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Characterization of thermophilic streptococci isolated from rustic white cheese

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The thermophilic streptococci are dominant members of the microflora of several types of cheese produced with a natural starter. A total of 205 isolates of thermophilic streptococci were obtained from 70 samples of white cheese which had been collected from producing areas of different regions of Syria during 2009 and 2010. The isolates were cocci gram positive and catalase negative. From these isolates, 120 were identified as *Enterococcus* spp. including: 72 isolates of *E. faecium*, 35 isolates of *E. faecalis* and 13 isolates of *E. durans*. In addition, 70 isolates were identified as *Streptococcus* including: 50 isolates of *S. thermophilus*, and 20 isolates of *S. equines*. Fifteen isolates of cocci grew at 45°C with spherical morphology, gram positive and catalase negative. The PCR technique could be efficiently used for identifying and typing the thermophilic streptococci.

Key Words—API technique; *Enterococcus*; PCR; *Streptococcus*; thermophilic; white cheese

Introduction

White cheese is a popular cheese, produced in different regions of Syria. It is traditionally manufactured from raw cow's or sheep's milk. The procedure of using unpasteurized milk without a starter in order to conserve microflora has received special attention (El-Baradei et al., 2007; Licitra et al., 2007; Randazzo et al., 2002; Terzic-Vidojevic et al., 2007). The thermophilic streptococci play an important role in the manufacture of white cheese (Stiles and Holzapfel, 1997), while other types (*Streptococcus pyogenes*, *S. sobrinus*, *S. mutans* and *Enterococcus faecalis*) have pathogenic properties (Molinari and Chhatwal, 1999). The thermophilic streptococci are ubiquitous and they can be

found in soil, on plants, in dairy products and as a part of the normal flora of the gastrointestinal tracts of humans and animals (Jett et al., 1994). *Enterococci* have been used in many different applications as a starter or adjunct culture. Moreover, they have a major role in improving flavor development in the food as well as the quality of cheese (Stiles and Holzapfel, 1997; Teanpaisan and Dahlen, 2006). Many kinds of cheese made from unpasteurized milk through traditional manufacturing procedures may possess a very diverse and rich microflora (Flórez et al., 2006). Some researchers have reported that the local microflora of raw milk influences the biochemical characteristics of cheese (Marino et al., 2003). Studies on the microbiota of traditional cheeses in the Mediterranean agro-zones have indicated that enterococci are of a significant importance in cheese ripening and contribute to its typical taste and flavor probably through proteolysis, lipolysis, and citrate breakdown (Felis and Dellaglio, 2007; Manolopoulou et al., 2003). *S. thermophilus* and other thermophilic streptococci, including *E. faecalis*

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and *E. faecium*, are among the dominant members of the microflora of many cheeses produced with natural cultures (Teng et al., 2002). Traditional bacterial classification methods based on morphology, physiology and biochemical tests could be time-consuming and misleading (Yanagida et al., 2007). Commercial Kits based on biochemical enzymatic and fatty acid analyses were found to be unstable due to their susceptibility to different bacterial physiological conditions (Teaupaisan and Dahlen, 2007). Therefore, efforts have been made to develop simple, fast and discriminating molecular methods for the identification of lactic acid bacteria (LAB); the 16S and 23S ribosomal are ubiquitous and composed of alternating conserved and hypervariable (V) regions, representing the prototype genetic marker in molecular microbial ecology (Huys et al., 2007; Yu et al., 2009). Several studies have rapidly classified LAB based on 16S and 23S rRNA restriction fragment length polymorphism analysis (Moschetti et al., 1998; Randazzo et al., 2004). The identification of thermophilic streptococci with phenotypic tests is often not conclusive, due to the frequent occurrence of abnormal biochemical patterns in strains isolated from natural populations (Moschetti et al., 1998). Because of the industrial importance of thermophilic streptococci and of the potential public health significance of some enterococci (Giraffa, 2002), rapid and reliable techniques for the identification and typing of these species are needed. The aim of this study was to evaluate morphological and biochemical methods in the identification of thermophilic cocci isolated from Syrian white cheese. Moreover, a direct analysis of total white cheese flora's DNA and the amplification of 16S, 23S rRNA and *LacZ* genes encoding by the Polymerase Chain Reaction (PCR) technique was performed.

Materials and Methods

Sample collection. Seventy samples of white cheese were collected from different Syrian regions dur-

ing 2009 and 2010. Samples were packed in sterilized bags, kept in ice boxes and transported to the laboratory for analysis.

Isolation of *Streptococci thermophilic*. Ten grams of samples were homogenized with 90 ml sterile peptone physiological saline solution (5 g peptone, 8.5 g NaCl, 1,000 ml distilled water, pH 7.0). The homogenate was diluted serially and the appropriate dilutions were surface plated on M17 agar media (MERCK, Germany), then plates were incubated at 45°C for 3 days.

Physiological and biochemical tests. Isolated bacteria were tested for gram reaction, catalase production, spore formation and cell morphology according to the methods described by Kebede et al. (2007). Growth in the presence of 4% and 6.5% NaCl was observed in M17 broth at 30°C for 2 days, and growth at 10°C and 45°C was also observed in M17 broth after incubation for 3 days. Production of acetoin from glucose was determined using the Voges-Proskauer test (Samelis et al., 1994).

API assay. The API 20 STREP (bioMérieux, Marcy l'Etoile, France) has been used to identify the enzymatic and carbohydrate fermentation patterns of *Enterococcus* and *Streptococcus* isolates and API 50 CHL (bioMérieux, Marcy l'Etoile, France) has been used to identify *S. thermophilus*. The API of all isolates were overnight cultured in M17 broth (MERCK, Germany), then added individually to the substrate in wells of the API strips. The inoculated strips were incubated at 37°C and then monitored for changes in the color of the medium after 24 h. Discrimination between isolates was based on the principle of a pattern matching manual as described by the manufacturer.

Genomic DNA isolation from bacteria. A single colony from each isolate was inoculated into 10 ml of the appropriate medium broth (kept in a 15 ml Falcon tubes) and incubated overnight at 37°C. The cultivated culture was harvested by centrifugation at 5,000 rpm for 5 min and the genomic DNA was isolated by a modified genomic DNA isolation protocol (Sambrook and Russell, 2001).

Table 1. Sequences of the primers used in the study.

Name	Gene	bp	Oligo sequence
<i>Streptococcus thermophilus</i>	<i>LacZ</i>	221	5'GAAGGTGTTGCATGGTGTGCG3' 5'CCACCACCAGAGTTACCCAT3'
<i>Enterococcus faecalis</i>	16S and 23S rRNA	200	5'ATTAATCGGTTGCCTGGACG3' 5'GCCAATTTCCAGGAACAGTG3'

PCR experiments. Amplification of DNA 16S RNA and 23S RNA regions was performed on a volume of 25 µl containing 1.5 µl PCR reaction buffer, 3 µl MgCl₂ (25 mM), 0.5 µl of each dNTP, 1 µl of each primer (see Table 1), 2 µl bacterial genomic DNA solution and 1 µl Taq DNA polymerase. The temperature profile was the following: 5 cycles consisting of 94°C for 5 min, 50°C for 1 min and 72°C for 2 min; and 30 cycles consisting of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s. A final extension at 72°C for 10 min was performed. All amplification reactions were performed in a GeneAmp® PCR System 9700 (Applied Biosystems). Following amplification, 2 µl of product was analyzed at 85 V, 150 A for 1 h by agarose electrophoresis (1.5% agarose gel, 0.2 µg ethidium bromide) and imaged under UV light.

16S RNA and sequence determination and phylogenetic analysis. Sequencing of the 16S RNA and LacZ were performed in collaboration. Sequences were submitted to the National Center for Biotechnology Information (Bethesda, MD) for similarity searches through BLAST (<http://www.ncbi.nlm.nih.gov/blast>) and were compared with type strains by multiple alignments.

Statistical analysis. Data were transferred to a Microsoft Excel spreadsheet for analysis. Using SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA), a chi-square test and Fisher's exact two-tailed test analysis were performed at the 5% level ($p < 0.05$).

Results

From the collected samples ($n = 70$), a total of 205 isolates of thermophilic streptococci were classified as gram positive and catalase negative cocci and ap-

peared mostly as pairs or forming chain that grew at 45°C (Table 2). They were tentatively referred as *Streptococcus* or *Enterococcus*.

Table 3. Carbohydrate fermentation by spherical bacteria isolated from white cheese using API systems.

No. of isolates	Bacteria	Carbon sources													
		VP	HIP	PYRA	AGAL	BGUR	BGAL	PAL	LAP	ADH	RIB	ARA	MAN	SOR	LAC
13	<i>E. durans</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+
35	<i>E. faecalis</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+
72	<i>E. faecium</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	<i>S. equines</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+
50	<i>S. thermophilus</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ = positive fermentation, - = negative fermentation, ± = partial fermentation.

VP = Voges-Proskauer, HIP = hippuric acid, PYRA = pyruvic acid, AGAL = α-galactosidase, BGUR = β-glucuronidase, BGAL = β-galactosidase, PAL = alkaline phosphatase, LAP = leucine aminopeptidase, ADH = arginine dihydrolase, RIB = ribose, ARA = arabinose, MAN = mannitol, SOR = sorbitol, LAC = lactose, TRE = trehalose, INU = inulin, RAF = raffinose, AMD = amide, GLYG = glycogen.

Table 4. Characteristics of Streptococci strains isolated from white cheese.

No. of isolates	%	Bacteria	Growth at 10°C	Growth at 45°C	Growth in a medium with 6.5% NaCl
13	7	<i>E. durans</i>	+	+	+
35	18	<i>E. faecalis</i>	+	+	+
72	38	<i>E. faecium</i>	+	+	+
20	11	<i>S. equines</i>	–	+	–
50	26	<i>S. thermophilus</i>	–	+	–

+ = positive growth, – = negative growth.

Table 5. Regional distribution of bacterial isolates in some cheese samples.

Regions	<i>E. durans</i>	<i>E. faecalis</i>	<i>E. faecium</i>	<i>S. equines</i>	<i>S. thermophilus</i>
Southern region	2	1	15	2	12
Central region	5	7	13	3	15
Eastern region	1	5	20	9	11
Coastal region	2	10	17	4	4
Northern region	3	12	7	1	8
Total	13	35	72	20	50

Fig. 1. Percentages of thermophilic bacteria isolated from white cheese.

API assay

A total of 205 isolates were screened for their performance regarding growth characteristics on carbon sources supplied by the manufacturer. Based on phenotypic characteristics and interpretation of the API database, 120 isolates were identified as *Enterococcus* spp. (58.53% of 205 isolates); among them, 72 isolates were identified as *E. faecium*, 35 isolates were identified as *E. faecalis* and 13 isolates were identified as *E. durans* (Table 3). The high percentage of *E. faecium* is an indicator to the key role played by these bacteria in the fermentation process. Seventy isolates (34.14% of 205 isolates) were identified as *Streptococcus* spp.; among them, 50 isolates were identified as *S. thermophilus* and 20 isolates were identified as *S. equines* (Table 4). Fifteen isolates (7.3% of 205 iso-

lates) were identified as cocci, grew at 45°C, with spherical morphology, and were gram positive and catalase negative (Table 4). Figure 1 and Table 5 revealed the distribution of thermophilic bacteria isolated from Syrian white cheese.

PCR results

Figure 2 demonstrates that the length of *LacZ* gene (221 bp) bands (bands 1–6) was similar to that of Hansen's bands (*S. thermophilus* DSM20617 strain, which was used as a positive reference), which confirmed

Fig. 2. Electrophoresis on 1.5% agarose gel showing the PCR products.

Lane 7: negative control (*E. faecalis*). Lane 1: positive control (*S. thermophilus* DSM20617, Hansen®). Bands 2–6: fragment of 221 bp obtained from Syrian white cheese. Lane MW: molecular weight marker (100-bp DNA ladder).

Fig. 3. Electrophoresis on 1.5% agarose gel showing the PCR products.

Bands 2–5: fragment of 200 bp obtained from Syrian white cheese. Lane 1: negative control (*S. thermophilus* DSM20617, Hansen®). Lane MW: molecular weight marker (100-bp DNA ladder).

that the isolates belong to the *S. thermophilus* type, whereas Fig. 3 demonstrates that the length of 16S RNA-23S RNA gene (200 bp) bands (bands 1 and 4) was similar to that of the *E. faecalis* strain (cf. the website www.ncbi.nlm.nih.gov), which indicates that the isolates belong to the *E. faecalis* type. The negative control (*S. thermophilus* TH-4) is shown on band 1. The sequences of primers used in the study are summarized in Table 1.

The statistical study indicated that no relationship was found between white cheese and thermophilic streptococci among sampling regions during the study period.

Discussion

Syria has several types of cheeses that form an integral part of its native cuisine. There is an old folk saying that cheese was born from a serendipitous discovery by the Bedouins of the Arabian Peninsula. White cheese is the most popular cheese in Syria and makes up about 60% of the cheese produced and consumed in the country. It differs chiefly from other pickled cheese in that the milk is salted at the first step of its manufacture. In the present study, the distribution of thermophilic streptococci was revealed by using classical and DNA techniques. *S. thermophilus* and other thermophilic streptococci, including *E. faecium*, are among the dominant members of the microflora of many types of cheeses (Morea et al., 1999). The milk can be curdled fresh (never after pasteurization) with the addition of two starters (Barthelemy and Sperat-Czar, 2004). The cheese can be consumed either fresh or after conservation in salted water, or alternatively

frozen at -18°C up to 6–8 months. The Syrian cheese actually comes in many different varieties, one of which is sort of like a braided mozzarella. The genus *Enterococcus*, which includes *E. faecalis* and *E. faecium*, was separated from *Streptococcus* on the basis of DNA hybridization data by Schleifer and Kilpper-Balz (1984). *S. thermophilus* and other thermophilic streptococci, including *E. faecalis* and *E. faecium*, are among the dominant members of the microflora of many cheeses produced using natural starter cultures (De Angelis et al., 2001; Koedel et al., 2002). The identification of thermophilic streptococci with phenotypic tests is often not conclusive, due to the frequent occurrence of abnormal biochemical patterns in strains isolated from natural populations (Moore et al., 2003; Moschetti et al., 1998). Our results indicate that 205 isolates that could grow on M17, and at 4% NaCl including isolates that can grow at 6.5% NaCl were referred as *Enterococcus* spp. (Abdullah and Osman, 2010). All the isolates could grow at 45°C , but only 198 isolates could grow at 10°C , and the isolates were different in carbohydrate fermentation. According to the API system, 58.53% of isolates were identified as *Enterococcus*; 35.1% of isolates were identified as *E. faecium*, 17.07% of isolates were identified as *E. faecalis* and 6.3% of isolates were identified as *E. durans*. Similar results had been reported in a large variety of Egyptian dairy products (EL-Soda et al., 2003). Moreover, 34.14% of isolates identified as *Streptococcus*, 24.39% of isolates identified as *S. thermophilus*, 9.75% of isolates identified as *S. equines*, and 7.3% of isolates identified as cocci grew at 45°C , with spherical morphology, and were gram positive and catalase negative. In traditional Moroccan soft white cheese, produced in eight different regions in Morocco, a total of 164 lactic acid bacteria were isolated, purified and identified (Ouadghiri et al., 2005). The majority of the strains belonged to the genera *Enterococcus* (*E. durans*, *E. faecalis*, *E. faecium* and *Streptococcus* spp.). Due to the industrial importance of thermophilic streptococci and of the potential public health significance of some enterococci (Giraffa, 2002; Licitra et al., 2007), rapid and reliable techniques for the identification and typing of these species are needed. Our study revealed that the PCR technique might be an effective tool in the identification and typing of the thermophilic streptococci. The 16S rRNA gene has been used in many scientific studies, to classify the bacterial types, amongst which is lactic acid bacteria (Moschetti et al.,

1998). As far as we are concerned, studies that used the *LacZ* gene are rare, and our results showed that by magnifying this gene, we could distinguish *S. thermophilus* from *E. faecalis* (Figs. 1 and 2). It is well known that these two types of species are dominant in dairy products flora and possess similarities in some of their biochemical properties (Giraffa, 2002; Morea et al., 1999; Moschetti et al., 2001); it is therefore necessary to find a rapid and reliable technique to differentiate between these two species in order to employ *S. thermophilus* as a starter and exclude *E. faecalis*.

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