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RESEARCH ARTICLE

# Identification of Nonstarter Indigenous Lactobacilli from Kars Gravier Cheese

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## Summary

The aim of this study was to identify indigenous nonstarter lactobacilli (NSLAB) isolated from Kars Gravier cheese. In this study, 120 isolates of NSLAB from Kars Gravier cheese were isolated and identified with the API 50 CHL system. The ratio of identified NSLAB strains were as follows; 30 (25%) were *Lactobacillus brevis type* 1, 22 (18.3%) were *Pediococcus pentosaceus* type 1, 17 (14.1%) were *Lactobacillus paracasei* ssp. *paracasei*, 16 (13.3%) were *Lactobacillus fermentum* type 1, 15 (12.5%) were *Lactococcus lactis* ssp. *lactis* type 1, 9 (7.5%) were *Lactobacillus delbrueckii* ssp. *lactis* type 1, 6 (5%) were *Lactobacillus plantarum* type 1 and 5 (4.16%) of them were *Lactobacillus rhamnosus*. In the light of the results obtained in this study, it could be said that *Lactobacillus* species formed the dominant microflora of Kars Gravier cheese.

***Keywords:*** *Kars Gravier Cheese, API 50 CHL, Lactobacillus, Identification*

# Kars Gravyer Peynirinden İzole Edilen Doğal Laktobasillerin Tanımlanması

## Özet

Bu çalışmanın amacı, Kars Gravyer peynirinin hakim mikroflarasını oluşturan doğal laktobasillerin tanımlanmasıdır. Araştırmada, Kars Gravyer peynirinden 120 adet laktobasil suşu izole edilmiş ve API 50 CHL sistemi ile tanımlanmıştır. Tanımlanan suşların sayı ve oranları aşağıdaki gibidir; 30 tanesi (%25) *L. brevis* tip 1, 22 tanesi (%18.3) *Pediococcus pentosaceus* tip 1, 17 tanesi (%14.1) *Lactobacillus paracasei* ssp *paracasei*, 16 tanesi (%13.3) *Lactobacillus fermentum* tip 1, 15 tanesi (%12.5) *Lactococcus lactis* ssp *lactis* tip 1, 9 tanesi (%7.5) *Lactobacillus delbrueckii* ssp *lactis* tip 1, 6 tanesi (%5) *Lactobacillus plantarum* tip 1 ve 5 tanesi (%4.16) *Lactobacillus rhamnosus* olarak tanımlanmıştır. Araştırmada elde edilen bulgular ışığında, Kars Gravyer peynirindeki dominant mikroflorayı *Lactobacillus* türlerinin oluşturduğu söylenebilir.

***Anahtar sözcükler:*** *Kars Gravyer Peyniri, API 50 CHL, Lactobacillus, İdentifikasyon*

## INTRODUCTION

Lactic acid bacteria (LAB) have been used around the world to improve the preservation, sensorial characteristics and nutritional value of a large variety of food products, such as milk, meat and vegetables [1]. LAB consist of a wide range of genera and include a considerable number of species. Their common characteristics are: Gram-positive, usually catalase negative, non-motile, non-spore-forming, growth under microaerophilic to strictly anaerobic conditions and lactic acid production. These bacteria are the major component of the starters used in fermentation, especially for dairy products, and some of them are also natural habitants of the gastrointestinal microflora. *Lactobacillus* is one of the most important genera of LAB [2-4]. Lactobacilli play an important role as starter bacteria in cheeses such as Parmesan, Mozzarella and Swiss types and are also found as adventitious microflora or nonstarter lactic acid bacteria in Cheddar cheese. They either survive pasteurisation or enter the cheese milk as post pasteurisation contaminants and may play an important role in flavour development during secondary ripening. Lactobacilli are naturally present or added intentionally, for technological reasons or to generate a health benefit for the consumer [2,5]. Lactobacilli play an important role during the

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ripening of cheeses. They may have entered adventitiously from the milk and the immediate surroundings during cheese manufacture. The nonstarter lactobacilli constitute the majority of the non-starter lactic acid bacteria (NSLAB) present in most types of cheese varieties during ripening [6-9]. NSLAB which are initially present in small numbers but increase to high numbers in cheese varieties that require long ripening times. Very few studies have been conducted to control the numbers and types of NSLAB, although these organisms are thought to have a significant influence on cheese flavour development and to participate directly in the production of some major aroma compounds such as acetic acid, formic acid and gas production [2,9].

In Turkey, more than 50 different types of cheese are produced. Many of these cheeses are produced only in specific geographical region [10,11]. One of the cheese varieties produced is Kars Gravier cheese a traditional very hard cheese variety and manufactured in the Kars province in Turkey. Gravier cheese originated from Switzerland Emmantel cheese and was brought to Kars by Russians in 1905 [12]. Kars Gravier cheese is produced in small dairyplant from raw cow's milk, using rennet enzyme, and without any addition starter culture. The ripening processes of Kars Gravier cheese are completely achieved by the indigenous microflora present in the raw cow's milk and coming from the environment during the cheese manufacture. There are a few studies related to Kars Gravier cheese. Previous studies focused on production technique [13] and microbiological, physical and chemical qualities of this cheese [12,14]. Kamber et al.[12] reported that, Kars Gravier cheese samples were found to be incompatible as compared to the Turkish Food Codex's microbiologic criteria. On the other hand Ulutaş [14] found that, Gravier cheese samples were found to be compliant to Turkish Gravier cheeese Standard (TSE 2174) [15] physical and chemical criteria.

The identification of *Lactobacillus* strains based on mainly morphology, fermentation of carbohydrates, genomic structure and phylogenetic relationships and all these methods are still used successfully [16]. There is no study relating the identification of the microflora in Kars Gravier cheese. The aim of this study was to determine species and ratios of the non starter LAB from Kars Gravyer cheeese to improve the technological production and to guarantee typical standart properties of this cheese and transfer the obtained knowledge to future generations.

## MATERIAL and METHODS

### *Gravier Cheese Samples*

This research was performed between the 2009-2010 years. Twelve samples of Gravier cheese were purchased from different retail markets in Kars province (Turkey), transported to the laboratory as soon as possible and stored at 3-4ºC before microbiological analysis.

### *Isolation of Lactic Acid Bacteria (LAB)*

Each cheese samples (10 g) were homogenized with stomacher (IVL Masticator, Spain) in 90 mL sterilized 2% (w/v) sodium citrate (Merck 567446) solution. Aliquots were then spread in the De Man Rogosa and Sharpe (MRS) agar (Merck 1.10660), which were the most suitable for isolating lactobacilli then incubated anaerobically (Gas generating kit, Anaerocult A, Merck 1.13829) at 35-37°C for 3 days [17]. After the incubation period, growing colonies (at least the square root of the CFU counted per plate) were randomly picked up from the petri plate with sterile loop. Purity of the cultures was confirmed by microscopic observation, Gram stain (KOH method) and catalase test (5% $H_2O_2$). Gram-positive and katalase- negative rots were subcultured to purity on MRS agar at 35-37°C for 3 days anaerobically. This rots were considered pure cultures of lactobacilli. The pure cultures were frozen and stored at -80°C in MRS broth (Merck 1.10660) containing 20% glycerol (Merck 356350) before the identification test.

### *Identification of LAB With API 50CHL*

The API 50 CHL (Biomerieux, Marcy l'Etoile, France) consisting of 50 biochemical tests to identify *Lactobacillus* and related genera strips according to the manufacturer's instructions was used. The isolated *Lactobacilli* strain was subcultured in the MRS agar anaerobically 30°C for 24 h. Anaerobically 24-hour growing colonies were collected with sterile swab stick and were inoculated into 2 ml of sterile suspension medium (Biomerieux, ref. 70700) to obtain 2 Mc Farland (Biomerieux, ref. 70900) density. This procedure were made for each isolate. The prepared suspension was distributed properly to the strip. Strips wells were filled with the addition of mineral oil. For composure of an atmosphere moist, 10 ml distilled water was disposed within the box incubator and the strip was installed in the incubation box. When carbohydrates were fermented, the pH drops in medium. Color changes occured in strips depend on the decrease in pH indicators. At the end of a 24 h incubation in strips blue, purplish color as negative, yellow, yellow-green color formation in the biochemical profile of isolates were evaluated as positive [18]. The API LAB PLUS database (Bio Merieux, France) was used for the interpretation of the result. This programme discriminates between species on the basis of a pattern-matching principle. Identification of an organism is accompanied by the % ID and and T index. % ID: an estimate of how closely the observed profile corresponds to the proposed taxon relative to all the other taxa in the database. The T index: an estimate of how closely the profile corresponds to the most typical set of reactions for the stated taxon [19].

## RESULTS

A total of 120 LAB strains were isolated from 12 Gravier cheese samples in our study. The LAB strains were identified

by API LAB PLUS software API 50 CHL systems version 5.0 database. Among the isolates, *Lactobacillus* spp formed the dominant microflora of Kars Gravier cheese. LAB isolates were classified as 83 isolates lactobacilli (69.16%), 22 isolates pediococci (18.3%) and 15 isolates lactococci (10%). These isolates were identified as follows: 30 strains *Lactobacillus brevis* type 1 (25%), 22 strains *Pediococcus pentosaceus* type 1 (18.3%), 17 strains *Lactobacillus paracasei* ssp. *paracasei* (14.1%), 16 strains *Lactobacillus fermentum* type 1 (13.30%), 25 strains *Lactococcus lactis* ssp. *lactis* type 1 (12.5%), 9 strains *Lactobacillus delbrueckii* ssp. *lactis* type 1 (7.5%), 6 strains *Lactobacillus plantarum* type 1 (5%) and 5 strains *Lactobacillus rhamnosus* (4.16%) (*Table 1*).

researchers reported that 32% *L. plantarum* isolates, 15% *L. brevis* isolates, 12% *L. paracasei* subsp. *paracasei* isolates, 9% *L. curvatus* isolates, 6% *L. fermentum* isolates, 6% *L. casei* subsp. *casei* isolates, 5% *L. pentosus* isolates, 3% *L. casei* subsp. *Pseudoplantarum* isolates, and 1% *L. rhamnosus* isolates. Oner et al.[20] studied isolation of LAB from Turkish tulum cheese, and was found that the Tulum cheeses could had been characterised with lactobacilli (48.5%). Turgut et al.[10] studied isolation of LAB from cheese called Karın Kaymak and identified 82 pieces lactobacilli as follows; 47.56% were *L. plantarum* type 1 and 2, 21.9% were *L. brevis* type 1 and 3, 1.97% were *L. delbrueckii* ssp *delbrueckii*, 9.75% were *L. acidophilus* type 3 and 2.43% were *L. fermentum*. Also,

***Table 1.*** *Distribution of the strains isolated and identified from the Kars Gravier cheese*
***Tablo 1.*** *Kars Gravyer peynirinden izole edilen ve tanımlan suşların dağılımı*

| Bacterial Strain/Sample | A | B | C | D | E | F | G | H | I | J | K | L | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *L. lactis ssp lactis* type 1 | 2 | - | 2 | 2 | 1 | 3 | - | 3 | - | 1 | 1 | - | 15 |
| *L. brevis* type 1 | 2 | 3 | 2 | 1 | 3 | 2 | 4 | 2 | 3 | 3 | 3 | 2 | 30 |
| *L. rhamnosus* | 2 | - | - | - | 2 | - | 1 | - | - | - | - | - | 5 |
| *L. plantarum* type 1 | - | 1 | 1 | 2 | - | - | - | - | - | - | - | 2 | 6 |
| *P. pentosaceus type* 1 | 4 | 3 | 2 | - | 2 | 2 | 3 | 2 | 1 | - | 2 | 1 | 22 |
| *L. delbrueckii* ssp *lactis* tip1 | - | 1 | 1 | 1 | - | - | - | 1 | 2 | - | - | 3 | 9 |
| *L. paracasei* ssp par*acasei* | - | 1 | 1 | 2 | - | 1 | - | 2 | 3 | 4 | 2 | 1 | 17 |
| *L. fermentum* type 1 | - | 1 | 1 | 2 | 2 | 2 | 2 | - | 1 | 2 | 2 | 1 | 16 |

# DISCUSSION

NSLAB usually increase from a low number in fresh curd to dominate the microflora of mature cheese. In contrast to starters, NSLAB tolerate the hostile environment of cheese during ripening [9]. Althought there are a few studies on chemical and microbiological quality of Kars Gravier cheese there is no study on isolation an identification of LAB from this cheese. The present study is the first research on this subject so far. In the our investigation, total of 120 LAB strains were identified. It was found that *Lactobacillus* strains composed the predominant microflora of the Kars Gravier cheese. Lactobacilli isolates (83 pieces) constituted 69.16% of all isolates. In the present study, number and rates of identified lactobacilli strains were as follows: 30 strains *L. brevis* type 1 (25%), 17 strains *L. paracasei* ssp *paracasei* (14.1%), 16 strains *L. fermentum* type 1 (13.30%), 9 strains *L. delbrueckii* ssp *lactis* type 1 (7.5%), 6 strains *L. plantarum* type 1 (5%) and 5 strains *L. rhamnosus* (4.16%). Laan et al.[5] reported that, species identified in Cheddar cheese as non-starter lactic acid bacteria are *Lactobacillus plantarum*, *L. casei* and *L. brevis* and to a lesser extent *Pediococcus* or *Micrococcus* species. Williams et al.[8] stated that the culturable non-starter population in Cheddar cheese is dominated by homo- and heterofermentative species of lactobacilli although other LAB genera can be present. Angelis et al.[9] *Lactobacillus* strains were isolated from 12 Italian ewe cheeses, hard or semihard Cheese, and 123 isolates and 10 type strains were subjected to phenotypic, genetic, and cell wall protein characterization analyses. As a result of this Şengül and Çakmakçı [21], obtained total 240 LAB isolates from Tulum cheese samples and reported that 92.08% were *Lactobacillus*, 7.08% *Pediococcus* and 0.83% *Leuconostoc*. In this regard, the results are compatible with our study. A similar results was obtained by Sağdıç et al. [22] for Van Herby Cheese. They reported that the dominant flora of Van Herby cheese consisted of *L. plantarum, L. casei* and *P. pentosaceus*. Öksüzepe et al. [23] studied on identification of total of 783 lactic acid bacteria from Tulum cheese and found that, lactic streptococci were predominant within the first month of ripening and, then, replaced by lactobacilli. As a result, lactobacilli strains were found to constitute the dominant indigenous flora of Kars Gravier cheese and *L. brevis* type 1, *L. paracasei* ssp *paracasei* and *L. fermentum* were predominant lactobacilli species. In this study, specific to our range of local cheese microbial diversity conservation and future generations will have been taken a step toward more.

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