cheeses (five samples): Manouri, Mizithra and Xinomizithra. Eight samples were produced from raw milk without the addition of starters (artisanal cheeses). These belonged to 4 cheese varieties (Feta, four samples; Kalathaki, two samples; Arseniko, one sample; Mizithra, one sample).

2.3. Determination of bacterial counts in cheese

Cheese samples (10 g) were homogenized in 90 mL of sodium citrate 2% w/v (Fluka, Buchs, Switzerland). Decimal dilutions were prepared in Ringer solution and plated on BBSM medium. Inoculated plates were incubated anaerobically in a CO_2 incubator (8% CO_2) at 44 °C for 48 h. Total counts as well as yellow colonies were enumerated, the latter corresponding to the presumptive *S. macedonicus* counts. In order to obtain the *S. macedonicus* counts on BBSM, species-specific polymerase chain reaction (PCR) was performed to a representative number of yellow colonies, equal to the square root of the number of the presumptive *S. macedonicus* colonies.

2.4. Identification of S. macedonicus by species-specific Polymerase Chain Reaction (PCR)

Cheese DNA extraction was performed according to Pacini et al. (2006) with the following modification. The isolated DNA was purified with the synthetic InstaGene™ Matrix (Bio-Rad Laboratories, Hercules, CA, USA). More specifically, 200 μL of the Matrix were added to the pellet and incubated at 56 °C for 30 min and then vortexed for 10 s, heated at 100 °C for 8 min, vortexed again, and centrifuged at 12,000 *g* for 3 min. The DNA concentration of the supernatant was measured photometrically at 260 nm (NanoDrop® ND-1000 Spectrophotometer, Wilmington, Delaware USA).

S. macedonicus species-specific PCR assay was performed according to Papadelli et al. (2003) using as template either DNA extracted from cheese (150 ng, cheese DNA PCR assay) or DNA extracted from presumptive *S. macedonicus* single yellow colonies (colony DNA PCR assay) picked from the BBSM medium.

3. Results and discussion

In the present study 68 cheese samples have been examined for the presence of *S. macedonicus*. These derived from various areas in Greece geographically well distributed, were prepared either from pasteurized milk with the addition of starters or from raw milk without the addition of starters (artisanal cheeses) and represented 20 Greek cheese varieties and 5 cheese types. For this, both culture dependent, namely enumeration on selective medium and identification of colonies by species-specific PCR, as well as culture independent, namely species-specific PCR using as template DNA isolated directly from the cheese samples, approaches were applied.

In the last decade it was shown that classical microbiological techniques do not accurately detect microbial diversity (Giraffa & Neviani, 2001). It is well documented for example, that stressed or injured cells do not recover in selective media and that cells present in low numbers are very often inhibited by microbial populations numerically more abundant. As a consequence, an increasing interest in the development and use of culture independent techniques emerged. A variety of new methods has been developed to directly characterise the microorganisms in particular habitats without the need for enrichment or isolation. Typically, these strategies examine the total microbial DNA (or RNA) derived from mixed microbial populations to identify individual constituents. This approach eliminates the necessity for strain isolation, thereby negating the potential biases inherent to microbial enrichment. Studies, which employed such direct analysis, have repeatedly demonstrated a tremendous variance between cultivated and naturally occurring species, thereby dramatically altering our understanding of the true microbial diversity present in various habitats.

In the present study, in order to optimize the selectivity of the SM medium reported by Pacini et al. (2006), the pH indicator bromocresol purple (pH range 5.2–6.8) was replaced by bromothymol blue (pH range 6.0–7.6), due to its different pH range sensitivity. This was expected to allow a more efficient detection of *S. macedonicus*, which is known for its low rate of acid production (Georgalaki et al., 2000), despite the concomitant decrease of the medium's selectivity and the appearance of higher number of false positive colonies.

The selectivity of the above medium was evaluated by streaking 19 LAB strains, representing nine different species, including *S. macedonicus*. The effect of growth temperatures (42 and 44 °C) and aeration conditions (aerobic and anaerobic) on the formation of yellow colonies were also considered. *S. macedonicus* strains did not

seem to be significantly affected by the various growth conditions, with the exception of the aerobic growth at 44 °C, where only one strain grew and produced yellow colonies. On the contrary, growth conditions affected mostly the other LAB tested (data not shown). Conditions similar to those reported by Pacini et al. (2006) have been finally chosen (temperature of 44 °C and anaerobic conditions), in order to promote the best growth of *S. macedonicus*, favor yellow colonies formation, and weaken the growth of the other species. Under these growth conditions, among the 8 LAB species tested, *S. gallolyticus*, *S. pasteurianus* and one *S. thermophilus* strain formed yellow colonies, while the rest, either grew without forming the characteristic yellow colonies (*S. bovis*, *E. faecium*, *E. faecalis*, *L. rhamnosus*), or did not grow at all (two *S. thermophilus* strains, *E. durans*). These results are not in complete agreement with the findings of Pacini et al. (2006), who reported that *S. thermophilus* did not grow on the SM medium, while *S. gallolyticus*, *S. lutetiensis*, *S. infantarius*, *S. bovis*, *L. rhamnosus* and most of the enterococcal strains tested were able to grow on the SM medium, with only the streptococci being however able to produce the typical yellow halo around the colony. It is obvious that, although the use of bromothymol blue slightly reduced the selectivity of the SM medium, yellow colony formation is not only species but also strain dependent, and thus yellow colonies' counts do not necessarily represent the real numbers of *S. macedonicus* in cheese. In this sense the use of another method is needed to confirm the identity of the yellow colonies.

All 68 samples examined, apart from three (Feta, Kalathaki and Katiki; one sample each), gave colonies on the BBSM medium, ranging from 2.48 to 9.75 log cfu g^{-1}. Among them, 28 gave yellow colonies with counts ranging from 2.0 to 6.97 log cfu g^{-1}, indicating the presence of *S. macedonicus*. However, species-specific PCR performed with DNA isolated from a well representative number of yellow colonies confirmed the presence of *S. macedonicus* in only 12 out of the 28 cheese samples (Table 1). On the basis of the colony DNA PCR results, *S. macedonicus* levels were determined and found to range from 1.82 to 6.84 log cfu g^{-1}. Among these 12 samples, only seven were found to be positive by the cheese DNA PCR assay as well. Concerning the remaining 16 samples, which gave yellow colonies but negative results with the colony DNA PCR assay, only two gave positive PCR results using the cheese DNA PCR assay (Table 1). Finally, among the 28 samples, which gave yellow colonies, 14 were negative for the presence of *S. macedonicus* by both cheese and colony DNA PCR (Tables 1 and 2). Concerning the 40 samples, which did not produce either any colonies (three samples) or produced yellow colonies (37 samples) on the BBSM medium, only one, a Mizithra sample, was found to give positive results with the cheese DNA PCR assay. Thus, the presence of *S. macedonicus* was confirmed in 15 out of the 68 cheese samples examined, with the colony DNA PCR assay being slightly more efficient in 12 samples and the cheese DNA PCR in 10 samples, while in seven out of the 15 positive samples both PCR assays were successful (Table 2). Improvement of cheese DNA extraction protocol could overcome the limitation. Indeed, species-specific PCR is a rapid and reliable molecular technique for the characterisation of bacterial communities without colony isolation. However, the sensitivity of PCR in foods can be reduced due to the complexity of the food matrix and the presence of many PCR inhibitors. Many substances have been proven to be PCR inhibitory (Rossen et al., 1992) and for this reason appropriate DNA extraction protocols, chosen on the basis of the food matrix under study, should be optimized and used.

Regarding the 20 cheese varieties examined in this study, *S. macedonicus* was detected in 13. In the remaining seven cheese varieties, comprising 17 samples, *S. macedonicus* was not detected neither by cheese nor by colony DNA PCR (no matter if any colonies appeared on the BBSM medium). These included Arseniko (2 samples), Kalathaki (3 samples), Katiki (2 samples), Kefalograviera (4 samples), Manouri (2 samples), Sfella (2 samples) and Xinomizithra (2 samples). The cheese manufacturing conditions did not influence significantly the presence of *S. macedonicus*, as it was detected in all five cheese types tested, and more specifically in two out of 15 white brine, one out of four soft, two out of 14 semihard, nine out of 30 hard and one out of five whey cheeses (Table 1). Only pH seemed to affect the presence of *S. macedonicus* in cheese. More specifically, *S. macedonicus* was not detected in all Feta cheese samples apart from one (pH 4.56), the two Katiki samples (pH 4.20–4.53), one (pH 4.81) out of the two Kopanisti samples and the two Xinomizithra samples (pH 4.15–4.60). Finally, among the eight artisanal samples, only one (the

Table 1
Cheese samples where the presence of *Streptococcus macedonicus* was confirmed by cheese and/or colony DNA PCR

Cheeses[a]	Total BBSM counts (log cfu g^{-1})	Number of samples with yellow colonies on BBSM (log cfu g^{-1})[b]	Number of samples positive by cheese DNA PCR	Number of samples positive by colony DNA PCR (log cfu g^{-1})[c]	Number of samples negative by both cheese and colony DNA PCR[d]
White brine cheeses (2/15)		**2**	**1**	**1**	**0**
Feta (1/9)	<1–8.30	1 (3.30)	0	1 (2.99)	0
Touloumotyri (1/1)	7.20	1 (2.30)	1	0 (<1)	0
Soft cheeses (1/4)		**1**	**1**	**1**	**0**
Kopanisti (1/2)	5.34–8.08	1 (6.97)	1	1 (6.36)	0
Semihard cheeses (2/14)		**7**	**2**	**2**	**5**
Kasseri (1/12)	3.00–7.78	6 (2.54–6.34)	1	1 (1.93)	5
Mastelo (1/2)	6.76–7.45	1 (5.70)	1	1 (5.70)	0
Hard cheeses (9/30)		**14**	**5**	**8**	**5**
Formaella (1/2)	8.08–9.75	1 (6.84)	0	1 (6.84)	0
Graviera (1/9)	2.60–7.85	3 (2.30–4.08)	0	1 (1.82)	2
Kefalotyri (3/5)	5.54–7.79	4 (3.70–5.34)	2	3 (<1–5.16)	1
Ladotyri (1/3)	2.48–6.95	3 (2.00–2.70)	1	0 (<1)	2
Metsovone (1/2)	4.45–5.00	1 (5.00)	0	1 (4.70)	0
San Giorgio (1/1)	7.93	1 (4.60)	1	1 (4.60)	0
San Michali (1/2)	6.28–7.08	1 (5.70)	1	1 (5.40)	0
Whey cheeses (1/5)		**0**	**1**	nyc	–
Mizithra (1/1)	5.36	0	1	nyc	–

[a] Total number of samples positive by cheese and/or colony DNA PCR per total number of samples tested.
[b] Presumptive *S. macedonicus* counts on BBSM.
[c] *S. macedonicus* counts on BBSM as determined by colony DNA PCR.; (nyc): no yellow colonies on BBSM.
[d] Additionally, three Kefalograviera samples and one Arseniko sample, which gave yellow colonies, were negative by both cheese and colony DNA PCR; (–): not applicable..

Table 2
Efficacy of enumeration on BBSM and species-specific PCR for the detection of *Streptococcus macedonicus* in Greek cheese samples

Number of cheese samples	Presence of yellow colonies on BBSM	Cheese DNA PCR	Colony DNA PCR
39	−	−	nyc
1	−	+	nyc
5	+	−	+
14	+	−	−
7	+	+	+
2	+	+	−
Total 68	28	10	12

whey cheese Mizithra) proved to be *S. macedonicus* positive by PCR, while among the 60 cheese samples produced from pasteurized milk 14 were found to be positive.

Since its description by Tsakalidou et al. (1998), *S. macedonicus* has been detected in various European cheeses but also in other food and non-food environments. *S. macedonicus* seems to be widespread in Italian cheeses, such as Asiago and Fontina (Andrighetto et al., 2002; Lombardi et al., 2004; Pacini et al., 2006), Montasio (Andrighetto et al., 2002), Monte Veronese (Andrighetto et al., 2002; Lombardi et al., 2004), Morlacco, Mozzarella, Ragusano and Spressa (Lombardi et al., 2004), Scamorza Altamurana (Baruzzi et al., 2002; Poznanski et al., 2004), Nostrano di Primiero (Poznanski et al., 2004) and Provolone del Monaco (Aponte et al., 2008). These cheeses derived from different areas all over Italy, they were prepared either with the addition of starters or from raw milk and were subjected to different thermal treatments. These all suggest that the occurrence of *S. macedonicus* in Italian cheeses is not dependent on geographical origin or cheese manufacturing conditions, which is in agreement with the findings of the present study. *S. macedonicus* was also detected in Salers cheese, a French traditional raw milk cheese (Callon et al., 2004). Interestingly, *S. macedonicus* was also detected via ribotyping in Pozol, a Mexican fermented maize beverage (Díaz-Ruiz et al., 2003).

4. Conclusions

This study reveals the occurrence of *S. macedonicus* in various Greek cheeses corresponding to different cheese types and varieties, independently of the geographical origin. Detection of *S. macedonicus* in both artisanal samples and samples produced from pasteurized milk indicates that its presence may be attributed not only to raw milk or deficient pasteurization, but also to conditions of hygiene prevailing during cheese making. On the other hand, survival to heat treatment is possible, as the presence of *S. macedonicus* has been confirmed in Kasseri cheese until the end of ripening, indicating its survival during scalding of the curd, at 75–80 °C for 10 min (Anastasiou et al., 2007; Poirazi et al., 2007). Additionally, it seems that occurrence of this microorganism in cheese is affected by the cheese pH.

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