African Journal of Biotechnology Vol. 11(28), pp. 7218-7226, 5 April, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.125
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Genotypic identification and technological characterization of lactic acid bacteria isolated from traditional Turkish Kargi tulum cheese

Buket Kunduhoglu[1]*, Ozlem Elcioglu[2], Yekta Gezginc[3], Ismail Akyol[4], Sevil Pilatin[1] and Asya Cetinkaya[5]

[1]Department of Biology, Faculty of Science and Arts, University of Eskişehir Osmangazi, 26480-Eskişehir, Turkey.
[2]Institute of Science, University of Eskişehir Osmangazi, 26480-Eskişehir, Turkey.
[3]Department of Food Engineering, Faculty of Agriculture, University of Kahramanmaraş Sütçü İmam, 46100-Kahramanmaraş, Turkey.
[4]Microbiology and Microbial Genetics Laboratory, University of Kahramanmaraş Sütçü İmam, USKIM, 46100-Kahramanmaraş, Turkey.
[5]Kars Vocational School, University of Kafkas, 36100-Kars, Turkey.

Accepted 2 March, 2012

Kargi tulum cheese is an artisanal cheese produced through the spontaneous fermentation of raw milk ripened inside a goat-skin bag. The objective of this study was to characterize the dominant species of natural lactic acid bacteria (LAB) found in Kargi tulum cheese. Some technologically important properties of LAB isolates were also determined. Seven samples of cheese of different levels of ripeness were taken from local producers and 97 isolates were obtained from these samples. Non-spore forming, Gram-positive, catalase- and oxidase-negative isolates were assessed as LAB. Based on genotypic characterization, the dominant LAB were identified as *Lactobacillus paracasei* (43.3%), *Lactobacillus plantarum* (23.7%), *Enterococcus durans* (6.2%), *Streptococcus thermophilus* (6.2%), *Lactobacillus brevis* (5.2%), *Enterococcus faecium* (5.2%), *Lactobacillus fermentum* (4.1%) and *Lactobacillus pentosus* (1%). Homofermentative strains such as *L. paracasei*, *L. plantarum*, *S. thermophilus*; *E. durans* and *E. faecium* were selected as probable starter cultures. These strains were tolerant to 6.5% salt. They are also characterized by acidification ability (from pH = 6.6 to 4.7 to 6.0, in 6 to 8 h), low proteinase and high peptidase, esterase, esterase-lipase, β-galactosidase and β-glucosidase activities. They also produce diacetyl and H_2O_2.

Key words: Lactic acid bacteria, genotypic characterization, technological characterization, tulum cheese.

INTRODUCTION

Kargi tulum cheese is manufactured as an organic food in the high plateau of Kargi, Çorum, in the Middle Anatolian region of Turkey. It is manufactured in summer months and marketed in public bazaars in autumn (Dinkci et al., 2007). Kargi tulum cheese is produced by small-scale dairies using raw whole sheep, goat, cow and buffalo milk or a mixture of these and no added lactic starters. The traditional production method of Kargi tulum cheese begins with fresh raw milk that is coagulated by adding rennin and salt (2 to 3% w/w). The indigenous microflora contained in raw milk increase the acidity of the curd (12

*Corresponding author. E-mail: bkunduh@gmail.com. Tel: +90-222-2393750. Fax: +90-222-2393578.

h). Afterwards, the curd is cut into small pieces by hand (in pea size, approximately 1 cm^3) and put into small cotton bags. A weight is then put over the bags to drain the whey (at 20°C for 24 h). The drained curds are kneaded with salt and put into larger cotton bags. Any remaining whey is drained by putting a weight over the cotton bags. The cheese is removed from the big cotton bags at certain intervals (once every 15 to 20 days), kneaded and put into new cotton bags. This process is repeated until the end of October. Afterwards, the curds are compressed into goat-skin bags so that no entrapped air remains. Goat-skin bags are the material traditionally used to package tulum cheese. Kargi tulum cheese prepared in this way is then incubated for at least 3 months (at 4 to 6°C) in the uplands. Kargi tulum is a semi-hard cheese with a crumbly texture, white-cream color and a quite favorable taste (personal communication with local producer). Because of their unique taste, artisanal cheeses produced through spontaneous fermentation of unpasteurized milk receive a great deal of attention from consumers, not only in Turkey but also in other countries around the world. However, it is difficult to control spontaneous cheese fermentation by indigenous microflora in raw milk and the fermentation process and practically impossible to standardize the quality and shelf-life of the product.

Furthermore, pathogens, such as *Staphylococcus aureus*, *Escherichia coli* type I (Efe and Heperkan, 1995), *Listeria monocytogenes* and *Salmonella* spp. (Colak et al., 2007), have been isolated from tulum cheeses. While pasteurization eliminates several pathogens that threaten consumer health, it also eliminates a large part of the indigenous lactic acid bacteria (LAB) contained in raw milk and changes the cheese flavor (Torres-Llanez et al., 2006). Therefore, when producing traditional cheeses using pasteurized milk and modern production processes, it is very important to provide a taste and flavor similar to that of traditionally produced raw milk cheeses (Perez-Elortondo et al., 1998). To ensure this similarity, the cheese's dominant LAB should be isolated, identified and positive technological characteristics determined. The main objective of this study was to characterize and identify the LAB that plays a predominant role in the spontaneous fermentation of Kargi tulum cheese. Also, technologically important characteristics such as acidification ability, enzymatic and proteolytic activities of these LAB strains were determined. The findings obtained from this study may aid in selecting LAB strains as potential starter cultures to improve the commercial value of Kargi tulum cheese, and in preserving the valuable bacterial strains as genetic resources of Turkey.

To the best of our knowledge, this is the first report on the LAB composition of traditionally processed Kargi tulum cheese.

MATERIALS AND METHODS

Cheese samples

Seven samples (samples 1 to 7) of Kargi tulum cheese at different stages of ripening (1, 2, 3 and 4 months) were obtained from local producers. Samples 1 and 2 (1 month old) were put into cotton bags. The other five samples (2 to 4 months old) were taken as being put into goat-skin bag. Maturation temperatures of cheeses were 4 to 12°C. All samples were transported to the laboratory at 4°C and analyzed within 24 h.

Chemical analysis of cheese samples

All Kargi tulum cheese samples were analyzed for moisture content (TS EN ISO 5534, 2006), titratable acidity as lactic acid-°SH and pH (TS 591, 2006), total N, total protein (TS EN ISO 8968-3, 2004), total fat (TS 3046, 1978), NaCl content and salt in the dry matter (TS EN ISO 5943, 2001). All chemical analyses were carried in the laboratories of the Ministry of Agriculture and Rural Affairs in accordance with the standard methods stated in parentheses.

Isolation of LAB

The cheese samples (25 g) were homogenized in sterile Ringer's solution (225 ml) with a blender for 2 min. Decimal dilutions were made in sterile 0.85% (w/v) saline. From each dilution, 0.1 ml was plated in duplicate on MRS or M17 agar medium, incubated for 6 days under anaerobic conditions at 37 or 30°C, respectively. Five colonies were randomly picked from plates with 30 to 300 colonies and purified by streaking on the same isolation medium. All isolates were tested for cell morphology, Gram staining, catalase, activity, oxidase activity and spore formation. Gram-positive, catalase-negative, oxidase-negative and non-spore-forming isolates with rod or cocci shapes were considered LAB and selected for further examination. The LAB isolates were phenotypically identified (data not shown) by the API identificaticon system (API 50 CHL and API 20 Strep test kits, bioMérieux, France). The pure cultures of these strains were stored in MRS or M17 broth supplemented with 25% glycerol at –80°C. When required, cultures were activated by two consecutive transfers to MRS or M17 broth incubated at 37 or 30°C, respectively.

Genotypic identification of LAB isolates

Molecular identification of isolated LAB strains was carried out according to the methods as described by Tabasco et al. (2007) and Walter et al. (2000). Genomic DNA was extracted using the Genomic DNA Extraction Kit (Fermentas) according to the supplier's specifications. DNA quantity and quality were determined by nano-drop and confirmed by agarose gel electrophoresis. Molecular identification of isolates was conducted using specific primers to amplify the 16S rRNA region. These primers were obtained from Iontek (Istanbul, Turkey) and sequences are listed in Table 1. The PCR amplification reaction was performed in a 40 µl solution containing 1 µl of each primer (20 pmol), 4 µl 10X reaction buffer, 1 µl of each dNTPs, 0.5 µl Taq DNA polymerase and 1 µl of

Table 1. Species-specific primers (16S RNA) used to identify LAB strains isolated from Kargi tulum cheese samples.

Species	Primer	Sequence (5'→3')	Length of product (bp)	Reference
E. durans	Dur. F Dur. R	CCTACTGATATTAAGACAGCG GGGCGGTGTGTACAAGGC	295	Walter et al. (2000)
E. faecium	Ef. F Ef. R	GAAAAAACAATAGAAGAATTAT TGCTTTTTTGAATTCTTCTTTA	215	Walter et al. (2000)
L. brevis	Lb. F Lb. R	CTTGCACTGATTTTAACA GGGCGGTGTGTACAAGGC	1340	Walter et al. (2000)
L. fermentum	Ferm. F Ferm. R	GCCGCCTAAGGTGGGACAGAT CTGATCGTAGATCAGTCAAG	301	Walter et al. (2000)
L. paracasei	Pcas. F Pcas. R	GCACCGAGATTCAACATGGAA GCCATCTTTCAGCCAAGAACC	142	Tabasco et al. (2007)
L. pentosus	Pent. F Pent. R	CAGTGGCGCGGTTGATATC TCGGGATTACCAAACATCAC	218	Walter et al. (2000)
L. plantarum	Plan. F Plan. R	GCCGCCTAAGGTGGGACAGAT TTACCTAACGGTAAATGCGA	318	Walter et al. (2000)
S. thermophilus	Therm. F Therm. R	ACGCTGAAGAGAGGAGCTTG GCAATTGCCCCTTTCAAATA	157	Tabasco et al. (2007)

the isolated DNA. The PCR products were generated using an initial denaturation step of 4 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 1 min, elongation at 72°C for 1 min and final at 72°C for 5 min. PCR reactions were performed in an Eppendorf Mastercycler Personal system.

All of the PCR amplification products were analyzed on 1% (m/v) agarose gels, and the separated fragments were stained with 0.5 µg/ml ethidium bromide (Sisto et al., 2009) and visualized under UV light.

Technological properties of LAB

Acidification ability

The isolates activated in MRS or M17 broth (30°C for 24 h) were centrifuged at 7500 rpm for 5 min, and the pellets were washed with peptone water and re-suspended in the same medium. Tubes containing 10 ml of whole fat UHT milk (pH = 6.6) were inoculated with the cell suspension (1% v/v) and incubated at 30°C for 48 h. Then, pH changes in the milk were measured using a pH meter (WTW 735, Germany) during incubation (after 2, 4, 6, 8, 24 and 48 h) (Olasupo et al., 2001).

Proteolytic activity

Surface-dried plates of skim milk agar (10% skim milk + 2% agar w/v) were used to determine the proteolytic activity (Gordon et al., 1973). The same cell suspensions discussed earlier were spotted (5 µl) into the plate surfaces. Plates were incubated at 30°C for 24 h and then examined for the appearance of halos of proteolysis around the spotted samples.

Diacetyl production

The production of diacetyl was performed qualitatively. Tubes containing 5 ml of whole-fat UHT milk were inoculated with the activated cell suspensions (1% v/v) and incubated at 30°C for 24 h. After incubation, 1 ml aliquot was transferred to another sterile test tube and reagents (0.5 ml of 1% alpha naphthol and 0.5 ml of 16% KOH) were added. Diacetyl-producing strains had a red ring at the top of the tube after incubation (King, 1948).

Hydrogen peroxide (H_2O_2) production

LAB isolates were grown at 30°C for 48 h in 5 ml MRS broth and diluted with 5 ml of sterile distilled water. The cells were removed by centrifugation at 7500 rpm for 5 min and the supernatant fluid was filtered (0.45 µm, Millipore Corporation, USA). Then, 0.5 ml citric acid, 0.5 ml ammonium molybdate and 0.5 ml KI (in that order) were added to 4 ml of supernatant and mixed vigorously. The optical density of this solution was determined at 350 nm. In order to determine the H_2O_2 concentration in the supernatants, standard curve of the H_2O_2 (30%) was used (Whittenbury, 1964).

Table 2. Chemical characteristics of Kargi tulum cheese at different ripening periods.

Chemical Property	Age of Kargi tulum cheese samples						
	1 month		2 months	3 months		4 months	
	1*	2	3	4	5	6	7
Moisture content (%)	53.9	46.4	42.5	35.0	38.0	29.0	25.0
Titratable acidity (°SH)	1.5	2.8	2.5	2.7	2.6	2.9	2.8
pH	5.05	5.09	5.13	5.2	5.15	5.20	5.25
Total Protein (%)	14.8	21.9	22.2	23.4	23.4	26.0	25.1
Total Nitrogen (%)	2.3	3.4	3.5	3.7	3.7	4.1	3.9
Fat (%)	24.0	25.0	29.0	35.0	32.0	39.5	44.3
Fat in dry matter (%)	52.0	46.6	50.4	53.8	51.6	55.6	59.1
NaCl in dry matter (%)	2.5	5.4	5.8	4.7	5.6	3.1	3.6

*1-7: Sample number of cheeses.

Enzymatic profiles of LAB isolates

The enzymatic profiles of the LAB were assayed using API-zym galleries (bioMérieux, France) according to manufacturer's instructions. These tests were replicated twice.

RESULTS AND DISCUSSION

Chemical properties of the cheese samples

Chemical properties of the 7 Kargi tulum cheese samples at different ripening periods are given in Table 2. As seen in the table, the moisture ratio in the younger cheese samples (samples 1 and 2) which were not put in goat-skin bags was high. However, due to the porous texture of the goat-skin bags, the moisture ratio of the cheese sampled during the ripening period gradually decreased. While the amounts of dry matter in the fresh cheese samples were 46.1% (sample 1) and 53.6% (sample 2), the amounts of dry matter in the cheese samples that were ripened for 4 months were 71 and 75%. During the ripening period, an increase in the amount of salt in dry matter in the samples was observed. For tulum cheese, 3 to 6% (w/w) salt in dry matter is acceptable (TSE 3001, 2006). The amount of salt in dry matter in our cheese samples varied between 2.5 to 5.8%. Ripened tulum cheese contains a high proportion of fat. The lowest fat and fat in dry matter ratios were found in 1 to 2-month-old cheese samples. This ratio gradually increased towards the end of the ripening period. In parallel with the ripening period, the total protein and nitrogen ratios in the fresh cheese samples also increased. Except for cheese sample no. 1, there were no apparent changes in the °SH values of the samples. However, as the ripening period increased, a slight elevation in the °SH values was measured. Similar to our study, Bayar and Ozrenk (2011) have also reported that during ripening period (max 90 days), the dry matter, salt, protein, fat and °SH values of tulum cheese samples increased.

The pH of tulum cheese during the ripening period usually varies from 4.8 to 5.2 (Hayaloglu et al., 2007a). Similar to these findings, in our study, the pH of cheese samples varied from 5.05 to 5.25.

Genotypic identification of LAB

Gel images obtained as a result of the genotypic characterization are shown in Figure 1. Identification of 92 isolates was performed using species-specific primers, but five isolates could not be identified with the primers used. The distribution of the LAB species isolated from different cheese samples and their isolation frequencies are shown in Table 3. In our study, it was found that the dominant population in Kargi tulum cheese samples was lactobacilli (77.3%; 75/97). The predominant species in almost all cheese samples was *Lactobacillus paracasei*, followed by *Lactobacillus plantarum*. Other lactobacilli were found during various stages of the incubation period. Although, their isolation frequency was not high, *Enterococcus faecium* and *Enterococcus durans* were isolated from the 1-, 2- and 3-month-old cheese samples. *Streptococcus thermophilus* was isolated from the 3- and 4-month-old samples. The low isolation frequency of these species maybe due to cell autolysis during the cheese ripening period. Various studies conducted on fermentative microflora of other 'tulum' cheese types (manufactured with different milk types, under different conditions, or in different regions) have reported different dominant LAB species in cheeses of different levels of ripeness. Bostan et al. (1992) found that *Lc. lactis* subsp. *lactis* and *Enterococcus faecalis* were dominant in tulum cheese made from cow milk at the beginning of its ripening period, and *E. faecium*, *Lc. lactis* subsp. *lactis,*

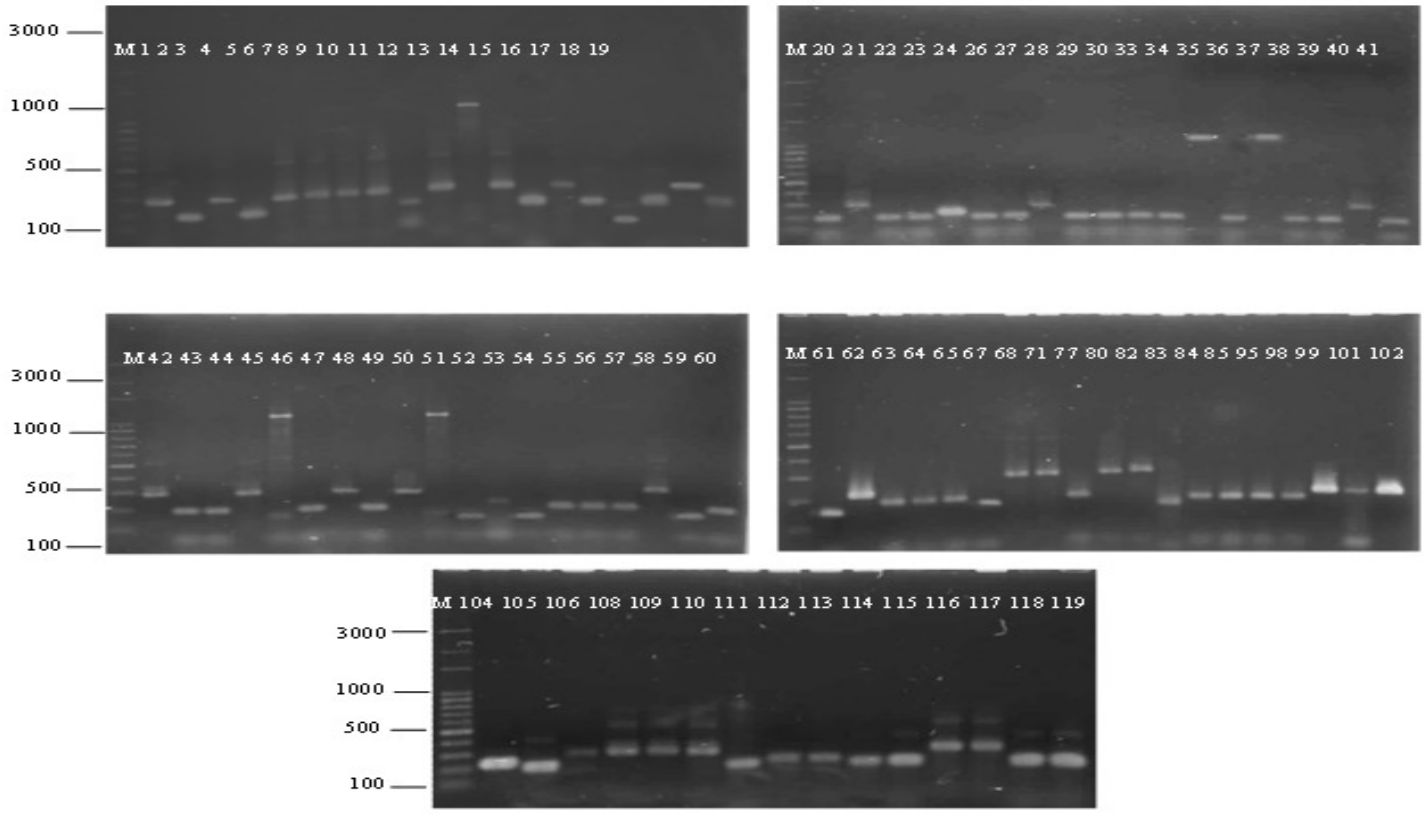

Figure 1. PCR amplification products obtained from pure culture. DNA sizes are enumerated according to the M: 100 bp DNA ladder. *L. paracasei* (lanes 2, 4, 13, 15, 17, 19, 20, 22, 23, 26, 27, 29, 30, 33, 34, 36, 38, 39, 41, 43, 44, 47, 49, 55 to 57, 60, 62 to 65, 77, 84, 85, 95, 98, 105, 111, 114, 115, 118 and 119), *L. plantarum* (lanes 1, 3, 5, 6 to 8, 10, 12, 14, 21, 40, 42, 45, 58, 68, 71, 80, 82, 108, 109, 110, 116 and 117), *St. thermophilus* (lanes 16, 52, 54, 59, 61 and 67), *E. durans* (lanes 9, 53, 101, 106, 112 and 113), *L. brevis* (lanes 11, 35, 37, 46 and 51), *L. fermentum* (lanes 18, 28, 48 and 50), *E. faecium* (lanes 24, 62, 99, 102) and *L. pentosus* (lane 83).

Lb. casei and *Lb. plantarum* became dominant over time. Similar to the findings in our study, Sengul and Cakmakci (2003) found that 92% of Savak tulum cheese isolates were *Lactobacillus* spp. They reported that the dominant species among such isolates were *Lb . parabuchneri* and *Lb . bifermentans*.

In a study conducted by Oksuztepe et al. (2005) using Savak tulum cheese, the number of lactococci and lactobacilli was high at the beginning and at the end of the ripening period, respectively and *Lb. casei* subsp. *casei*, *Lb. plantarum*, *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris* and *Leu. mesenteroides* subsp. *cremoris* were the predominant species.

Technological characterization of LAB

In this part of the study, the potential of the LAB isolated from Kargi tulum cheese to be used as starter cultures was evaluated. Starters selected from among the native LAB of Kargi tulum cheese could be used to produce Kargi tulum cheese safer in terms of human health and standardized in quality, while preserving its traditional aroma. Starters used in cheese production must have a high acidifying capacity and be able to develop at the salt concentration and temperature at which the cheese is produced. To avoid rapid ripening of and bitterness in the cheese, its proteolytic activity should be low and should produce a pleasant taste and smell at the desired dosage and combination (Halkman, 1991; Arora et al., 1990). The extent to which our isolates had these characteristics and their potential as starter cultures were determined by performing the tests shown in Tables 4 and 5. In addition to coagulation, rapid acidification of milk inhibits several pathogens and saprophytic organisms. Garabal et al. (2008) stated that the use of LAB which reduce the pH of milk below 6 within the first 6 h, as a starter culture in cheese production provides an advantage. In our study, *Lb. plantarum*–5, 7, 71 and *Lb. paracasei*–30 reduced the pH of UHT milk to pH = 5.8 to 6.0 within 6 h. *Lb.*

Table 3. Genotypic species characterization of the LAB isolates using species-specific primers and distribution of these species in cheese samples. Isolation percentages (%) are indicated in parenthesis.

Genotypic identification		Age of Kargi tulum cheese samples						
		1 month		2 months	3 months		4 months	
	n	1*	2	3	4	5	6	7
Lb. paracasei	42	11 (42.3)	4 (33.3)	6 (46.2)	4 (66.7)	1 (25)	7 (53.8)	9 (39.1)
Lb. plantarum	23	7 (26.9)	2 (16.7)	2 (15.4)	–	1 (25)	1 (7.7)	10 (43.5)
Lb. brevis	5	4 (15.4)	–	–	–	–	–	1 (4.3)
Lb. fermentum	4	2 (7.7)	–	–	1 (16.7)	–	–	1 (4.3)
Lb. pentosus	1	–	–	1 (7.7)	–	–	–	–
E. faecium	5	–	2 (16.7)	2 (15.4)	1 (16.7)	–	–	–
E. durans	6	1 (3.8)	1 (8.3)	2 (15.4)	–	–	1 (7.7)	1 (4.3)
S. thermophilus	6	–	–	–	–	1 (25)	4 (30.8)	1 (4.3)
Unidentified	5	1 (3.8)	3 (25.0)	–	–	1 (25)	–	–
Total	97	26	12	13	6	4	13	23

*1-7: Sample number of cheeses.
– : No isolation

plantarum–3, 7 and 14 and *Lb. paracasei*–15 reduced the pH of milk to 4.7 to 5.2 at the end of 8 h (Table 4). After 24 h, the milk pH decreased from 3.7 to 3.9 with *Lb. plantarum*–5, 7 and *Lb. paracasei*–39 and 40 (Table 4). It has been reported that lactobacilli with a high caseinolytic activity play a critical role in the ripening of cheese (Broome and Hickey, 1991). Almost all of our isolates hydrolyzed casein (Table 4).

It has been reported that lactobacilli with a high caseinolytic activity play a critical role in the ripening of cheese (Broome and Hickey, 1991). Almost all of our isolates hydrolyzed casein (Table 4). The strains with the highest caseinolytic activity (21 *to* 24 mm in diameter) were *E. faecium*–104> *S. thermophilus*–54= *E. durans*–112 > *9* = *Lb. plantarum*–3 = *Lb. brevis*–46. *Lb. plantarum*–82 and *Lb. paracasei*–98 were the lowest caseinolytic strains (0 to 6 mm in diameter). Other strains that showed low caseinolytic activity (7 to 13 mm in diameter) were *Lb. plantarum*–7, 71 and 110 and Lb. *paracasei*–19, 27, 47, 85.

It is known that LAB produces various natural antimicrobials such as organic acids (lactic acid, acetic acid, formic acid, etc.), CO_2, H_2O_2, diacetyl and bacteriocins (Messens and De Vuyst, 2002). In our study, all of our LAB isolates produced varying amounts of H_2O_2. The isolates that produced the highest amount of H_2O_2 (1.5 to 3.0 µl/ml) were *Lb. paracasei*–27, 29, and 39 and *Lb. plantarum*–*6* (Table 4). Citrate is the primary organic acid contained in milk, and diacetyl and acetoin are formed as a result of citrate fermentation by LAB such as *Leu. citrovorum, Str. faecalis, Str. diacetylactis, Lb. casei, Lb. plantarum, L. brevis* and *L. bulgaricus*. Diacetyl is also a major substance that gives cheese a distinctive flavor and butter smell (Hegazi and Abo-Elnaga, 1980). Except for the *L. brevis* and *Lb. fermentum* strains, all of our LAB strains produced diacetyl (Table 4).

The enzymatic characteristics of the LAB used in cheese production are among the factors that most affect cheese flavor (McSweeney, 2004). For ripened hard cheeses, starter cultures with high proteolytic activity are used. However, low proteolytic activity is required for fresh unripened cheeses and semi-hard ripened cheeses. On the other hand, if proteases are not balanced with peptidases and are found in cheese at high ratios, this can cause bitterness and texture defects (Coskun , 2000; Halkman and Taşkın, 2001). In our study, the enzymatic profiles of LAB strains were assayed using the API zym galleries. Each LAB strain produced a wide spectrum of enzymes (Table 5). While the leucine arylamidase and valine arylamidase (peptidases) enzyme activities were observed in 97 to 100% of LAB strains, α-chymotrypsin activity was found in only 8.3% of the strains and none of the strains showed trypsin activity. In terms of proteolytic activity, the majority of our LAB strains will provide a positive contribution to flavor formation in cheese. In addition, for cheeses produced by ripening, starter species with low lipase but high esterase and esterase-lipase enzyme activities are preferred for their flavor and texture formation (Arora et al., 1990). Controlled lipolysis during ripening positively affects the flavor development in tulum cheese (Hayaloglu, 2007b). In our study, the

Table 4. Some technological characteristics of LAB strains isolated from the cheese samples.

Strains identified	*n*	pH of UHT Milk (Acidifying activity) 6 h Mean	6 h Range	8 h Mean	8 h Range	24 h Mean	24 h Range	Proteolytic activity (zone of proteolysis in mm) Mean	Proteolytic activity Range	Production of H_2O_2 (µl/ml) Mean	Production of H_2O_2 Range	Diacetyl producers
Lb. paracasei	42	6. 3	5.9– 6.5	6.1	5.2– 6.5	4.8	3.8– 6.5	13.0	6.0– 20	1.1	0.3– 3.0	18
Lb. plantarum	23	6.2	5.8– 6.5	5.9	4.7– 6.5	4.7	3.7– 6.5	12.9	0– 21	1.2	0.4– 2.9	7
S. thermophilus	6	6.4	6.3– 6.6	6.2	5.8– 6.6	4.6	3.9– 6.4	15.3	12– 22	1.2	1.0– 1.3	3
E. durans	6	6.3	6.1– 6.5	6.2	5.9– 6.5	5.2	4.5– 6.5	16.0	7.0– 22	1.0	0.9– 1.1	4
Lb. brevis	5	6.2	6.0– 6.4	5.8	5.4– 6.3	4.8	4.6– 5.1	15.0	11– 21	0.8	0.5– 1.1	–
Lb. fermentum	4	6.4	6.2– 6.5	6.3	6.1– 6.4	4.8	4.5– 5.3	15.8	19– 14	1.3	1.1– 1.6	–
E. faecium	5	6.2	6.1– 6.3	6.0	5.9– 6.0	4.5	4.5– 4.5	15.7	10– 24	1.1	1.0– 1.1	1
Lb. pentosus	1	6.4	6.4	6.1	6.1	4.7	4.7	15.0	15.0	1.0	1.0	1
Unidentified	5	6.1	5.9– 6.3	5.9	5.3– 6.1	4.5	4.0– 4.9	9.7	3– 14	0.7	0.5– 1.1	4

Table 5. Enzymatic profiles of LAB strains isolated from the cheese samples.

Enzyme name	Number of strains showed enzyme activity *Lb. par. (n=42)*	*Lb. pla. (n=23)*	*S. ther. (n=6)*	*E. dur. (n=6)*	*Lb. bre. (n=5)*	*Lb. fer. (n=4)*	*E. fae. (n=5)*	*Lb. pen.(n=1)*	*Un-identified (n=5)*	Total
Alkaline phosphatase	9	5	1	–	–	1	–	–	–	16
Esterase	30	11	5	4	3	4	5	–	5	67
Esterase Lipase	34	13	4	6	5	4	5	–	5	76
Lipase	3	–	–	–	–	–	–	–	–	3
Leucine arylamidase	42	23	6	6	5	4	5	1	5	97
Valine arylamidase	42	22	6	6	5	4	5	1	5	96
Cystine arylamidase	14	16	2	1	3	2	1	–	1	40
Trypsin	–	–	–	–	–	–	–	–	–	–
α- Chymotrypsin	3	2	1	–	–	1	–	–	1	8
Acid phosphatase	39	20	6	6	5	4	5	–	5	90
Naphthol-AS-BI-phosphohydrolase	42	23	6	6	5	4	5	1	5	97

Table 5 cont.

α- Galactosidase	32	10	3	5	5	3	5	–	5	68
β- Galactosidase	41	22	6	6	5	4	2	1	5	92
β- Glucuronidase	24	6	2	2	4	1	1	–	5	45
α- Glucosidase	41	21	6	6	5	4	5	1	5	94
β- Glucosidase	42	21	6	6	5	4	5	1	5	95

–: No activity.

majority of lactobacilli showed esterase (65%) and esterase-lipase (75%) enzyme activities within 24 h, but lipase activity was observed in only 3 (3.1%) isolates. While all enterococci had esterase-lipase activity, only 78% showed lipase activity. Esterase and esterase-lipase enzyme activities were observed in 60 and 90% of streptococci, respectively. When we address the situation in terms of Kargi tulum production on an industrial scale, a large number of isolates which are likely starter cultures and have low lipase, high esterase and esterase-lipase enzyme activities were obtained in our study.

β-Galactosidase (lactase) is an enzyme found in milk that hydrolyzes lactose to galactose and glucose monosaccharides. Almost all of our isolates showe β-galactosidase and β-glucosidase enzyme activities.

Conclusions

Kargi tulum cheese is an artisanal cheese produced by small family dairies using raw milk. It is consumed locally, after being ripened in the uplands for 3 to 4 months in a goat-skin bag. Such products produced though natural fermentation are an important part of Turkey's cultural heritage. However, no scientific research has been conducted on the indigenous LAB that play a role in the fermentation of this cheese. In our study, homofermentative strains isolated from Kargi tulum cheese samples such as *L. paracasei*–19 and 111; *L. plantarum*–10, 45 and 71; *S. thermophilus*–59; *E. durans*–9 and 106; and *E. faecium*–24 and 37 were selected as potential starter cultures. These strains were tolerant to 6.5% salt; had a fast acidifying capacity; produced diacetyl and H_2O_2; had low proteinase and lipase activities but high peptidase, esterase and esterase-lipase activities; and had β-galactosidase and β-glucosidase activities. Starter cultures prepared from various combinations of the aforementioned indigenous LAB strains can be used in Kargi tulum cheese production with pasteurized milk. The most appropriate starter culture combination to give the traditional texture, flavor and taste to artisanal Kargi tulum cheese should be determined and safe production of the cheese for consumers at industrial scale with standardized quality will be possible. However, further study is required to evaluate the selected strains, individually and in mixed cultures, on a pilot scale.

Before use in industrial production, it would also be beneficial to determine the phage resistance of the selected starter strains.

ACKNOWLEDGEMENT

This research was supported by the Scientific Research Projects Commission of Eskişehir Osmangazi University (Project number: 2007-19027).

REFERENCES

Arora G, Lee BH, Lamoureux M (1990). Characterization of enzyme profiles of *Lactobacillus casei* species by a rapid API ZYM system, J. Dairy Sci. 73: 264-273.

Bayar N, Ozrenk E (2011). The effect of quality properties on Tulum cheese using different package materials, Afr. J. Biotechnol. 10: 1393-1399.

Bostan M, Ugur M, Ciftcioglu G (1992). Tulum peynirinde laktik asit bakterileri ve kuf florasi. IU Veteriner Fakultesi Dergisi. 17: 111-118.

Broome MC, Hickey MW (1991). Peptidase activity of non-starter lactobacilli, Aust. J. Dairy Technol. 46: 19-23.

Colak H, Hampikyan H, Bingol EB, Ulusoy B (2007). Prevalence of *L. monocytogenes* and *Salmonella* spp. in

Tulum cheese. Food Control, 18: 576-579.
Coskun H (2000). Peynirlerin olgunlaşmasında starterlerin rolü. Süt Mikrobiyolojisi ve Katkı Maddeleri. VI. Süt ve Süt Ürünleri Sempozyumu Tebliğler Kitabı, pp. 47-56, Tekirdağ.
Dinkci N, Akalin AS, Gonc S, Unal G (2007). Isocratic Reverse-Phase HPLC for determination of organic acids in Kargi Tulum Cheese. Chromatographia Supplement. 66: S45-S49.
Efe A, Heperkan D (1995). *Tulum peynirlerinde* patojen bakteriler, In: II. Gida Muhendisligi Ulusal Sempozyumu, Ankara, Turkey, pp. 45-54.
Garabal JI, Rodríguez-Alonso P, Centeno JA (2008). Characterization of lactic acid bacteria isolated from raw cows' milk cheeses currently produced in Galicia (NW Spain). Food Sci. Technol.-LEB, 41: 1452–1458.
Gordon RE, Haynes WC, Pang CHN (1973). The Genus *Bacillus*, Handbook United States Department of Agriculture,Washington, D.C. p 427.
Halkman AK (1991). Tarim Mikrobiyolojisi. Ankara Üniversitesi Ziraat Fakültesi Yayınları No: 1214. Ankara Üniversitesi Baskı Ofset Ünitesi. Ankara, p. 82
Halkman AK, Taskın Y (2001). Süt Endüstrisinde Starter Kültür. Gıda Mühendisliği Dergisi. 5: 13-18.
Hayaloglu AA, Cakmakci S, Brechany EY, Deegan KC, McSweeney PLH (2007a). Microbiology, biochemistry and volatile composition of *Tulum cheese* ripened in goat's skin or plastic bags. J. Dairy Sci. 90: 1102–1121.
Hayaloglu AA, Fox PF, Guven M, Cakmakcı S (2007b). Cheeses of Turkey: 1. Varieties ripened in goat-skin bags. Lait. 87: 79-95.
Hegazi FZ, Abo-Elnaga IG (1980). Production of acetoin and diacetyl by lactic acid bacteria in skimmed milk with added citrate and pyruvate. Z. Lebensm. Unters. F. A. 171: 367-370.
King N (1948). Modification of the Voges– Proskauer test for rapid colorimetric determination of acetylmethyl carbinol plus diacetyl in butter cultures. Dairy Ind. 13: 860-866.
McSweeney PLH (2004). Biochemistry of cheese ripening. Int. J. Dairy Technol. 57: 127-144.
Messens W, De Vuyst L (2002). Inhibitory substances produced by Lactobacilli isolated from sourdoughs-a review. Int. J. Food Microbiol. 72: 31-43.
Oksuztepe G, Patir B, Calicioglu M (2005). Identification and distribution of lactic acid bacteria during the ripening of Savak *Tulum cheese*. Turk. J. Vet. Anim. Sci. 29: 873-879.
Olasupo NA, Schillinger U, Holzapfel WH (2001). Studies on some technological properties of predominant lactic acid bacteria isolated from Nigerian fermented foods. Food Biotechnol. 15: 157-167.
Perez-Elortondo FJP, Aldamiz EP, Albisu M, Barcina Y (1998). Indigenous lactic acid bacteria in Idizabal ewes' milk cheese. Int. Dairy J. 8: 725-732.
Sengul M, Cakmakci S (2003). Characterization of natural isolates of lactic acid bacteria from Erzincan (Savak) *Tulum cheese*. Milchwissenschaft . 58: 510-513.
Sisto A, Bellis P, Visconti A, Morelli L, Lavermicocca P (2009). Development of a PCR assay for the strain-specific identification of probiotic strain *Lactobacillus paracasei* IMPC2.1. Int. J. Food Microbiol. 136: 59-65.
Tabasco R, Paarup T, Janer C, Pelaez C, Requena T (2007). Selective enumeration and identification of mixed cultures of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *Bifidobacterium lactis* in fermented milk. Int. Dairy J. 17: 1107-1114.
Torres-Llanez MJ, Vallejo-Cordoba B, Diaz-Cinco ME, Mazorra-Manzano MA, Gonzalez-Cordova AF (2006). Characterization of the natural microflora of artisanal Mexican Fresco cheese. Food Control, 17: 683-690.
TS 591 (2006). Standard of White Cheese. Turkish Standards Institution, Ankara, Turkey.
TS 3001 (2006). Tulum Cheese. Turkish Standards Institution, Ankara, Turkey.
TS 3046 (1978). Cheese-Determination of Fat Content-Van Gulik Method. Turkish Standards Institution, Ankara, Turkey.
TS EN ISO 5534 (2006). Cheese and processed cheese-Determination of the total solids content. Turkish Standards Institution, Ankara, Turkey.
TS EN ISO 5943 (2001). Cheese and processed cheese products Determination of chloride content- Potentiometric titration method. Turkish Standards Institution, Ankara, Turkey.
TS EN ISO 8968-3: ISO 8968-3 (2004). Milk. Determination of nitrogen content. Part 3: Block-digestion method. Turkish Standards Institution, Ankara, Turkey.
Walter J, Tannock GW, Tilsala-Timisjarvi A, Rodtong S, Loach DM, Munro K, Alatossava T (2000). Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. Appl. Environ. Microbiol. 66: 297-303.
Whittenbury R (1964). Hydrogen peroxide formation and catalase activity in the lactic acid bacteria. J. Gen. Microbiol. 35: 13-26.