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Bacteriocinogenic bacteria isolated from Civil, Kashar and White cheeses in Erzurum, Turkey

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Summary: This study was carried out in order to identify the bacteriocinogenic lactic acid bacteria (LAB) isolated from civil, kashar and white cheeses produced by traditional methods in Erzurum, Turkey. LAB were isolated from 80 samples of cheese collected from the markets of Erzurum. Antimicrobial activities of the isolates were determined using agar spot and well diffusion methods. LAB that showing antimicrobial activity were characterized phenotypically and genotypically, and the bacteriocin-producing strains were determined. The susceptibilities of bacteriocins to different temperatures, pH and enzymes were tested. While 48.29% of the 381 LAB isolated from cheese samples had antimicrobial activity, only 4.35% of them were determined producing bacteriocin. While 168 of 184 isolates which were showing antimicrobial activity were identified by phenotypical methods at a genus level, and 11 of were at a species level, 135 of 184 isolates were identified by genotypical methods at a genus level, and 26 of were at a species level. *Lb. plantarum* (24.36%) and *Lb. brevis* (23.08%) in lactobacilli, *E. faecium* (38.89%) and *E. durans* (20.37%) in enterococci, and *Lc. lactis subsp. lactis* (100%) in lactococci were identified as the dominant species. All bacteriocin producing exhibited antimicrobial activity against *Micrococcus luteus*. It has been determined that bacteriocin producing *Lc. lactis* subsp. *lactis* and *Lb. pentosus* strains have inhibition impact on *Staphylococcus aureus* and *Listeria monocytogenes.* It has been concluded that the bacteriocin-producing isolates, due to not losing their activities in a wide range of pH and different temperature-time values, could be used as a bio-protective culture in food production and storage.

Keywords: Bacteriocin, Civil cheese, Kashar cheese, VITEK 2, 16S rDNA.

Erzurum ilindeki Civil, Kaşar ve Beyaz peynirlerden izole edilen bakteriyosinogenik bakteriler

Özet: Bu çalışma, Erzurum ilinde geleneksel yöntemlerle üretilen beyaz, civil ve kaşar peynirlerinden izole edilen bakteriyosinogenik laktik asit bakterilerinin (LAB) belirlemesi amacıyla yapıldı. Erzurum piyasasından toplanan 80 peynir örneğinden LAB izole edildi. İzolatların antimikrobiyal aktiviteleri agar spot ve kuyu difüzyon yöntemleri kullanılarak belirlendi. Antimikrobiyel aktivite gösteren LAB fenotipik ve genotipik olarak tanımlandı ve bakteriyosin üreten suşlar tespit edildi. Bakteriyosinlerin farklı sıcaklık, pH ve enzimlere karşı duyarlılıkları test edildi. Peynir örneklerinden izole edilen 381 izolatın %48.29'u antimikrobiyal etkiye sahipken bunlardan yanlızca %4.35'inin bakteriyosin ürettiği tespit edildi. Antimikrobiyal aktivite gösteren 184 izolattan 168 tanesi cins ve 11 tanesi tür düzeyinde fenotipik yöntemlerle tanımlanırken, 184 izolattan 135 tanesi cins ve 26 tanesi tür düzeyinde genotipik yöntemle belirlenmiştir. Laktobasillerde *Lb. plantarum* (%24.36) ve *Lb. brevis* (%23.08), enterokoklarda *E. faecium* (%38.89) ve *E. durans* (%20.37), laktokoklarda ise *Lc. lactis subsp. lactis* (%100) baskın türler olarak tespit edildi. Bakteriyosin üreten izolatların tamamı *M. luteus*'a karşı antimikrobiyel aktivite gösterdi. Bakteriyosin üreten *Lc. lactis subsp. lactis* ve *Lb. pentosus* suşlarının *S. aureus* ve *L. monocytogenes* üzerine inhibe edici etkisinin olduğu belirlendi. Bakteriyosin üreten izolatların geniş bir pH aralığında ve farklı ısı-zaman değerlerinde aktivitelerini kaybetmemelerinden dolayı gıda üretimi ve muhafazasında biyokoruyucu kültür olarak kullanılabileceği sonucuna varıldı.

Anahtar sözcükler: Bakteriyosin, Civil peynir, Kaşar peyniri, VITEK 2, 16S rDNA.

Introduction

LAB are Gram positive, non-spore forming, catalase negative, aerotolerant, acid-tolerant, and strictly fermentative rod or cocci, producing lactic acid as a major catabolic end product from glucose (23). Owing to their widespread existence in nature and playing a role in the production and ripening of some foods, these bacteria are very crucial for food technology. LAB are used to produce new foods by fermentation, provide desired characteristics, and add more durable structure to fermented foods (6, 10, 11). LAB, which are naturally found in many foods or used as a starting culture, exhibit antagonistic activity against saprophytes and pathogen microorganisms as a food preservative. The protective effect of these bacteria is mainly due to the organic acids they produce, which it shows itself by the decrease in pH. Likewise, antimicrobial compounds such as hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde, D-

isomer amino acids, and bacteriocin, produced during LAB fermentation, also contribute to flavor and aroma development, texture and shelf life of fermented foods (4, 29, 38).

This study is conducted in order i) to isolate the LAB from civil, kashar and white cheeses produced using traditional methods in the province of Erzurum, Turkey, ii) to define antimicrobial activities of the isolates, iii) to identification of the isolates showing antimicrobial activity, and to define whether the antimicrobial substances produced exist in the structure of bacteriocin.

Materials and Methods

Cheese samples and indicator bacteria: In this study, 30 civil cheese, 25 kashar and 25 white cheese samples produced by traditional methods in small-scale dairies and family enterprises in Erzurum, Turkey were used. Indicator bacteria (*Listeria monocytogenes* ATCC 19115, *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC, *Micrococcus luteus* RSK1123, *Enterococcus faecalis* ATCC 29212, *Lactobacillus plantarum* DSM 2601) obtained from Refik Saydam Hygiene Institute Culture Collection were used in determining antimicrobial activities of the isolates obtained from cheese samples.

***Isolation of LAB*:** Man Rogosa Sharpe (MRS-Merck 1.10660) MRS, M17 (Merck 1.15108) and Chromocult Enterococci (CE-Merck 100950) agar medium were used to increase the chance of isolation of LAB strains from the cheese samples. 0.1 mL of the appropriate dilutions were transferred to MRS, M17 and CE agar medium and plated by spread technique. At the end of the incubation, as far as possible morphologically different colonies were selected. Gram positive, catalase, and oxidase-negative, cocci or rod-shaped bacteria were determined as pure cultures for identification purposes (22, 30).

***Determination of antimicrobial activities of isolates*:** Agar spot (1, 31, 37) and agar well diffusion (8, 21, 27) methods were used in determining the antimicrobial activities of the isolates.

Phenotypic identification of isolates: In addition to biochemical and physiological tests at the identification of isolates, VITEK 2 compact system and VITEK 2 GP ID cards were used to identify phenotypically cocci-shaped isolates, and API 50 CHL test kit was used to identify phenotypically basil-shaped isolates.

***Genotypic identification of isolates*:** 16S rDNA regions on isolated genomic DNA; was bred in PCR device using 16S forward (5'-CCG TCA ATT CCT TTG AGT TT -3') and 16S reverse (3'-AGA GTT TGA TCC TGG CTC AG -5') primers (3). For this purpose, 25 µl molecular sterile water, 20 µl PCR master mix (Fermentas K0171), 1 µl 16S forward and 1 µl 16S reverse primer and 3 µl templete DNA were added to give a total volume of 50 µl. PCR protocol; 30 cycles consisting one cycle denaturation (double chain opening) at 94ºC for 30 s, after one cycle of initial denaturation step (pre-denaturation) for 120 s at 94ºC, 60 s at 55ºC primer binding and 90 s elongation steps at 72ºC and lastly one cycle at 72ºC and 10 min at the last elongation stages were composed (15). The amplified 16S rDNA PCR fragments' electrophoresis was made at a gel prepared in 1% agarose and the size of the fragment was calculated using a DNA ladder (Fermantas SM 321) marker of 100 bp. DNA sequence analysis of the PCR products was carried out at the Pendik Veterinary Control and Research Institute using automatic gene sequencing device (Perkin Elmer). 16S rDNA sequence similarity was determined using the National Center for Biotechnology Information (NCBI) BLAST program.

***Determining bacteriocin-producing isolates*:** For the determination of the presence of bacteriocin, 18 h of active cultures of isolates at MRS broth grown at 30°C, were centrifuged at 10,000 rpm for 15 min at 4°C. Neutralized supernatants were added catalase enzyme and then, were incubated for 2 h at 37°C in order to decompose possible hydrogen peroxide. At the end of this period, the enzyme effect was inhibited by standing in a water bath at 60ºC for 10 min (32). Then proteolytic enzyme (proteinase K) was applied to determine whether the substances produced by the isolates were in the protein structure. Samples with no added enzyme were used as controls. The antimicrobial activity of the neutralized supernatants with and without the enzyme was determined using the well diffusion method (35). The disappearance of antimicrobial activity as a result of proteolytic enzyme application showed that this effect originated from a substance having protein nature.

***The effect of enzyme, pH and temperature applications on bacteriocin activity*:** The effect of different pH, enzyme and temperature applications on the stability of bacteriocins produced by isolates in various conditions was examined (17, 35). In susceptibility tests, *M. luteus* was used as an indicator test bacteria.

Results

In this study, 450 isolates were obtained from 80 cheese samples collected from the markets of Erzurum, Turkey. Of these, 381 isolates with Gram positive, catalase and oxidase negative were selected as a probable LAB. From these, 184 (48.29%) isolates were found to have antimicrobial activity on all or a few of the indicator bacteria. Only eight (4.35%) of 184 isolates with antimicrobial activity were found to have an antimicrobial substances in protein structure.

While 168 of 184 isolates showing antimicrobial activity were identified by phenotypical methods at a genus level, and 11 of were at a species level, 135 of 184 isolates were identified with genotypical methods at a genus level, and 26 of were at a species level. As shown in Table 1., 77 of 102 cocci-shaped isolates, identified by phenotypic tests and the VITEK 2 system, and could have been verified genotypically. However, 52 of 77 rop-shaped isolates, identified by phenotypic tests and the API 50 CHL kit, and could have been verified genotypically (Table 2.).

In the white cheese samples *Lc. lactis subsp. lactis, E. faecium, Lb. plantarum* and *Lb. brevis*, in the civil cheese samples *E. faecium* and in the kashar cheese samples *Lb. paracasei* subsp. *paracasei* and *Lb. brevis* were determined as the most common types. While in white and civil cheese the dominant species were *Lactobacillus* spp. and *Enterococcus* spp., in kashar cheese the dominant species were *Lactobacillus* spp.

In this study 3 of strains producing bacteriocin were *Lc. lactis subsp. lactis*, 3 of strains producing bacteriocin were *Lb. pentosus*, others were defined as *Leu. lactis* and *Lb. paracasei subsp. paracasei*. The activity results of 8 bacteriocin producing isolates against indicator test bacteria are given in Table 3.

In Table 4, susceptibility test results of strains producing bacteriocin were given. It was determined that the isolates' bacteriocin activities stayed stable against *M. luteus* at different pH. As a result of different temperature and enzyme applications, it was observed that some of the isolates' bacteriocin activity was stabilized and did not lose their effects, but some of them has lost their effects.

Table 1. 16S rDNA sequence analysis verification of cocci-shaped isolates identified by VITEK 2.
Tablo 1. VITEK 2 ile tanımlanan kokların 16S rDNA dizi analizi ile doğrulanması.

Types of bacteria	**VITEK 2**	**16S rDNA**	**%**
Enterococcus durans	11	11	100
Enterococcus faecalis	7	6	85.71
Enterococcus faecium	21	21	100
Enterococcus lactis	7	4	57.14
Enterococcus spp.	11	8	72.72
Lactococcus lactis subsp. *lactis*	21	16	76.19
Lactococcus raffinolactis	1	0	0
Pediococcus pentosaceus	12	0	0
Leuconostoc mesenteroides	3	3	100
Leuconostoc mesenteroides subsp. *mesenteroides*	3	3	100
Leuconostoc pseudomesenteroides	1	1	100
Leuconostoc lactis	4	4	100
Total	102	77	75.49

Table 2. 16S rDNA sequence analysis verification of rod- shaped isolates Identified with API 50 CHL.
Tablo 2. API 50 CHL ile tanımlanan basillerin 16S rDNA dizi analizi ile doğrulanması.

Types of bacteria	**API CH50**	**16S rDNA**	**%**
Lactobacillus plantarum	16	14	87.50
Lactobacillus plantarum subsp. *plantarum*	3	1	33.33
Lactobacillus brevis	18	18	100
Lactobacillus casei	8	4	50
Lactobacillus paracasei	6	4	66.67
Lactobacillus paracasei subsp. *paracasei*	10	9	90
Lactobacillus curvatus spp. *curvatus*	4	0	0
Lactobacillus pentosus	7	4	57.14
Lactobacillus fermentum	5	0	0
Total	77	54	70.13

Table 3. Bacteriocinogenic effect of isolates against various pathogens.
Tablo 3. Çeşitli patojenlere karşı izolatların bakteriyosinojenik etkisi.

Indicator test bacteria	Bacteriocinogenic isolates							
	Lc. lactis* subsp. *lactis (B10)	***Lc. lactis* subsp. *lactis*** (B11)	***Lb. pentosus*** (C52	***Lb. pentosus*** (C56)	***Lc. lactis* subsp. *lactis*** (B47)	***Leu. lactis*** (K33)	***Lb. pentosus*** (B55)	***Lb. paracasei* subsp. *paracasei*** (C59)
L. monocytogenes	+	+	+	+	-	-	-	-
M. luteus	+	+	+	+	+	+	+	+
B. subtilis	+	+	+	+	+	+	-	-
E. coli O157:H7	+	+	+	+	-	-	-	-
S. aureus	+	+	+	+	+	-	-	-
E. faecalis	+	+	+	+	-	-	+	+
Lb. plantarum	-	-	-	-	-	-	-	-

(+):Bacteriocinogenic effect positive; (-):Bacteriocinogenic effect negative
B10, B11, B47, B55: White cheese isolates; C52, C59: Civil cheese isolates; K33: Kashar isolate

Table 4. The effect of pH, temperature/time and enzyme applications on bacteriocin activity.
Tablo 4. Bakteriyosin aktivitesi üzerine pH, sıcaklık/zaman ve enzimlerin etkisi.

	Lc. lactis* subsp. *lactis (B10)	***Lc. lactis* subsp. *lactis*** (B11)	***Lb. pentosus*** (C52	***Lb. pentosus*** (C56)	***Lc. lactis* subsp. *lactis*** (B47)	***Leu. lactis*** (K33)	***Lb. pentosus*** (B55)	***Lb. paracasei* subsp. *paracasei*** (C59)
pH								
2	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+
Temperature/time								
100ºC/5 min	+	+	+	+	+	+	-	-
100ºC/10 min	+	+	+	+	+	-	-	-
65ºC/30 min	+	+	+	-	+	+	-	+
121ºC/15 min	+	+	+	-	-	-	-	-
4ºC/7 day	+	+	+	+	+	+	+	+
Enzyme								
α-chymotrypsin	-	-	-	-	-	-	-	+
Protease	-	-	-	-	-	+	+	+
Trypsin	-	-	-	-	-	-	+	+
Lipase	-	-	-	-	-	+	+	-
Pepsin	-	-	-	+	+	+	+	+
Catalase	-	-	-	-	-	-	+	-
α-amylase	+	-	-	+	-	+	+	+
Proteinase-K	-	-	-	-	-	-	-	-

(+): Insensitive to; (-): Sensitive to
B10, B11, B47, B55: White cheese isolates; C52, C59: Civil cheese isolates; K33: Kashar isolate

Discussion and Conclusion

The transformation of milk to cheese is very complex and occurs in a dynamic microbial ecosystem. The most important part of this microbial system is constituted by LAB. While 48.29% of the 381 isolates, isolated from cheese samples, has shown to have antimicrobial activity, only 4.35% of that produced bacteriocin. 95.65% of the 184 isolates showing antimicrobial activity were found to have an antimicrobial effect due to low pH and organic acids. There are many studies that indicate that the protective effect of LAB is due to the organic acids they produce (18, 28, 32, 34).

In general, phenotypic methods that allow the identification of LAB at the genus-species level are based on the morphological, physiological, metabolic and biochemical characteristics of the bacteria (24, 25, 33). However, some species of bacteria do not show similar characteristics. Some lactococcal strains are known to tolerate 6.5% NaCl and/or 45°C temperature, as enterococci (12). In this study, it was determined that 3 lactococci isolates showed different characteristics. It was determined that one of them was tolerant to both salt and heat, while the second one tolerant to salt and the last one was tolerant to heat. This indicated that some LAB genus were difficult to distinguish from each other due to their similar phenotypic characteristics. Therefore, it was concluded that phenotypic descriptions based solely on morphological and biochemical characteristics were not reliable by themselves.

The 16S rRNA regions of LAB contain highly conserved sequences. Therefore, sequences of rRNA encoding genes (rDNA) are used to identify the taxonomic group of that organism. All types involved in any bacterial species can be precisely identified by determining sequence analyzes of variable regions in 16S rDNA (24). In this study, only 16 of 20 LAB species determined by phenotypic methods, could have been confirmed by genotypic methods (16S rDNA sequence analysis). It is thought that these differences between phenotypic and genotypic methods may be due to gene changes between strains in the natural microflora of the cheese. Gunay-Esiyok et al. (19) suggest that the formation of moderate prophylactic, linear plasmids and repeat regions that can accommodate chromosomes by horizontal gene transfers among strains found in the natural microflora of food may cause these differences. Molecular methods that give more precise and certain results should be preferred in the studies for the identification of LAB.

In our study, it is determined that 10.87% of the LAB isolated from cheese samples were *Lactococcus* spp., while 44.56% of them were *Lactobacillus* spp., 28.26% *Enterecoccus* spp. and 12.49% *Leuconostoc* spp. According to these results, the dominant flora in cheese samples was LAB not as the starter (*Lactobacillus* spp., *Enterecoccus* spp., *Pediococcus* spp., *Leuconostoc* spp.). These bacteria have also been reported in many studies (5, 7, 36) that affect proteolysis and lipolysis during cheese ripening and contribute to the taste and flavor of the cheese.

It is stated that the enterococci which were known to be very resistant to environmental conditions cause pathogenicity in humans as well as the positive effects for traditional cheeses on maturation and aroma development (20, 26). In our study, the second dominant flora in cheeses was enterococci. *E. faecium, E. durans, E. faecalis* and *E. lactis* are the most abundant species in cheese samples. *E. lactis* isolated from civil cheese samples in our study was also determined in a study by Morandi et al. (26) as a new enterococcus species.

Although the practice of many bacteriocins identified up to now is limited due to their narrow inhibitor spectra, bacteriocins produced by LAB are of interest in food preservation. Only the 8 LAB isolated from cheese samples were found to produce bacteriocin in our study. While all of the antimicrobial substances produced by these strains are effective against *M. luteus*, they did not affect *Lb. plantarum*. On the other hand, it was detected that they had inhibitory effects on some important foodborne pathogens. The inhibitory effect of *Lc. lactis subsp. lactis* and *Lb. pentosus* strains on *S. aureus* and *L. monocytogenes* was determined.

Although the antimicrobial effect of LAB is usually against Gram positive bacteria, we have identified 4 strains that have bactericidal activity against Gram negative bacteria namely *E. coli* O157: H7. Two of them are described as *Lc. lactis* subsp. *lactis* and 2 of them as *Lb. pentosus.* In a study conducted by Caridi (9), the cheese was isoleted *Lb. paracasei* subsp. *paracasei and Lb. curvatus* of which are reported to exhibit antimicrobial activity against *E. coli*.

The main reasons for the use of bacteriocins, produced by LAB, as food preservatives are that they are tolerant to pH and temperature (13). All of the bacteriocin-producing strains isolated from the cheese samples were found to retain antimicrobial activity at pH values between 2 and 11. In addition, many isolates found to maintain their antimicrobial activity in different temperature-time applications, demonstrating that bacteriocins produced by these isolates may maintain their stability, especially at pasteurization and sterilization temperatures.

Consequently, the identification of bacteriocin-producing LAB genus to improve quality and reliable dairy products is of utmost importance for human health as far as the milk industry. In this study 3 of strains producing bacteriocin were *Lc. lactis* subsp. *lactis*, 3 of them were *Lb. pentosus*, and others were defined as *Leu. lactis* and *Lb. paracasei* subsp. *paracasei*. Bacteriocin-producing *Lc. lactis* subsp. *lactis* and *Lb. pentosus* strains

were found to have inhibitory effects on *S. aureus* and *L. monocytogenes*. It has been concluded that the bacteriocin-producing isolates, due to not losing their activities in a wide range of pH and different temperature-time values, can be used as a bio-protective culture in food production and storage. It is concluded that these bacteria and the bacteriocins produced by them could be used in the production of fermented dairy products to prevent the development of pathogen or saprophyte microorganisms and to contribute as a food preservative to the dairy industry.

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