Short communication: Technological and genotypic comparison between *Streptococcus macedonicus* and *Streptococcus thermophilus* strains coming from the same dairy environment

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ABSTRACT

The species *Streptococcus thermophilus* is widely used for the preparation of several dairy products, and its technological contribution is clear. On the other hand, although *Streptococcus macedonicus* was first described more than 10 yr ago and, despite the scientific interest around this issue, the exact role of *Strep. macedonicus* in cheese making has yet to be clarified. In this study, 121 strains belonging to both species and isolated from the same dairy environment were genetically characterized by random amplification of polymorphic DNA (RAPD)-PCR and compared for the main biochemical features of technological interest, such as acid production, galactose utilization, citrate metabolism, exopolysaccharide production, and lipolytic, ureolytic, exocellular proteolytic, and decarboxylase activities. Analysis by RAPD-PCR highlighted a remarkable genotypic heterogeneity among strains in both species, and, at a similarity level of 78%, all the isolates and reference strains of *Strep. thermophilus* grouped together and were well separated from the strains of *Strep. macedonicus*, confirming that these 2 species are different microbial entities. Comparison between genetic and phenotypic or biotechnological data did not reveal any relationships.

Key words: *Streptococcus thermophilus*, *Streptococcus macedonicus*, random amplification of polymorphic DNA (RAPD)-PCR, Provolone del Monaco cheese

Short Communication

During a survey on the evolution of the lactic acid bacteria microbiota throughout the manufacturing and ripening process of Italian Provolone del Monaco cheese, 121 strains belonging to the species *Streptococcus macedonicus* and *Streptococcus thermophilus* were

isolated (Aponte et al., 2008). The genus *Streptococcus* includes pathogenic species widely distributed in humans and animals, together with “generally recognized as safe” (GRAS) species found in raw milk, dairy products, and plant materials. The species *Strep. thermophilus* is widely used in the preparation of several dairy products, such as fermented milks, yogurt, and cheeses. The role of *Strep. thermophilus* in milk fermentation is related not only to the production of lactic acid that allows a pH decrease of the medium, but also to several other important technological properties that must be considered when selecting strains for starter preparation. *Streptococcus macedonicus* possesses a food-grade and nonpathogenic status (Poyart et al., 2002). Strains of *Strep. macedonicus* are weak milk acidifiers and generally possess low proteolytic and citrate-catabolizing activities but moderate lipolytic activity (De Vuyst and Tsakalidou, 2008). Several strains produce exopolysaccharides (Vincent et al., 2001) or exhibit antimicrobial activity (Georgalaki et al., 2000; Zoumpopoulou et al., 2008). These physiological and technological properties make *Strep. macedonicus* a multi-functional candidate coculture for dairy food applications. Since its description by Tsakalidou et al. (1998), *Strep. macedonicus* has been detected in various cheeses (Andrighetto et al., 2002; Baruzzi et al., 2002; Callon et al., 2004; Lombardi et al., 2004; Poznanski et al., 2004; Pacini et al., 2006; Aponte et al., 2008; Georgalaki et al., 2009) and in one Mexican fermented maize beverage (Díaz-Ruiz et al., 2003). However, the exact role of *Strep. macedonicus* in cheese making has yet to be elucidated.

In this study, 71 strains of *Strep. macedonicus* and 50 strains of *Strep. thermophilus* from the same food ecosystem (Aponte et al., 2008) were genetically characterized and compared for the main biochemical features of technological interest (Table 1). Acid production in pasteurized cow milk after 6 h at 37°C (1% vol/vol inoculum) was evaluated according to Georgalaki et al. (2000), whereas the utilization of galactose was tested as described by Moschetti et al. (1998). Determination of lipolytic, ureolytic, and exocellular proteolytic

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Table 1. *Streptococcus* spp. strains isolated during manufacture and ripening of Provolone del Monaco cheese (Aponte et al., 2008)

Species	Sample ¹								Medium ²
	M	C ₀	C _F	C _S	C _B	C ₁	C ₅	C ₉	
<i>Strep. thermophilus</i>	13 (–5) ³	305, 307, 308, 309, 310pg, 312, 314 (–6); 315 (–7)	57 (–7); 68, 69 (–8)	75, 80 (–7); 82a, 82b (–8)	242, 243 (–7); 246, 247 (–8)		381 (–5)		M ₃
<i>Strep. thermophilus</i>	26, 27, 30, 33 (–5); 49, 50 (–6)	34, 35, 40, 41, 43, 44, 46, 48, 49, 50 (–6); 53 (–7)	83, 84, 85, 87 (–7); 92, 94, 95 (–8)	101, 103, 104, 105 (–7)	230, 232 (–7)	333 (–6)			M ₄
<i>Strep. macedonicus</i>		310pb (–6)	56, 58, 59 (–7); 65, 67 (–8)	70, 71, 72, 73, 74, 78 (–7); 81 (–8)	237, 238, 239, 240, 241 (–7); 245 (–8)	371 (–6); 373, 374, 376, 377, 378 (–7)	380, 382 (–5); 383, 384, 385, 386, 387, 388, 389 (–6); 390 (–7)	448 (–5)	M ₃
<i>Strep. macedonicus</i>		42, 45 (–6); 51 (–7)	88, 90, 96, 97 (–8)	98, 100 (–7); 106, 108, 109, 111 (–8)	227, 228, 229, 231, 234 (–7)	331 (–6); 335 (–7)	392, 393, 394, 395, 396, 397 (–5); 399, 400 (–6)	456, 458, 460, 461, 462 (–5); 464 (–6)	M ₄
<i>Strep. macedonicus</i>							414 (–5)		SB
<i>Strep. macedonicus</i>					282 (–6)				I

¹Samples were collected during cheesemaking of Provolone del Monaco: M = milk before cheesemaking; C₀ = curd after cooking; C_F = curd at the end of fermentation; C_S = cheese after stretching; C_B = cheese after brine; C₁ = cheese after 1 mo of ripening; C₅ = cheese after 5 mo of ripening; C₉ = cheese after 9 mo of ripening.

²Media and incubation conditions. M₃ = M17 agar incubated at 30°C; M₄ = M17 agar incubated at 44°C; SB = Slanetz & Bartley agar incubated at 37°C; I = Isolini agar (Isolini et al., 1990) incubated in anaerobiosis at 37°C.

³Strains of each sample with isolation dilution in parentheses. Reference strains used in this study included *Strep. thermophilus* DSM20259, DSM20479, DSM 8713, and DSM20617 obtained from DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

activities was carried out according to Georgalaki et al. (2000), Lanyi (1987), and Franciosi et al. (2009), respectively. Screening for decarboxylase activity was performed as reported by Joosten and Northolt (1989) with ornithine, histidine, and tyrosine (Sigma, Milan, Italy) as decarboxylase substrates. Strains were screened for citrate metabolism using Simmons citrate agar (Oxoid, Basingstoke, UK). Exopolysaccharide (EPS) production was tested in ST1 medium (Gancel and Novel, 1994) supplemented with filter-sterilized solutions of different carbohydrates (1% vol/vol) and ruthenium red (0.08 g/L). Glucose, galactose, lactose, saccharose, fructose, and skim milk powder were added one by one.

For genotypic characterization, DNA from pure cultures was extracted according to a procedure developed ad hoc. Briefly, cells grown in 10 mL of de Man, Rogosa, and Sharpe medium to the exponential phase were washed in saline-Tris-EDTA (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.00), suspended in 250 μ L of lysis solution (50 mM Tris-HCl, 5 mM EDTA, pH 8.00) containing 1.6 mg/mL of lysozyme, 40 U/mL of mutanolysin, and 0.4 mg/mL RNase, and incubated overnight at 37°C. Twenty-five microliters of 25% (vol/vol) SDS and 2 μ L of pronase (20 mg/mL) were added, and incubation was allowed to proceed at 37°C for 1 h. A quantity of sodium acetate (5 M) equal to the volume of the water phase was added, and, after cooling at 0°C, DNA was precipitated by centrifugation (14,000 $\times$ g for 10 min at 4°C) with 0.7 volumes of isopropanol. Finally, the pellet was washed with 70% ethanol and, after drying, nucleic acids were dissolved in 50 μ L of rehydration buffer (Promega, Madison, WI; 55°C for 1 h). Concentration of DNA of samples was estimated by Nanodrop (Thermo Scientific, Wilmington, DE). All reagents, unless otherwise noted, were from Sigma.

Random amplification of polymorphic DNA (RAPD)-PCR reactions were performed with primer XD9 (5'-GAAGTCGTCC-3'). Temperature profile was as follows: 40 cycles consisting of 94°C for 1 min, 31°C for 1 min, and 72°C for 2 min; the final extension was continued for 7 min at 72°C. The PCR products were separated by electrophoresis (2 h at 130 V) on 1.5% (wt/vol) agarose gel (Invitrogen, Cergy-Pontoise, France), and the DNA was detected by UV transillumination after staining with ethidium bromide (0.5 μ g/mL). Photopositives were analyzed using the Bionumerics software, version 5.1 (Applied Maths, Kortrijk, Belgium). The RAPD-PCR patterns were grouped by means of cluster analysis with the Pearson product-moment correlation coefficient and the unweighted pair group method using arithmetic averages (UPGMA).

Depending on the pH reached after 6 h of incubation in milk, strains were arbitrarily classified into 3 categories: good acidifiers (pH values <5.00), fair acidifiers (pH values ranging between 5.00 and 6.00), and poor acidifiers (pH values >6.00). According to this arbitrary classification, no *Strep. macedonicus* strain could be considered a good acidifier and just 19 strains out of 71 (about 27%) could be reported as fair acid producers in cow milk (Figure 1). Among these 19 strains, 17 (about 24% of the entire group) exhibited extracellular proteolytic activity on skim milk (Figure 2). In other words, *Strep. macedonicus* was confirmed to be a moderately acidifying species (Georgalaki et al., 2000; Lombardi et al., 2004; De Vuyst and Tsakalidou, 2008), but the percentage of proteolytic strains recorded in this survey appeared to be significantly higher than that obtained by other authors (Georgalaki et al., 2000; Lombardi et al., 2004). On the other hand, *Strep. thermophilus* strains showed better performance in milk and just 18 out of 50 (approximately 36%) led to weak acidifica-

Figure 1. Acid production of *Streptococcus macedonicus* and *Streptococcus thermophilus* strains expressed as pH values after 6 h in pasteurized cow milk.

Figure 2. Cluster analysis-based dendrogram (unweighted pair group algorithm with arithmetic averages method) showing diversity of *Streptococcus* strains with respect to the utilization of carbon sources, enzyme activities, and exopolysaccharide (EPS) and biogenic amine (BA) production. Shaded boxes indicate the presence of an attribute. Type strains of the species *Strep. thermophilus* were included for comparison.

Figure 3. Dendrogram obtained from XD9 random amplification of polymorphic DNA (RAPD)-PCR fingerprints of 125 *Streptococcus* strains. Patterns were grouped with the unweighted pair group algorithm with arithmetic averages (UPGMA). Type strains of the species *Strep. thermophilus* were included for comparison.

tion after 6 h (Figure 1). Despite the higher number of strains with good or fair acidifying ability, the percentage of strains able to express proteolytic activity was less than 39% (Figure 2).

About 50% of *Strep. macedonicus* strains showed lipolytic activity toward tributyrin (Figure 2), confirming the hypothesis that *Strep. macedonicus* could have a greater effect on lipolysis than on proteolysis during cheese ripening (De Vuyst and Tsakalidou, 2008). Lipolysis was widespread even among *Strep. thermophilus* strains (34 strains out of 50), proving that thermophilic

streptococci possess a high esterase activity among lactic acid bacteria. All *Strep. macedonicus* strains were able to produce acid from galactose (Figure 2), as was reported in the first description of the species provided by Manachini et al. (2002). Surprisingly, only 4 strains of *Strep. thermophilus* proved unable to ferment this sugar (Figure 2). According to the literature, *Strep. thermophilus* is not capable of metabolizing galactose and thus expels this sugar into the medium during lactose fermentation (Mora et al., 2002; de Vin et al., 2005; Vaillancourt et al., 2008). In fact, galactose-fermenting

Strep. thermophilus strains have been reported (Giraffa et al., 2001; Mora et al., 2002), but the high occurrence of galactose-fermenting strains recorded in this survey has never been reported before.

Based on the obtained results, tyrosine was the most decarboxylated amino acid by 17 out of 50 *Strep. thermophilus*, and 13 out of 71 *Strep. macedonicus* strains (Figure 2). In *Strep. thermophilus* species, in addition to tyrosine-decarboxylation, the first evidence was found in a limited number of strains with activity on ornithine and histidine. Previous studies investigating the production of biogenic amines by this species showed no activity or only tyrosine-decarboxylating activity (Straub et al., 1995; Bukková et al., 2009). Similar results were reported by Marino et al. (2008) in *Strep. thermophilus* strains isolated from Montasio cheese. None of the *Strep. macedonicus* strains could decarboxylize ornithine, and only 1 of 71 produced histamine. The percentage of strains able to decarboxylize tyramine (about 18%) was higher than that recorded during a previous survey on this species (Georgalaki et al., 2000). No strain was able to utilize citrate (data not shown). This finding was not surprising for *Strep. thermophilus* because this species does not possess genes for citrate, malate, or gluconate transport and utilization (Hols et al., 2005). Conversely, according to Georgalaki et al. (2000), some *Strep. macedonicus* strains should grow on Simmons citrate agar with citrate as the sole carbon source. This trait in particular, together with fat and casein hydrolysis, would indicate the usefulness of this species as an adjunct or coculture (De Vuyst and Tsakalidou, 2008). With reference to EPS production, *Strep. macedonicus* strains exhibited a reduced variability, with more than 50% of the strains unable to produce EPS independently from the carbon source (Figure 2). In fact, Georgalaki et al. (2000) and Lombardi et al. (2004) did not find any EPS-producing strains during their surveys; and this capability was exclusively observed in one strain coming from the Nestlé Culture Collection (Vincent et al., 2001). In *Strep. thermophilus*, just 6 strains did not produce EPS under any conditions, and 64% of the strains were positive for EPS production in every medium (Figure 2). Moreover, the high variability in the patterns of sugar utilization could represent a further approach for strain characterization. Biochemical results allowed 49 different patterns to be detected: 33 out of 50 for *Strep. thermophilus* and 16 out of 71 for *Strep. macedonicus*. These characteristic profiles were used to construct a dendrogram on the Jaccard coefficient matrix via a modified UPGMA cluster analysis (Figure 2).

To evaluate if the detected phenotypic variability was reflected at the genetic level, a multilocus typing approach was carried out by analyzing all *Streptococcus* spp.

strains by RAPD-PCR analysis. In preliminary RAPD experiments, primers XD5 (5'-CTGGCGGCTG-3'), OPI-02MOD (5'-GCTCGGAGGAGAGG-3'), REP-1 (5'-IIICGICGICATCIGGC-3'), REP-2 (5'-ICGICTATCIGGCCTAC3'), and XD9 were tested for their discrimination ability on a limited number of strains (data not shown). Primer XD9 was chosen because it gave the most reproducible band patterns and the higher strain differentiation, yielding 15 different RAPD profiles out of 50 strains in *Strep. thermophilus*, and 19 out of 71 strains in *Strep. macedonicus* (Figure 3). In other words, a remarkable genotypic heterogeneity between strains was highlighted in both species, confirming previous results obtained within *Strep. thermophilus* (Moschetti et al., 1998; Giraffa et al., 2001; Mora et al., 2002) and *Strep. macedonicus* species (Lombardi et al., 2004; Franciosi et al., 2009). Moreover, at a similarity level of 78%, all the isolates and reference strains of *Strep. thermophilus* were grouped together and were well separated from the strains of *Strep. macedonicus*, confirming that these 2 species are different microbial entities, as previously indicated by both taxonomic and genotypic studies (Tsakalidou et al., 1998; Andrighetto et al., 2002).

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