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Lactic Flora of Local Foods Produced in Kars Region*

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Abstract: The aim of this study was to analyze the indigenous lactic microbiota of local food produced in the Kars region for identification and for creating a culture collection of the natural flora strains obtained. A total of 3700 samples were collected from a total of 80 points at the Kars city center and districts. From 3700 food samples collected, 9160 isolates were obtained. Upon basic identification tests, it was determined that out of 9160 isolates, 3846 (41.9%) were lactic acid bacteria (LAB) among which 1249 could not be cultured. In a total of 3700 samples, 2597 LAB were isolated. These traditional products made from the milk and meat of animals grazed out in the fresh pasture during the high plateau period were found to be quite rich in lactic flora. No commercial starter culture was used in the production of such products; the natural raw milk and meat flora was utilized. The identification of LAB, known as probiotics, in food samples reveals the importance of these foods for the local people in terms of nutrition.

Keywords: Cheese, Lactic acid bacteria, PCR, Traditional food, Yogurt.

Kars Yöresinde Üretilen Yerel Gıdaların Laktik Florası

Öz: Bu çalışmada Kars ve yöresinde geleneksel yöntemler ile üretilen yöresel fermente gıdaların doğal laktik asit bakteri florası araştırılarak bu laktik mikrofloranın tanımlanması ve elde edilen doğal suşlardan bir laktik asit bakterileri kültür koleksiyonu oluşturulması amaçlanmıştır. Bu amaçla Kars il merkezi ve bağlı ilçelerinde olmak üzere toplam 80 farklı noktadan 3700 adet örnek toplanmıştır. Toplanan 3700 adet geleneksel gıda örneğinden toplam 9160 adet izolat elde edilmiştir. Kültürel yöntemler ile yapılan temel identifikasyon testleri sonucunda 9160 adet izolattan 3846'sı (%41.9) laktik asit bakterisi olarak belirlenmiş olup bunlardan da 1249'u kültüre edilememiştir. Toplam 3700 adet örnekten kültürel ve genetik yöntemler ile 2597 adet laktik asit bakterisi izole edilmiştir. Meralarda açık havada taze otla beslenen hayvanlardan yayla döneminde iken alınan süt ve et ile yapılan bu geleneksel ürünlerde laktik flora oldukça zengin bulunmuştur. Bu geleneksel ürünlerin yapımında ticari starter kültür kullanılmamakta, çiğ sütte ve çiğ ette bulunan doğal floradan yararlanılmaktadır. Toplanan gıda örneklerinde probiyotik olarak da bilinen laktik asit bakterilerinin identifiye edilmiş olması bu fermente gıdaların beslenme açısından yöre halkı için önemini ortaya koymaktadır.

Anahtar Kelimeler: Geleneksel gıdalar, Laktik asit bakterileri, PCR, Peynir, Yoğurt.

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INTRODUCTION

Animal husbandry is the principal source of income in the Kars region located in northeast Turkey. The milk collected from animals at high plateaus is processed into various types of cheese at the temperature of raw milk at collection without heat treatment. For kashar cheese (raw milk), butter and yogurt, production is typically conducted at households or dairy farms. As ripening is carried out by indigenous microorganisms coming from raw milk, the quite different microbiota the animals receive during vegetation by grazing at high altitudes also passes to the products. As for sausage production, no heat treatment is applied during the process and is ripened by the natural lactic flora of meat. There are thousands of different dairy and meat products produced across the world by fermenting raw milk by nonstarter lactic acid bacteria (NSLAB). Studies have investigated many characteristics of these bacteria in fermented food, such as their effect on flavor, aroma, and taste (1-3). The aroma of dairy products produced from raw milk is more dominant than that of cheese coming from pasteurized milk, hence more preferable for consumers. Geographical region and technological process are also quite effective on the number and type of NSLAB in fermented food (4-5). Although standard production is mandatory for simplifying the production process, improving quality and consumer trust, many known food producers are now inclined towards natural products and have launched new products identified as “home-made” or “village-made.” However, although food production using indigenous flora satisfies the requirements in terms of flavor, it will nevertheless be unacceptable due to the risks natural fermentation may entail (6). The most corrected method to overcome this existing deficiency is to identify the main microbiota of traditional food, create a culture collection through such flora and conduct safe food production by using this collection in a way agreeable to the local palate. This study aimed to analyze the indigenous lactic microbiota of the local food produced in the Kars region, which is in high demand, identify such microbiota, and create a culture collection of the indigenous strains obtained.

MATERIALS and METHODS

Food Samples

Food samples to be analyzed in this study were collected from the villages of the Kars’s provincial center and its districts. In each collection point determined, the sampling was conducted to cover only food of animal origin “produced by traditional methods” (chechil cheese, white cheese, tulum cheese, butter, yogurt, kashar cheese, sausage). In this region, dairy products and sausages were produced from raw milk and meat without adding any starter culture. Food samples were collected between 2009 and 2010 from May to December (the pastoral animal husbandry period) (Table 1). The traditionally produced sausage and kashar cheese samples were supplied from the dairy stores and local butchers’ located at Kars city center. All other samples were collected not from commercial sales points but individually from the households producing the respective foods.

Table 1. Sample distribution.
Tablo 1. Örnek dağılımı.

At least 9 samples for each food type (7 different foods) were collected from each village	
Kars (9 villages and the city center)	450 samples (chechil cheese, white cheese, tulum cheese, butter, yogurt) 50 sausage samples and 50 kashar cheese samples (Sausage and kashar cheese samples were collected only at Kars city center)
Akyaka (9 villages and the district center)	450 samples (chechil cheese, white cheese, tulum cheese, butter, yogurt)
Arpaçay (9 villages and the district center)	450 samples (chechil cheese, white cheese, tulum cheese, butter, yogurt)
Digor (9 villages and the district center)	450 samples (chechil cheese, white cheese, tulum cheese, butter, yogurt)
Kağızman (9 villages and the district center)	450 samples (chechil cheese, white cheese, tulum cheese, butter, yogurt)
Sarıkamış (9 villages and the district center)	450 samples (chechil cheese, white cheese, tulum cheese, butter, yogurt)
Selim (9 villages and the district center)	450 samples (chechil cheese, white cheese, tulum cheese, butter, yogurt)
Susuz (9 villages and the district center)	450 samples (chechil cheese, white cheese, tulum cheese, butter, yogurt)
80 collection points in total	3700 samples

Reference Strains

In PCR, as positive control, the reference strains used for tests are shown in Table 2. A PCR mix without genomic DNA was used as negative control in PCR.

Table 2. Primers of the study.
Tablo 2. Çalışmada kullanılan primerler.

LAB	Primers	Reference
Lb.plantarum NRRL-B 4496	plan F (5'- CCG TTT ATG CGG AAC ACC TA-3') pREV (5'- TCG GGA TTA CCA AAC ATC AC-3')	12
Lc.lactis spp lactis CECT4432	LacreR (5'-GGGATCATCTTTGAGTGAT-3') LacF (5'-GTACTTGTACCGACTGGAT-3')	13
Lb.acidophilus ATCC 4356	LacidoF (5'-CACTTCGGTGATGACGTTGG-3') LacidoR (5'-CGATGCAGTTCCTCGGTTAAGC-3')	14
Lb.brevis NRRL-B 4327	LbLMA1-rev (5'-CTCAAAACTAAACAAAGTTTC-3') Lbbrv-F (5'-TTGAAACAATGTTCAGTTTTGAGGGGC-3')	15
Lb.rhamnosus ATCC 53103	Rham1 (5'-GTCGAACGAGTTCTGATTATTG-3') RhamR (5'-GAACCATGCGGTTCTTGGAT-3')	14
Lb.johnsonii ATCC 33198	LbLMA1-rev (5'-CTCAAAACTAAACAAAGTTTC-3') Lbjoh-F (5'-GAGAAACTTTGTTTAGTTTTGAGGGTA-3')	15
Lb.bulgaricus ATCC 7993	LbLMA1-rev (5'-CTCAAAACTAAACAAAGTTTC-3') Lbbul-F (5'-AAGAAACTTTGTTCAGTTTTGAGAGTA-3')	15
Lb.casei CH1	LbLMA1-rev (5'-CTCAAAACTAAACAAAGTTTC-3' Lbca-F (5'-ACGAAACTTTGTTTAGTTTTGAGGGGA-3')	15
Lb.helveticus ATCC 15009	LbLMA1-rev (5'-CTCAAAACTAAACAAAGTTTC-3' Lbhlv-F (5'-GAGAAACTTTGTTTAGTTTTGAGGGTA-3')	15
Leuc.mesenteroides ATCC 8293	Lmes-F (5'-AACTTAGTGTCGCATGAC-3') Lmes-R (5'-AGTCGAGTTACAGACTACAA-3')	16
Lc.cremoris NRRL-B 634	LacreR (5'-GGGATCATCTTTGAGTGAT-3') CreF (5'-GTGCTTGCACCGATTTGAA-3')	13
S.thermophilus ATCC 19258	LbLMA1-rev (5'-CTCAAAACTAAACAAAGTTTC-3') Sther-F(5'-TAAGGAAAAACGGAATGTACTTGAGTTTC-3')	15

Isolation of LAB

For isolation, decimal dilutions of samples were prepared by using ¼ Ringer or buffered peptone water. In order to increase the chance of isolation, double petri dishes for each sample were inoculated into MRS Agar, modified Chalmers Agar (7), and M17Agar mediums. After incubation, the entirety of the typical colonies in each medium, as well as 5 random colonies from each petri dish with non-typical colonies, were chosen. The isolates and the reference strains were incubated in MRS Broth and M17 Broth at 30℃ for 18 hours. After incubation, inoculation was performed into Modified Chalmers Agar and M17 Agar and incubated at the same temperature, then they were controlled for purity and maintained in the BHI Agar Slant at 4℃ throughout the study.

Identification of LAB By Cultural Method

For the aim of identifying and characterizing LAB isolates, classical identification tests were performed. The isolates were identified and named according to the test results (8-11). Throughout the study, the isolates were reproduced in BHI Agar Slant (Oxoid, CM1136) and maintained at 4℃.

Identification of LAB By Molecular Method

DNA isolation kit was used for genomic DNA isolation from bacteria. The procedure was designed as recommended by the manufacturer (QIAGEN-QIAamp 51304). The DNAs were maintained at -20℃ until use. The classical PCR method was used for the identification of isolates. The isolates were individually screened for each species according to the groups divided upon the cultural methods and biochemical tests, starting from those LAB species with a higher likelihood of existence in fermented food. The primers and methods used for PCR (alphaDNA-Canada) are shown in Table 2 below.

RESULTS

A total of 3700 samples were collected from a total of 80 points (villages, dairy stores, and local butchers) at Kars province and districts (Table 1). From the 3700 food samples collected, 9160 isolates were obtained. Upon basic identification tests, 3846 (41.9%) out of 9160 isolates were identified as LAB. Among these LAB isolates, 1249 could not be cultured during and/or after the stock culture preparation phase. In a total of 3700 samples, 2597 LAB were isolated. The isolates were collected under 6 groups of microscopic morphology, gas production from glucose, arginine hydrolysis, and tolerance tests (salt, temperature, pH).

The isolates were individually screened for each species according to the groups divided upon the cultural methods and biochemical tests, starting from those LAB species with a higher likelihood of existence in fermented food. Eighteen isolates (0.5%) could not be genetically identified. As a result of cultural tests, it was determined that 5 of these isolates were *Lactobacillus spp* (betabacterium group), 5 were *Leuconostoc spp*, 2 were *Streptococcus spp,* and 6 were *Lactococcus spp*. The distribution of LAB by food samples is provided in Table 3 below.

Table 3. Distribution of LAB by samples.
Tablo 3. LAB'nin örneklere göre dağılımı.

LAB flora of Food	Number of samples	isolates	LAB
Yogurt: 38% *Lb.bulgaricus* (124 isolates), 26% *S.thermophilus* (83 isolates), 13% *Lb.plantarum* (41 isolates), 10% *Lb.rhamnosus* (32 isolates), 8% *Lb.johnsonii* (26 isolates), 3% *Lc.lactis* (9 isolates), 2% *Lb.acidophilus* (7 isolates)	450	475	322
Butter: 37% *Lc.lactis* (171 isolates), 25% *Lb.plantarum* (116 isolates), 23% *Lc.cremoris* (107 isolates), 9% *Lb.casei* (42 isolates), 6% *Leuc.mesenteroides* (28 isolates)	450	2409	464
Chechil cheese: 35% *Lc.lactis* (182 isolates), 26% *Lb.bulgaricus* (136 isolates), 19% *Lb.plantarum* (99 isolates), 14% *S.thermophilus* (73 isolates), 4% *Lb.casei* (20 isolates), 2% not determined (10 isolates)	450	2587	520
Tulum cheese: 22% *Lb.plantarum* (121 isolates), 19% *Lc.lactis* (104 isolates), 14% *Lb.brevis* (76 isolates), 10% *Leuc. mesenteroides* (55 isolates), 9% *Lc.cremoris* (49 isolates), 7% *Lb.helveticus* (36 isolates), 6% *Lb.bulgaricus* (32 isolates), 4% *S.thermophilus* (22 isolates), 3% *Lb.rhamnosus* (16 isolates), 3% *Lb.acidophilus* (16 isolates), 2% *Lb.johnsonii* (13 isolates), 1% not determined (8 isolates)	450	2505	548
White cheese: 34% *Lc.lactis* (123 isolates), 23% *Lb.casei* (83 isolates), 17% *S.thermophilus* (62 isolates), 16% *Lb.plantarum* (58 isolates), 10% *Lb.bulgaricus* (36 isolates)	450	740	362
Kashar cheese: 32% *S.thermophilus* (70 isolates), 29% *Lb.bulgaricus* (64 isolates), 15% *Lc.lactis* (33 isolates), 13% *Lb.plantarum* (29 isolates), 7% *Lb.brevis* (15 isolates), 4% *Lb.acidophilus* (9 isolates)	50	260	220
Sausage: 39% *Lb.plantarum* (63 isolates), 32% *Lc.lactis* (52 isolates), 15% *Lb.casei* (24 isolates), 14% *Lc.cremoris* (22 isolates)	50	184	161
Total	3700	9160	2597

DISCUSSION and CONCLUSION

This study which analyzed the indigenous flora of fermented meat and dairy products manufactured by traditional methods from meat and milk not subjected to heat treatment determined a rich and varied lactic flora. In the production of traditional foods in the region, the natural microflora of the raw material (meat or milk) is used without the use of a starter culture to maintain the desired taste of the products. The indigenous microbiota gets involved in the food through the raw material and the production and/or ripening medium. Another method frequently used by producers is "back-slopping" i.e., using a piece of the favored product of the previous production as a starter in the next production. Compared to commercial starter cultures, indigenous flora has far better adaptation to the environment, and thanks to its high competitive power, can rapidly and positively affect the product quality sensory-wise (17). NSLAB are very effective on the product texture, consistency, aroma and flavor of fermented foods by the way of many metabolic products they produce such as organic acids, aroma compounds, carbon dioxide etc. Tulum cheese and chechil cheese are not commercially produced at large scale in Turkey. Therefore, there are no commercial starter cultures for these two traditional cheese types. As for white cheese and kashar cheese, they are the most widely produced types in Turkey. In the industrial-scale production of these types of cheese, yogurt cultures for kashar cheese and various combinations of *Lc.lactis, Lb.casei, Lb.plantarum,* and *Lb.brevis* for

white cheese are typically used. In this study, we have detected a quite rich lactic flora in tulum cheese. This group included not only those aromatic forming materials and affecting acidity but also probiotic bacteria. In tulum cheese made of raw milk, lactic flora varies depending on the ripening period and the packaging material (plastic bin, goatskin casing) to be used for ripening. In the rich lactic flora of tulum cheese made of raw milk, *Lb. brevis, Lb paracasei, Lb.casei, Lb.plantarum, Lb.bulgaricus, Lb.lactis, Lb.rhamnosus, Lb.curvatus, Lb.salivarius, Lb.helveticus, Lb.fermentum, Lb.coryniformis, Lc.cremoris, Leu.dextranicum* were isolated (18,19). Although white cheese production usually utilizes commercially prepared *Lc.lactis* and *Lc.cremoris* strains, different LAB have been identified in studies on white cheese made of raw milk. However, it has been reported that in ripened white cheese, the dominant flora is *Lc.cremoris*, *Lb.casei*, *Leu.paramesenteroides*, *Lb.pantarum*, *Lb.brevis,* and *Leu.dextranicum*, including *Lc.lactis* at the forefront (20,21). In chechil cheese, it has been observed that the flora is greatly affected by the ripening instrument being an animal skin casing or a plastic bin. It is known that mostly yogurt bacteria are detected in chechil cheese, as has been the case in our study. Şengül (22) determined that lactic flora in chechil cheese made of raw milk by traditional methods involved *Lb*. *bulgaricus* and *Lb. fermentum.*

Kars kashar cheese has been especially famous across Turkey, which has also been registered with a geographical indication. Although raw milk is used in the production of this type of cheese, a natural decrease in microbiota occurs at the stage of scalding. However, since this cheese is ripened under natural conditions in the region, NSLAB develops as the secondary flora, thereby providing the main taste. In cheese, NSLAB is more resistant than starter lactic acid bacteria (SLAB) to the absence of oxygen, high salt concentrations, and acidity. Thus, NSLABs are quite effective in the ripening phase of cheese (17). Yuvaşen et al. (3) identified 13 different types of NSLAB in kashar cheese produced by traditional methods and identified *Lb. paracasei*, *Lb. rhamnosus, Lb. curvatus, Lb. plantarum* and *Lb. fermentum*.

We isolated lactococcus, lactobacillus and *leuconostoc spp,* which is especially effective on aroma, in the butter made of the milk of animals grazed in pasturage in May, also called “the spring butter”. *Lb.plantarum* and *Lb.casei* are responsible for acid production in butter, whereas *Leuconostoc spp* produces diacetyl which is the final product in the citrate metabolism providing the strong aroma of butter. Commercial starter cultures for yogurt consist of *Lb.bulgaricus* and *S.thermophilus* strains. In the homemade yogurt analyzed, Demir et al. (23) identified *Lb.bulgaricus, Lb.helveticus, Ent. faecium*, and *Pediococcus acidilactici,* and reported that the ability of *Lb.bulgaricus* to produce exopolysaccharides is crucial in yogurt. It has been determined that the homemade yogurt analyzed in this study included *Lb.bulgaricus*, *S.thermophilus, Lb.plantarum, Lb.rhamnosus*, *Lb.johnsonii, Lc.lactis,* and *Lb.acidophilus* strains which cover the most significant probiotic cultures. It has been determined that in the production of the other samples analyzed (tulum cheese, chechil cheese, white cheese and kashar cheese), the locals prefer to use yogurt for fermentation. This may explain why the other cheese samples include similar flora and have probiotic culture. The dominant flora in sausage has been determined as *Lb.plantarum* followed by *Lc.lactis, Lb.casei,* and *Lc.cremoris*. The LAB used as a starter or protective culture in fermented sausage production are responsible for the sensory quality and microbiological safety of the product. The dominant flora frequently reported in fermented meat products include *Lb.plantarum, Lb.sake, Lb.curvatus, Lb.brevis* (24-27). Therefore, dominant flora differences may result from many variables such as the fermentation temperature-duration process in production, pH of the meat, water activity, air conditions (humidity, airflow, etc.), and storage conditions (28).

The results of this study have shown that the foods analyzed have a more different and richer flora than ready-made food produced by commercial starter cultures. The foods analyzed are particularly rich in terms of probiotic LAB. There are many studies on probiotic LAB and public health. It is important for the health of the local people that these foods, which are consumed without heat treatment, are rich in probiotic cultures. Determining the function and composition of the indigenous flora of fermented foods, the production methods of which are followed for the aim of protecting and improving traditional methods, is very important in the production of high quality (sensory characteristics, textural structure, safety) fermented products. The strains obtained from traditional foods may be analyzed for significant characteristics such as antibiotic resistance, biogenic amine production, and resistance to various salt concentrations in order to determine novel starter culture combinations. Determining starter culture combinations that can adapt well to ideal ripening temperature and pH values and create a strong aroma will contribute to the development of products at an industrial scale.

Conflict of interest

The authors declare that they have no conflict of interest.

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