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Research Article

Identification and Distribution of Lactic Acid Bacteria During the Ripening of Şavak Tulum Cheese

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Abstract: This study was undertaken to determine changes in the flora of lactic acid bacteria during the ripening of Şavak tulum cheese and to identify the major species playing an important role in the ripening process. Tulum cheese was produced from raw sheep milk using traditional methods and ripened in plastic containers at 4 °C for 90 days. Samples were taken on days 0, 15, 30, 60 and 90 and analyzed for isolation and identification of lactic acid bacteria. A total of 783 isolates of lactic acid bacteria were identified. The results indicated that, in general, lactic streptococci were predominant within the first month of ripening and, then, replaced by lactobacilli. It is concluded that *Lactobacillus casei* subsp. *casei*, *Lactobacillus plantarum* from the family Lactobacillaceae and *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, and *Leuconostoc mesenteroides* subsp. *cremoris* from the family Streptococcaceae were the predominant species among the isolates, indicating that these species may play a major role in the ripening of tulum cheese.

Key Words: Tulum cheese, ripening, lactic acid bacteria.

Şavak Tulum Peynirinin Olgunlaşması Sırasında Laktik Asit Bakterilerinin Dağılımı ve İdentifikasyonu

Özet: Bu çalışma Şavak tulum peynirinin olgunlaşmasında önemli rol oynayan türleri belirlemek ve olgunlaşma esnasında laktik asit bakteri florasında meydana gelen değişimleri araştırmak amacıyla yapıldı. Tulum peyniri geleneksel metotlar kullanılarak çiğ koyun sütünden üretildi ve plastik bidonlarda 4 °C'de 90 gün olgunlaştırıldı. Örnekler, 0, 15, 30, 60 ve 90.günlerde alındı ve laktik asit bakterilerinin izolasyon ve identifikasyonu için analiz edildi. Toplam 783 laktik asit bakteri izolatı identifiye edildi. Sonuçlar gösterdiki; genel olarak olgunlaşmanın ilk ayında laktik streptekoklar, daha sonra laktobasiller baskın bulundu. İzolatlar içerisinde Lactobacillaceae familyasından *Lactobacillus casei* subsp. *casei* ve *Lactobacillus plantarum*, Streptococcaceae familyasından *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, ve *Leuconostoc mesenteroides* subsp. *cremoris*'in predominant olduğu ve tulum peynirinin olgunlaşmasında önemli bir rol oynayabileceği sonucuna varılmıştır.

Anahtar Sözcükler: Tulum peyniri, olgunlaşma, laktik asit bakterisi.

Introduction

Şavak tulum cheese is a popular semi-hard cheese (e.g., 43% moisture) in Turkey manufactured from unpasteurized sheep milk. This particular type of cheese is the third most produced among the cheese types manufactured in Turkey. Şavak tulum cheese used to be produced and distributed locally. At present, demand for this cheese has increased and the annual production reached 4000 t due to its high nutritional value and highly recognized flavor and aroma. Since 1987, tulum cheese has been among the export products of Turkey (1-3).

Şavak tulum cheese is generally produced from raw sheep milk in rural areas in Eastern Turkey. Villagers in this region produce tulum cheese using traditional methods and market them after 15 to 90 days of ripening, depending on the market demand. Traditionally, the packing material is a container made of goat skin. However, in the recent years, ripening of the cheese in plastic containers has become widespread in the industry. Microbiological, chemical, and physical aspects of the cheese may vary depending on the plant, experience of the personnel, and quality of raw milk. As a result, a standard quality of product cannot be produced (4-8).

Since tulum cheese is produced by traditional methods in small-scale establishments, starter cultures are usually not used in production and fermentation depends on indigenous bacterial flora. There are very limited data on flora of lactic acid bacteria during the manufacture and ripening of tulum cheese. For example, Karasoy (9) reported that *Streptococcus lactis* played a role in the initial ripening while *Streptococcus lactis*, *Thermobacterium bulgaricum*, and *Oidium lactis* together were the essential flora of the actual ripening. In another study, Bostan et al. (10) experimentally produced tulum cheese from raw cow's milk and isolated a total of 684 strains of lactic acid bacteria at various stages of ripening. Their results showed that *Streptococcus lactis* and *Streptococcus faecalis* were the predominant flora during the initial phase whereas *Streptococcus faecium*, *Streptococcus lactis*, *Lactobacillus casei*, and *Lactobacillus plantarum* predominated in later stages of the ripening period. In the study, it was noted that Leuconostoc and Pediococcus species existed but at low levels. In some studies, tulum cheese was produced using various starter culture combinations. For example, Bostan (4) reported that the combination of *Lactobacillus casei* and *Streptococcus lactis* was the most appropriate among many other cultures tested. In another study, Patır et al. (8) reported that *Streptococcus cremoris*, *Streptococcus lactis*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus curvatus*, *Leuconostoc cremoris*, *Streptococcus faecium*, and *Streptococcus faecalis* might play an important role in ripening of tulum cheese. Bostan et al. (10) surveyed some commercial tulum cheese samples of premium quality for isolation and identification of lactic acid bacteria and reported that, of the 426 isolates, the majority was *Streptococcus faecium*, *Lactobacillus casei*, *Streptococcus faecalis*, *Streptococcus lactis*, *Lactobacillus plantarum*, *Leuconostoc cremoris*, and *Lactobacillus curvatus*. In a similar study by Sürmeli et al. (11), it was indicated that bacterial species found in commercial tulum cheese was in the order of enterococci, lactic acid bacteria, pyogenic and viridians streptococci and Leuconostoc spp.

Results of the above studies on lactic acid bacterial flora are quite diverse and there are some conflicting data. The objective of the present study was to determine the species of lactic acid bacteria most important for flavor and aroma development during the ripening of traditionally produced Şavak tulum cheese for providing useful data to develop a starter culture blend that can be used by the manufacturers of this particular type of cheese.

Materials and Methods

Raw Sheep Milk

Raw sheep milk was obtained from the Dairy Sheep Unit of the Research and Training Farm of the Faculty of Veterinary Medicine, Fırat University, Elazığ, Turkey. The milk was transferred to the milk-processing laboratory within approximately 1 h after morning milking. A 200 ml portion was taken for microbiological and chemical analysis of the raw milk. Briefly, numbers of total aerobic mesophile bacteria, Lactobacillus-Leuconostoc-Pediococcus, lactococci, enterococci, Staphylococcus-Micrococcus, coliform, and mold-yeast were determined by pour-plate method on to appropriate media as described previously (12-14). The samples were analyzed for titrable acidity (% lactic acid) and levels of fat and dry matter (15). The remaining volume of milk was used for making Şavak tulum cheese.

Determination of Inhibitory Substances in Raw Milk

Inhibitor substances in the milk was detected using fermentation test (16). Milk that contained any inhibitor was rejected.

Manufacture of Traditional Tulum Cheese

Milk that was free of any inhibitory substance was filtered and sufficient amount of rennet (approximately 1.85 ml of rennet at the strength of 1/6000 per 25 kg of milk) was added according to the manufacturer's recommendation. The milk coagulated within approximately 90 min. The resulting curd was cut into 5 x 5 cm pieces and poured onto cheesecloth on a colander for draining of whey and then pressed (first press) using metal weights. After 24 h under press, curd was manually broken to pieces as small as a chickpea and 2% (w/w) salt was added and mixed followed by a second pressing in cheesecloth for 24 h at ambient temperature. Steps at first press were repeated for a third press. At the end of the third press, curds were broken to small pieces one more time and air-dried for approximately 24 h at room temperature. The resulting curd then was filled into plastic containers (750 g) and squeezed tightly to remove excessive air using a wooden stick. The plastic containers

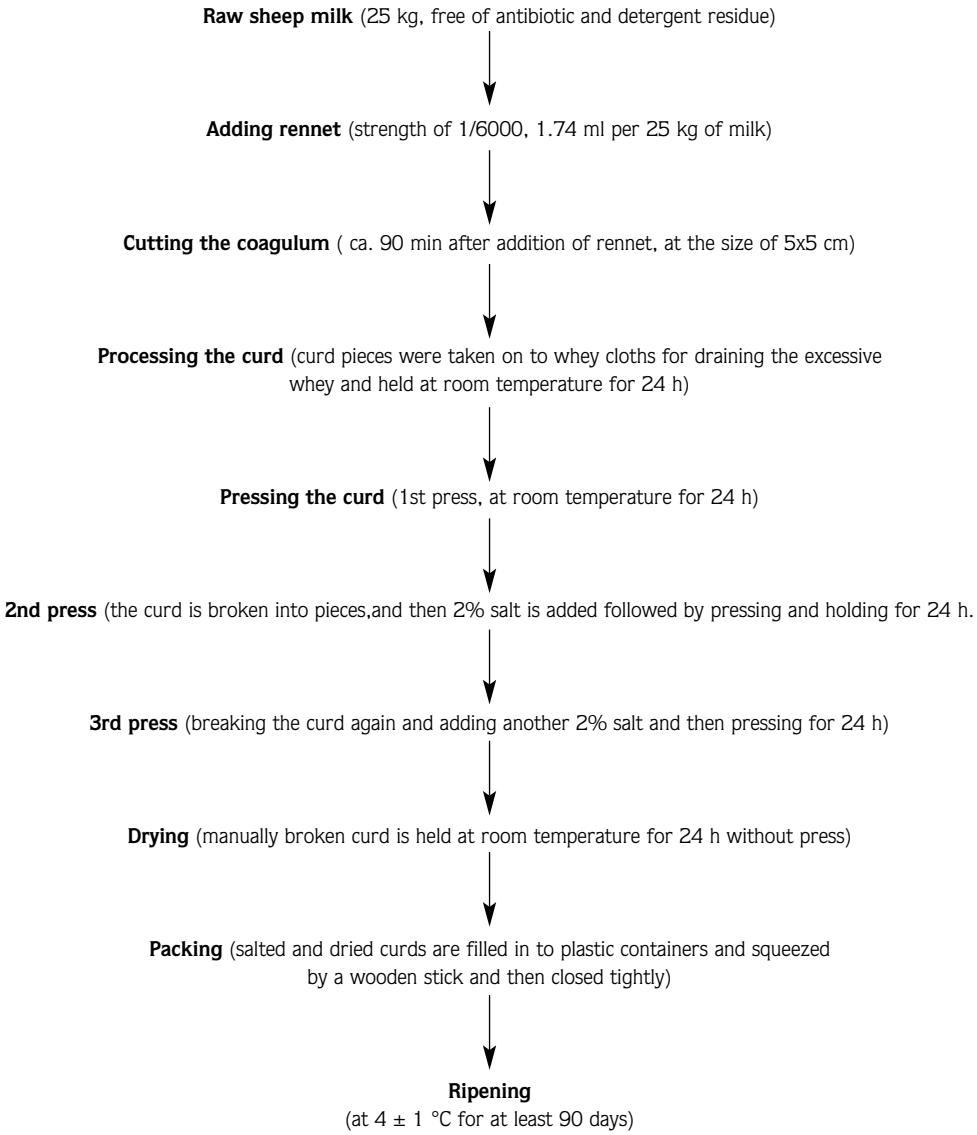

Figure. Flow chart for manufacture of Savak tulum cheese.

were closed up tightly with aluminum foil and stored at 4 ± 1 °C for 90 days for ripening (15,17,18). The same cheese container was used for sampling at different intervals. The study was conducted in 5 replicates.

Preparation of Samples

Samples were taken for microbiological analysis on days 0, 15, 30, 60, and 90 during the ripening period. Portions of 150-200 g were taken on each sampling interval using a sterile knife and transferred into a sterile jar. The samples were homogenized by a sterile spatula in the jar prior to weighing a 10 g portion and transferring to a sterile container of homogenizer (Buhler 51800/00). A 90 ml volume of sterile 2% sodium citrate was added to the 10 g sample and homogenized for 1/10 dilution. This homogenate was then decimally diluted using Ringer solution up to 10^{-8}. One ml from each dilution was pour-plated in duplicate onto M17 agar (Merck - 15108) and onto Rogosa's acetate agar (Oxoid – CM 627). Both types of plates were incubated at 30 ± 1 °C for 72 h and 5 days for M17 plates and Rogosa's acetate agar plates, respectively (12,13,16).

Identification of Lactic Acid Bacteria

Colonies from M17 or Rogosa'a acetate agar plates were selected from plates with colony numbers between 30 and 300. These plates were divided into 8 equal areas and all individual colonies were transferred into separate tubes of yeast extract containing 0.5% glucose (YGB) for colonies isolated from M17 agar plates and into MRS broth (LABM – LAB 94) for colonies isolated from Rogosa acetate agar plates. YGB and MRS broths were incubated at 30 °C for 48 h. All cultures were tested for Gram staining and catalase activity. Cultures that were Gram (+) and catalase (-) were evaluated as lactic acid bacteria (13,14,19). Cultures of lactic acid bacteria were further tested for growth temperature (10 °C, 15 °C, 45 °C) (13,20-23), for catalase activity ammonia formation from arginine (13), for Voges-Proskauer (13,24), CO_2 from glucose litmus milk (13), for acid production from L-arabinose, dextrose, maltose, mellebiose, saccarose, sellebiose, sorbitol, and trehalose (13).

Results

Results of microbiological and chemical analysis of raw sheep milk used in the production of tulum cheese are presented in Table 1. Microbiological analysis indicated that the milk was of poor hygiene, due especially to high coliform counts. A total 783 of 851 isolates obtained from 5 replicates during the ripening period were identified as lactic acid bacteria. Sixty-eight isolates (approximately 5% to 12% of isolates, depending on the sampling day) could not be identified. In general, the percentage of the species in Streptococcaceae was higher at the beginning of the ripening and then gradually decreased while that of species in Lactobacillaceae was low but slightly increased as the ripening progressed. Detailed distributions of species according to the taxonomic family and ripening period are listed in Tables 2 and 3.

Discussion

The use of raw milk with high coliform count for production of Şavak tulum cheese can never be recommended as part of hygienic cheese manufacturing. The high level of coliform found in milk used for tulum cheese production reflects the hygienic condition of milk commonly used for production of tulum cheese in Eastern Turkey. However, it has been reported that the level of coliform bacteria gradually decreases during ripening of traditional tulum cheese (4,25).

Our results indicated that the flora of lactic acid bacteria was changing as the ripening period progress, as expected. In general, a floral shift occurred between members of the family Streptococcaceae that was predominant within the first month of ripening and Lactobacillaceae family that over dominated after day 30 (Tables 2, 3). This floral shift might be the result of a synergism between members of the two families, as occurs in yogurt.

Among members of the family Streptococcaceae, Enterococcus spp., *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, and *Leuconostoc mesenteroides* subsp. *cremoris* possibly played important roles in the ripening as the major species (Table 3). Enteroccoci, in particular, was found at high levels, as estimated by the percentage (ca. 19% to 34% of all isolates), during the entire ripening period (Table 3). This finding is consistent with the results reported by Bostan et al. (10) that 22% to 33% of isolates were identified as enterococci during the ripening of tulum cheese produced from raw milk. Relatively high-level presence of enterococci might be due to acid-tolerant and halophilic members of enterococci such as *Streptococcus faecium*, *Streptococcus faecalis*, and *Streptococcus thermodurans.* The role of enterococci in the ripening of cheese, particularly in aroma and flavor development, has not been clearly established yet (26). However, there are

Table 1. Results of the microbiological and chemical analysis of raw sheep milk used for production of Şavak tulum cheese (means, n=5).

Numbers of Microorganisms (CFU/ml)							Chemical Analysis (%)		
Total Mesophilic Aerobes	Lactobacillus Leuconostoc Pediococcus	Lactococcus	Enterococcus	Staph.-Micrococ	Coliforms	Mold Yeast	TA lactic acid)	Fat	Dry matter
2.2×10^7	3.7×10^5	1.0×10^7	3.3×10^3	1.4×10^4	6.2×10^5	2.7×10^4	0.176	7.1	18.22

Table 2. Distribution of species in the family *Streptococcaceae* isolated from Şavak tulum cheese during ripening at 4 °C.

Species	Ripening (days)					
	Curd	0	15	30	60	90
Lactococcus lactis subsp. *cremoris*	13[a] (11.4)[b]	6 (5.0)	11 (9.9)	5 (6.9)	1 (1.6)	1 (1.5)
Lactococcus lactis subsp. *lactis*	22 (19.3)	26 (21.7)	15 (13.5)	10 (13.5)	4 (6.5)	2 (3.1)
Lactococcus lactis subsp. *lactis* biovar diacetylactis	1 (0.9)	1 (0.8)	6 (5.4)	3 (4.2)	1 (1.6)	–
Leuconostoc mesenteroides subsp. *cremoris*	9 (7.9)	6 (5.0)	9 (8.1)	7 (9.7)	10 (16.1)	10 (15.4)
Leuconostoc lactis	6 (5.3)	11 (9.2)	7 (6.3)	2 (2.8)	1 (1.6)	2 (3.1)
Leuconostoc mesenteroides subsp. *dextranicum*	12 (10.6)	7 (5.8)	8 (7.2)	4 (5.6)	1 (1.6)	–[c]
Leuconostoc mesenteroides subsp. *mesenteroides*	2 (1.8)	1 (0.8)	1 (0.9)	–	–	–
Pediococcus spp.	1 (0.9)	3 (2.5)	7 (6.3)	6 (8.3)	4 (6.5)	2 (3.1)
Enterococcus	31 (27.2)	46 (38.3)	33 (29.7)	27 (37.5)	34 (54.8)	39 (60)
Unidentified isolates	17 (14.9)	13 (19.8)	14 (12.6)	8 (11.11)	7 (11.3)	9 (13.8)
Total numbers of isolates	114	120	111	72	62	65

[a], Total numbers of isolates for a particular family or genus from 5 trials of tulum cheese production.
[b], Percentage of numbers of isolates based on total numbers of *Streptococcaceae* isolated at a particular sampling interval.
[c], None detected in 10 g sample

quite a few studies claiming that enterocci play an essential role in the ripening of cheese by their proteolytic, lipolytic and acidulating effects, and therefore can be used as starter cultures in cheeses (27-30). *Lactococcus lactis* subsp. *cremoris* apparently more rapidly lost its viability, as estimated by the percentage, compared to *Lactococcus lactis* subsp. *lactis*, and *Leuconostoc mesenteroides* subsp. *cremoris*. All the other species found at low percentages in the family Streptococcaceae including Pediococcus spp. and *Leuconostoc mesenteroides* subsp. *mesenteroides* might be considered to have minor effects in the ripening of tulum cheese (Table 2).

In general, the percentage of species in Lactobacillaceae increased after 30 days of ripening. This increase was quite evident for *Lactobacillus casei* subsp. *casei* (Table 3). *Lactobacillus plantarum* did not appreciably change during the ripening. All other lactobacillus species were either found at low levels, or identified at only some parts of the ripening (Table 3).

Table 3. Distribution of species in the family *Lactobacillaceae* isolated from Şavak tulum cheese during ripening at 4 °C.

Species	Ripening (days)					
	Curd	0	15	30	60	90
Lactobacillus casei subsp. *casei*	11[a] (36.7)[b]	5 (29.4)	13 (32.5)	35 (49.3)	37 (44.0)	33 (50.8)
Lactobacillus plantarum	10 (33.33)	6 (35.3)	8 (20.0)	16 (22.5)	25 (29.8)	21 (32.3)
Lactobacillus curvatus	–[c]	1 (5.9)	6 (15)	5 (7.0)	7 (8.3)	6 (9.2)
Lactobacillus helveticus	1 (3.33)	1 (5.9)	1 (2.5)	1 (1.4)	–	–
Lactobacillus delbrueckii subsp. *lactis*	2 (6.66)	2 (11.8)	6 (15.0)	2 (2.8)	2 (2.4)	–
Lactobacillus delbrueckii subsp. *bulgaricus*	6 (20.0)	2 (11.8)	3 (7.5)	4 (5.6)	–	–
Lactobacillus fermentum	–	–	2 (5.0)	3 (4.2)	7 (8.3)	4 (6.2)
Lactobacillus buchneri/brevis	–	–	1 (2.5)	5 (7.0)	5 (6.0)	1 (1.5)
Total numbers of isolates	30	17	40	71	84	65

[a], Total numbers of isolates for a particular family or genus from 5 trials of tulum cheese production.
[b], Percentage of numbers of isolates based on total numbers of *Lactobacillaceae* isolated at a particular sampling interval.
[c], None detected in 10 g sample.

In conclusion, the results of the present study indicate that the floral shift between Streptococcaceae and Lactobacillaceae suggest a synergism and that a potential starter culture blend for tulum cheese should include species from each of the two families. Species for such combination could be *Lactobacillus casei* subsp. *casei*, *Lactobacillus plantarum* for Lactobacillaceae and *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis* and *Leuconostoc mesenteroides* subsp. *cremoris* for Streptococcaceae. Nevertheless, further studies are required for testing pure cultures of these species and sensory attributes should be evaluated. The results of the present study may provide useful data for development of starter culture blends suitable for production of Şavak tulum cheese.

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