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Research Paper / Araştırma Makalesi

Presence of Carotenoid Gene in Lactic Acid Bacteria Isolated from White Cheese

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ABSTRACT

Carotenoids are organic pigments with antioxidant properties that are commonly found in nature. Various types of carotenoids are produced by microorganisms. In this study, we aimed to determine the microorganisms with potential carotenoid production and yellow-orange pigment production during the storage of white cheese below 10°C. Five different white cheeses with pigmentation problems were obtained from the provinces of Istanbul and Kocaeli. Colonies with a typical yellow-orange color and morphological differences were selected on MRS and M17 media. The presence of carotenoid genes in 136 selected colonies was determined by agarose gel electrophoresis using colony PCR, and carotenoid genes were detected in 6 colonies. According to the 16S rRNA sequence results, one of the 6 bacterial colonies carrying the carotenoid gene was *Lactococcus lactis*, another was *Enterococcus faecium*, and the rest were *Lactobacillus plantarum*. In addition to genotypic identification, Gram-staining was performed to determine the phenotypic characteristics of bacteria carrying the carotenoid gene, and it was found that six bacteria had Gram-positive and bacilli morphology. These results showed that some carotenoid producer strains existed in the microbioata of cheeses during cold storage.

Keywords: White Cheese, Carotenoid, *Lactococcus lactis*, *Lactobacillus plantarum*, *Enterococcus faecium*

Beyaz Peynirden İzole Edilen Laktik Asit Bakterilerinde Karotenoid Gen Varlığı

OZ

Karotenoidler antioksidan özellik gösteren ve doğada yaygın olarak bulunan organik pigmentler olarak bilinmektedir. Çeşitli karotenoid türleri mikroorganizmalar tarafından üretilmektedir. Bu çalışmada, beyaz peynirin 10°C'nin altında depolanması sonucu ortaya çıkan, potansiyel karotenoid üreticisi olup sarı-turuncu pigment oluşturan mikroorganizmaların belirlenmesi amaçlanmıştır. İstanbul ve Kocaeli illerinden toplamda 5 farklı sari pigmentasyon olan beyaz peynir temin edilmiştir. Peynirlerden MRS ve M17 besiyerleri üzerinden tipik sarı-turuncu renkli ve morfolojik bakımdan farklılık gösteren koloniler seçilmiştir. Seçilen 136 adet kolonide karotenoid geni varlığı koloni PZR yapılarak agaroz jel elektroforezde incelenmiş ve sonuca göre 6 adet kolonide karotenoid geni bulunduğu belirlenmiştir. Karotenoid geni varlığı tespit edilen kolonilerin 16S rRNA gen bölgesi PZR ile çoğaltılarak sekansa gönderilmiştir. 16S rRNA sekans sonucuna göre karotenoid geni taşıyan 6 adet bakteri kolonisinden bir tanesinin *Lactococcus lactis*, diğerinin *Enterococcus faecium* ve kalan diğerlerinin ise *Lactobacillus plantarum* olduğu tespit edilmiştir. Genotipik tanımlamanın yanında, karotenoid geni taşıyan bakterilerin fenotipik özelliklerinin belirlenmesi için gram boyama yapılmış ve 6 adet bakterinin Gram-pozitif ve çubuk hücre yapısında olduğu belirlenmiştir. Tüm bu sonuçlar peynirin soğuk muhafazasında muhtemel karatenoid üreticisi suşların varlığını göstermiştir.

Anahtar Kelimeler: Beyaz peynir, Karotenoid, *Lactococcus lactis*, *Lactobacillus plantarum*, *Enterococcus faecium*

INTRODUCTION

Milk is an important food source in terms of essential amino acids, fats, mineral substances, lactose, and vitamins. Milk is a perishable food owing to its rich nutritional content. This situation necessitates the application of heat treatments, such as pasteurization or sterilization of milk or converting milk into various fermented foods [1], [2]. Cheese, a product obtained by milk fermentation, preserves the main components of milk and is produced in various aroma, flavor, and textural forms [3]. Cheese is obtained by coagulating the milk protein casein with rennet, separating the whey, and salting the curd obtained by shaping or keeping it in brine [4]. There are more than 1000 types of cheese worldwide, of which 193 are produced in Turkey [3]. In Turkey, where there is a wide variety of cheese production, white cheese, cheddar cheese, and Tulum cheese are preferred among the people. In recent years, laited, curd, cottage, herby, and mihalic cheeses have attracted a great deal of attention [5].

White cheese is defined in the Turkish Food Codex No. 29261 as brined cheese with characteristic features, which can be defined as fresh or ripened according to the differences in production stages, produced by processing curd obtained by coagulating the raw material using rennet [6]. Studies have shown that *Lactococcus lactis* is the most dominant species in the microbiota of white cheese, followed by *Enterococcus feacalis, Enterococcus faceium, Lactiplantibacillus plantarum, Lacticaseibacillus casei, L. brevis, Leuconostoc lactis* and *Leu. Mesenteroides* ssp. *Dextranicum* species. It has also been shown that the microflora of white cheese changes during the ripening process. As cheese ripening progresses, a decrease in *Lactococcus* species and an increase in Lactobacillus species are observed [7]. At the end of maturation, *Lacticaseibacillus casei* and *Lactiplantibacillus plantarum* species were dominant [8]. In Turkey, white cheese is produced from milk pasteurized at 72°C for 20 s, and *Lactococcus lactis* subsp. and *Lactococcus lactis* subsp. *cremoris* strains are considered suitable [9]. White cheese is a semi-hard cheese obtained by mixing cow, sheep, goat, or milk in an appropriate ratio [10]. To provide the desired quality characteristics in white cheeses, they are matured in cold stores for at least 3 months and 6-12 months and presented to the consumer [11]. White cheese, which initially has a soft texture, is called semi-hard or semi-soft after it has matured in brine for 3 months [12].

For cheese production, raw milk is standardized and pasteurized for 2-3 seconds at 80-85°C, 30 min at 63°C, or 5 min at 65°C. After the temperature of the cooled milk after pasteurization reached 32°C, it was transferred to the fermentation tank. Starter culture and $CaCl_2$ are added to the pasteurized milk and left for incubation for 30 minutes, and at the end of this period, rennet is added. to 30-45 minutes after the yeast is added, the milk starts to take its gel form and after 75-90 minutes it becomes a firmer curd. The cheese pieces obtained by cutting the curd into cubes were kept in whey for 5-10 minutes. After soaking, the whey was removed and the cheese curd was transferred to stainless steel molds. After the transfer, a cloth was laid on the surface of the cheese and pressure was applied to the cheese at room temperature by placing a weight on it to tighten the curd. This process continues for 3-6 hours until the whey reached low levels. When the weight was lifted, the cloth was opened, and the cheese mass was cut into 7 × 7 × 7 or 7 × 7 × 10 cm^3 pieces and kept in brine at 15-16°C for 6-12 hours. Cheese molds were placed under the cans and filled with 14-16% brine to the brim, and the mouths of the cans were closed. Cheeses kept in tin cans at 12-15°C mature within 1-2 months and are ready for consumption [5]. During the ripening stage, various biochemical reactions occur in the cheese as a result of physical, chemical, and enzymatic interactions. These reactions can cause undesirable odor, flavor, and color changes as well as release components that have positive effects on flavor and aroma [13].

Carotenoid pigments, commonly found in milk, are important. Carotenoids are pigments synthesized in plant and animal cells, as well as in fungi and photosynthetic and non-photosynthetic microorganisms. It plays an important role in reducing oxidation reactions by removing free radicals with conjugated double bonds in Gram-positive bacteria [14]. It is necessary to determine the components that cause color changes during food spoilage and to reveal the factors that cause this change. Therefore, effective preventive methods should be developed. Microorganisms should be kept in the first place in deteriorations that cause color changes [13].

Color change is one of the leading quality issues in food. This study was carried out to identify the microorganisms that cause color changes in white cheese and carry a potential carotenoid gene. In this context, five white cheeses were collected from Istanbul and Kocaeli provinces. Dilutions were prepared from cheese samples and inoculated onto M17 and MRS media. Strains with a typical yellow-orange color and thought to differ in colony morphology due to incubation were selected. The aim of this study was to determine whether the selected strains carried the carotenoid gene. Genotypic and phenotypic identification of the strains carrying this gene was then performed.

MATERIALS and METHODS

Materials

Five unpackaged cheeses with yellow pigmentation problems were collected from the Istanbul and Kocaeli district bazaars. White cheese samples were stored at 4°C until further analysis (Figure 1).

Methods

Microbiological Analyses of White Cheese Samples

In this study, the counts of Total Aerobic Mesophilic Bacteria (TAMB), yeast mold, and Lactic Acid Bacteria (LAB) in cheese samples were determined using standard microbiological enumeration methods [15]. Appropriate dilutions were prepared from the cheese

samples brought to the laboratory, and microbiological inoculations were performed using the spread plate method on Plate Count Agar (PCA, Neogen, NCM0010A) for TAMB, Dichloran Rose Bengal Chloramphenicol (DRBC, Neogen, NCM0082A) for yeast mold, De Man, Rogosa and Sharpe (MRS, Neogen, NCM0079A) for Lactobacilli, and M17 (Liofilchem, 610505) for *Lactococcus*. The prepared Petri dishes were incubated at 37°C for 48 h for TAMB, 20-25°C for 5-7 days for yeast mold, and 37°C for 48 h for LAB.

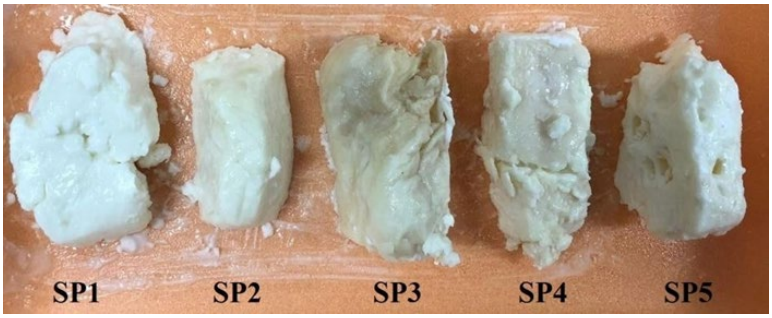


Figure 1. White cheese samples and their codes used in this study

pH and Titratable Acidity Values of Cheese Samples

The pH was determined using a compound electrode digital pH meter (PL-700PV, model pH meter). (Mettler Toledo, USA) [16]. For titratable acidity values, filtrate obtained from the cheese sample was titrated with 0.1 N sodium hydroxide solution using phenolphthalein indicator, and the titratable acidity of the cheese was expressed as percent lactic acid [17].

Isolation of Bacterial Strains

Physiological saline solution (90 mL, 0.85% NaCl) was added to 10 g of cheese sample and homogenized in a stomacher device. Dilutions of up to 10^6 were prepared from homogenized samples. For white cheese samples, MRS agar was used for *Lactobacillus* spp., and M17 agar was used for *Lactococcus* spp. Cells were cultured in MRS and M17 agar at 37°C for 48-72 hours. Typical yellow and orange colonies growing on M17 and MRS media, and colonies with different morphological features were selected.

Phenotypic Identification of Selected Strains by Gram-staining

Gram-staining was performed using strains selected from the colonies grown on MRS and M17 media.

Identification of Selected Colonies by Molecular Methods

Detection of Strains Carrying the *crt* Gene by Colony PCR

Glycerol stocks of the isolated colonies were plotted on MRS and M17 Agar media. The cells were then incubated for 24 h at 37°C. Colony PCR was performed to detect the presence of carotenoid genes in selected colonies. PCR reactions were performed on a PCR BIO-RAD T100™ Cycler using the FIREPol MasterCard Mix enzyme mixture, by preparing the carotenoid gene forward primer 5'-CGCGGAATTC TGAAGCAAGT TCGATTATTGGC and reverse primer-3' and 5'-GATCGAATTCTTAAGCCTCCTTAAGGGCTAGTTC-3, respectively. The PCR conditions for amplification of the carotenoid gene were as follows: initial denaturation at 95°C for 3 min, denaturation at 95°C for 30 s, adhesion at 58°C for 30 s, and elongation at 72°C for 150 s. Amplification, from denaturation to elongation, was repeated for 30 cycles. PCR reactions were loaded on a gel containing 1% agarose and carried out for approximately 90 min by applying a 100 Volt current. At the end of the run, the gel was kept in an ethidium bromide solution for approximately 1 h and then visualized on the BIO RAD GelDoc™ XR+ device under UV light. Colonies carrying the carotenoid gene were selected and genomic DNA was isolated.

Identification of strains using 16S rRNA

The genomic DNAs of the isolated strains was isolated using the Gene MATRIX Tissue & Bacterial RNA Purification kit. Because the 16S rRNA identification method was used for bacterial strain identification, the 16S rRNA region was chosen as the target region. For this purpose, 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCCAGCCGCA-3' were used as forward and reverse primers, respectively. Amplification of the 16S rRNA gene region was performed using a BIO RAD T100™ Thermal Cycler device. The PCR conditions for amplification of the 16S rRNA gene were as follows: initial denaturation at 95°C for 3 min, denaturation at 98°C for 30 s, adhesion at 58°C for 30 s, and elongation at 72°C for 150 s. Amplification, from denaturation to elongation, was repeated for 30 cycles. 16S rRNA reactions were loaded onto a gel containing 1% agarose and carried out for approximately 90 min by applying a 100 Volt current. At the end of the run, the gel was kept in an ethidium bromide solution for approximately 1 h, and the result was visualized on the BIO RAD GelDoc™ XR+ device under UV light.

RESULTS and DISCUSSION

In white cheese samples, *Lactococcus* spp. counts varied between $1.17x10^6$ and $9.9x10^7$, while *Lactobacillus* spp. counts varied between $2.1x10^5$ and $1.69x10^7$ (Table 1). This indicates that white cheese is a rich source of lactic acid bacteria.

Table 1. Microbiological analysis results (CFU/g) of white cheese samples

Sample code	*Lactococcus* spp.	*Lactobacillus* spp.	Yeast-Mold	Total Aerobic Mesophilic Bacteria
SP-1	$9.90x10^7$	$1.69x10^7$	$5.00x10^4$	$7.60x10^6$
SP-2	$1.92x10^7$	$1.60x10^7$	$1.00x10^2$	$1.68x10^7$
SP-3	$1.17x10^6$	$2.10x10^5$	0	$6.60x10^5$
SP-4	$7.00x10^2$	$2.90x10^5$	0	$2.90x10^5$
SP-5	$9.90x10^5$	$1.60x10^6$	0	$9.70x10^4$

The titratable acidity values of the five cheeses analyzed varied between 1.05% and 2.96% in terms of lactic acid. The average value was found to be 1.80%. The pH values of the analyzed white cheese samples varied between 4.55 and 5.97, with an average value was found to be 5.20 (Table 2). The cheese samples complied with the standards in terms of pH and titratable acidity.

Table 2. pH and titratable acidiy values of white cheese samples

Sample code	pH	Titration Acidity (% lactic acid)
SP 1	4.55	1.70
SP 2	5.97	1.05
SP 3	4.64	1.93
SP 4	5.91	1.43
SP 5	5.07	2.96

A total of 136 yellow-colored colonies with different colony morphologies were selected from white cheese samples sold in Istanbul and Kocaeli provinces and cultured on M17 and MRS agar medium. Examples are named SP1, SP2, SP3, SP4, and SP5. After screening, 62 colonies from MRS medium and 74 colonies from M17 medium were selected: 30 from SP1, 25 from SP2, 33 from SP3, 25 from SP4, and 23 from SP5. Colony PCR was performed to determine whether the selected colonies harbored the carotenoid gene. PCR analysis revealed that six strains carried the carotenoid gene (Figure 2).

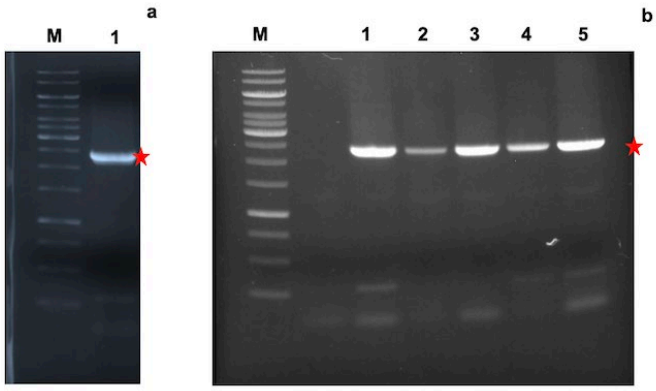
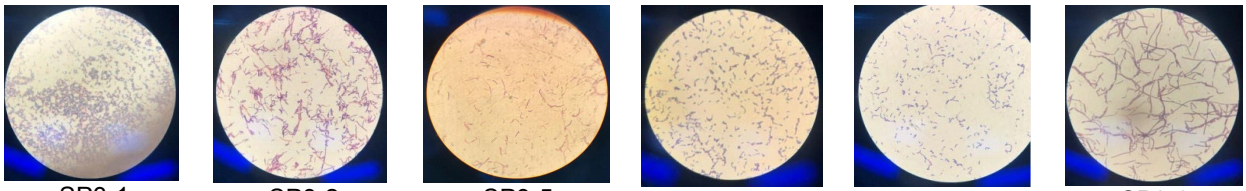


Figure 2. Colony PCR results of carotenoid gene screening of strains isolated from cheese samples. M: Ladder, a) First gel of Colony PCR, 1: SP3-1, b) Second gel of Colony PCR, 1: SP3-2, 2: SP3-3, 3: SP3-5, 4 : SP3-7, 5: SP3-9. The expected carotenoid gene length of 2379 bp is indicated with an asterisk.

Gram-staining was performed to determine phenotypic characteristics of the bacteria. Under the microscope, blue violet-colored cells were evaluated as Gram-positive and pink-colored cells as Gram-negative. All the strains found to carry the carotenoid gene were Gram-positive and bacil-positive (Figure 3).



Cheese Code

Figure 3. Gram-staining images of microorganisms

16S rDNA sequence analysis was used to identify bacterial strains. Sequence results were analyzed with the help of a plasmid editor program and converted to the FASTA format. Sequence data were analyzed using Nucleotide BLAST: nucleotide databases were searched using a nucleotidequery (nih.gov) [18]. According to the blast scan of the 16S rDNA sequence results for the identification of the strains carrying the carotenoid gene; It was determined that 1 strain (SP3-7) was *Lactococcus lactis*, 1 strain (SP3-3) was *Enterococcus faecium* and 4 strains (SP3-9, SP3-5, SP3-1, SP3-2) were *Lactobacillus plantarum* (Table 3).

Table 3. Blast scan results

JobTitle	ScientificName	MaxScore	TotalScore	QueryCover	E value	Percent Identification
SP3-1	*Lactobacillus plantarum*	2108	2108	%100	0.0	97.67
SP3-2	*Lactobacillus plantarum*	2071	2071	%99	0.0	98.64
SP3-3	*Enterococcus faecium*	1142	1142	%98	0.0	87.96
SP3-5	*Lactobacillus plantarum*	2217	2217	%99	0.0	98.57
SP3-7	*Lactococcus lactis*	2194	2194	%99	0.0	98.18
SP3-9	*Lactobacillus plantarum*	1988	1988	%98	0.0	97.20

Similar to our findings, Garrido-Fernández et al. [19] reported that 18 strains of *Lactobacillus plantarum* and *S. aureus* isolated from olive fermentation were carotenoid producers. Colonies isolated on MRS agar showed a dark yellow pigmentation. PCR studies have shown that *Lactobacillus plantarum* carries *crtM* and *crtN* genes in its genome.

In a study by Turpin et al. [20], it was determined that among 158 bacteria cultured in MRS medium, 36 strains produced carotenoid-like compounds. Among these strains, the carotenoid-producing bacterium *Lactobacillus plantarum* WCFS1 was also present, along with *Limosilactobacillus fermentum* and *Pediococcus acidilactici*, which are known to produce carotenoid-like compounds.

In a study by Kim et al. [21], 79 strains forming yellow colonies from various fermented foods were screened, and the 16S rDNA gene was identified by PCR amplification. According to the identification results obtained through 16S rDNA sequencing analysis, it was determined that among the identified bacterial strains, the *Lactilactobacillus plantarum* strain also carried the carotenoid gene.

In a study by Nam et al. [22], bottom portion of mevalonate partway of *Enterococcus faecium* VTCC-B-935 was transferred to *E. coli* and it was found that the biosynthesis of β-carotene increased 3 times.

In this study, we determined that the strains producing carotenoids were *Lactobacillus plantarum*, *Enterococcus faecium*, and *Lactococcus lactis*. A review of the literature led to the conclusion that *Lactobacillus plantarum* and *Enterococcus faecium* strains are carotenoid producers. However, whether *Lactococcus lactis* possesses the carotenoid gene was inconclusive. In this study, based on the identification of 16S rDNA, it was established that the *Lactococcus lactis* strain possesses the carotenoid gene.

This study highlights potential strategies involving the use of *Lactobacillus plantarum*, *Enterococcus faecium*, and *Lactobacillus plantarum* strains to meet the demand for carotenoid pigments in the food industry. The ability of these bacteria to produce carotenoids should be considered in future research to address the demand for natural coloring agents and to enhance the value of food products.

CONCLUSION

Five samples of white cheese were inoculated into MRS and M17 media. A total of 136 colonies with distinct yellow-orange color and morphological characteristics were selected, and their carotenoid gene was determined through colony PCR. Subsequently, six strains bearing the carotenoid gene were identified through 16S rRNA sequence analysis. The strains *Lactobacillus plantarum, Enterococcus faecium,* and *Lactococcus lactis* were found to carry the carotenoid gene. This finding provides an explanation for the yellow pigmentation observed in the cheese samples during cold storage, which is likely due to the stress response of certain strains within the microbiota.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

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