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Investigation of CRISPR anti-phage systems of *Lactobacillus plantarum* from pickled olives

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Abstract

Lactobacillus plantarum is the most commonly used microorganism in industrial food fermentations. The multidrug resistance, bacteriocin production, various enzyme activities, probiotic properties and the resistance of the strain against bacteriophages, are important for application in industrial field. Microorganisms have developed various survival strategies in the evolutionary process. Genomic anti-phage role of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR-Cas) is one of the defense strategies. This is an RNA-based immune system involved in the collaboration of CRISPR-related (cas) genes with DNA sequences of foreign genetic elements inserted between the repeated sequences.

In this study, a rapid diagnosis of *L. plantarum* was carried out among 59 pickled olives by Real-Time Polymerase Chain Reaction (PCR) with species-specific probes and primers. Twenty-five isolates were identified as *L. plantarum*. The presence of CRISPR-Cas loci of the genomic anti-phage system in these identified species was investigated using DNA based and bioinformatics methods such as PCR, sequencing, and molecular software. Specific primer design for *L. plantarum* CRISPR arrays was performed. CRISPR loci were detected in 14 *L. plantarum* strains via classical PCR method. The results were analyzed using NCBI-Blast, CRISPRFinder databases. In one of the isolates, 6 repeats, and 5 spacers nucleotide arrays were found. CRISPR-related proteins (Dead/Deah box helicases) were detected in remaining isolates.

Keywords

Anti-phage system, CRISPR-cas, CRISPRFinder, *L. plantarum*, pickled olives, TaqMan probe.

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INTRODUCTION

Table olives are of great importance among fermented vegetables worldwide. Turkey has a significant potential in the world's olive production, accounting for 7% of total olive and 17% of total table olive production (Cetin *et al.,* 2004). Izmir province is one of the leading production areas in Turkey. Table olive is very important for local people in Turkey and is fermented by lactic acid bacteria (LAB) and yeasts, which are natural biota of olives. The fermentation process is carried out by competition between these two groups of microorganisms. Yeasts play a minor role in the development flavor and aroma of table olives and in the improvement of LAB.

Lactobacillus spp. is the most frequently isolated bacteria from table olives. The predominant species in most natural and treated fermentation of table olives are *L. plantarum* and *L. pentosus*. In food fermentation, lactobacilli, especially *Lactobacillus* spp., play a major role as starters cultures, bio preservatives, and antimicrobial compounds (de Vries *et al.*, 2006). *L. plantarum* is one of the members of healthy human microbiota and has anti-microbial or anti-tumorigenic activities (Bevilacqua *et al.*, 2010; Chiu *et al.*, 2008; Mathara *et al.*, 2008). *L. plantarum* has an extended environmental niche, including fermented dairies, meat, and vegetables (Kleerebezem *et al.,* 2003).

Microorganisms have developed various strategies to avoid exposure to foreign genetic elements. Although abundant and ubiquitous viruses infect them, microorganisms routinely survive and thrive in the competitive environments. Continuous exposure to exogenous DNA through transduction, conjugation, and transformation has enabled microorganisms to recognize and distinguish "foreign" DNA from their "own" DNA, and develop a set of defense mechanisms that allow them not to be exposed to invasive elements. These systems not only preserve genetic integrity, also allow the uptake of external DNA and conservation of advantageous genetic material for adaptation to the environment. Some strategies, such as adsorption prevention, injection blocking, and abortive infection are effective against viruses. Other defense systems specifically target the invasive nucleic acid, such as the restriction-modification system (R-M) and the use of specific nucleases. Recently, an adapted microbial immune system that provides short palindromic repeats (CRISPR) at regular intervals has been identified and the system is reported to be an element of acquired immunity against viruses and plasmids (Barrangou and Horvath, 2012). CRISPR system includes DNA repeats from archaeal (~90%) and bacterial (~40%) genomes. CRISPR loci are

typically characterized by spacers (mostly phage and plasmid sequences) and *cas* genes are generally associated with CRISPR. Considering the importance of CRISPR loci in the adaptation and persistence of bacteria against viruses, this system clearly provides information about the evolution of genomes belonging to phages and hosts (Horvath *et al.,* 2008).

In this study, the TaqMan 5' nuclease assay method was utilized as a fast and secure vehicle for the detection, identification, and molecular characterization of *L. plantarum.* The presence of CRISPR-Cas loci of the genomic anti-phage system in *L. plantarum* isolates was investigated by molecular and bioinformatics methods.

MATERIALS AND METHODS

Bacterial isolation and media

In the present study, 59 samples were collected from Aydin-İzmir, Turkey. The samples were cultured by spread plate method on Mann Rogosa Sharp (MRS) agar containing 40% cycloheximide. The media were incubated at 30 °C for 48-72 hours under atmosphere containing 10% CO_2. Single colonies were subcultured in MRS broth supplemented with 10% ethanol (EMRS). Colonies were primarily identified based on Gram characteristics and cell morphologies. The strains were further identified by *L. plantarum* specific real-time PCR (RT-PCR). Purified cultures were stored in 30% (v/v) glycerol at -20 °C until further processing.

DNA isolation

QIAamp DNA mini kit was used for extracting DNA of the isolates. Isolated DNAs were used for the identification of the strains by species-specific qualitative PCR. PCR analyzes were carried out by Roche Light Cycler PCR. Amplification products were controlled by gel electrophoresis where the agarose is stained with GelRed.

Species-specific quantitative real-time PCR (TaqMan5' Nuclease Assay)

The optimized probe and primers of *Lactobacillus* spp. and *L. plantarum* specific to 16S-23S intergenic spacing regions that were designed based on EMBL and GenBank DDBJ databases were used (Haarman and Knol, 2006). 6.8 µl of nuclease-free ultra-pure water; 0.5 µL forward primer (20 µM); 0.5 µL reverse primer (20 µM); 0.2 µL of TaqMan probe (10 mM); 10 µL of enzyme and dNTP mixture were used for each reaction. The total volume of PCR reaction was 20 µL with 2 µL of template DNA. Sequences of primers and probes are listed in Table 1. PCR reaction conditions are listed in Table 2.

Table 1: Probe and primers for TaqMan5' Nuclease assay.

Forward primer	TGG ATC ACC TCC TTT CTA AGG AAT
Reverse primer	TGT TCT CGG TTT CAT TAT GAA AAA ATA
Probe	FAM-ACA TTC TTC GAA ACT TTG T NFQ-MGB

Table 2: RT-PCR reaction conditions for identification.

Program	**Denaturation**	**Amplification**			**Cooling**
Analysis mode	None	Quantification mode			None
Cycle	1	45			1
Target [°C]	95°C	95°C	57°C	72°C	40°C
Time[hh:mm:ss]	00:10:00	00:00:10	00:00:30	00:00:10	00:00:30
Rate[°C/s]	**20**	**20**	**20**	**20**	**20**

CRISPR loci analysis in *L. plantarum*

CRISPR sequences belonging to *L. plantarum* were scanned using the CRISPRFinder program and CRISPR site specific primers were designed using the Primer3web database. PCR application was performed using primers specific to CRISPR gene regions of *L. plantarum* (Table 3). Reaction mixture was as follows: 25 µl PCR Master-Mix (2X); 1 µl forward primer, 1 µl reverse primer; 5 µl of template DNA; 18 µl of nuclease-free ultrapure water. PCR reaction conditions are shown in Table 4. PCR products were visualized by agarose gel electrophoresis. PCR products were sent to Altigen-Bio Biotechnology Company (Izmir-Bornova, Turkey) for sequence analysis. Arrangement and analysis of DNA sequences were analyzed by DNA Baser program. The results of the PCR products were analyzed on the CRISPRFinder and NCBI-Blast database.

Table 3. CRISPR loci primers specific to *L. plantarum.*

Forward Primer	5'-TGG CGT GAT ATG AAT TAA TGA GT-3'
Reverse Primer	5'-GGG AAA AGA TGG CGT GAT TG-3'
Degenerate Forward Primer	5'-TCG AAT GGA AAA GTT MAA AA-3'
Degenerate Reverse Primer	5'-AAC CTR TWT TRG TTG GTG AG-3'

Table 4. PCR reaction conditions for CRISPR array.

Stages	**Temperatures**	**Times**	**Cycles**
Initial denaturation	95 °C	2 min	
Denaturation	95 °C	0:30 sec	
Annealing	48 °C	0:45 sec	35
Elongation	72 °C	1:30 min	
Final elongation	72 °C	5 min	

RESULTS AND DISCUSSION

As a result of microscopical examination of isolates, it was determined that 40 isolates from 59 saline samples were Gram positive and rod-shaped. The remaining isolates were cocci or large yeast cells. In previous studies, it was reported that LAB developed spontaneously in processed olives. Also the presence of yeast populations was demonstrated in natural olives. (Hurtado *et al.*, 2008). The isolation of *Lactobacillus* spp. from table olive brine samples and the differentiation of yeasts from these strains are quite difficult. To address this problem, 20 mL/L 1% (w/v) cycloheximide was added to the MRS agar medium for isolation (Sharp, 1962). However, the amount of cycloheximide was not enough to inhibit the yeasts. To overcome this problem, higher concentrations of cycloheximide were tested and the concentration of 40 mL/L 1% (w/v) was detected to have optimal potential to inhibit the yeast population. However, additional procedures were required to separate bacilli and yeasts. EMRS broth was utilized to inhibit yeasts and stimulate the growth of bacilli in a misture of yeasts and lactobacilli. After 3 days of incubation with shaking at 30 °C, high ethanol concentration in EMRS liquid medium was detected. The growth of yeasts was limited, and pure lactobacilli colonies were observed on MRS agar plates. According to the results of the study, the use of EMRS liquid medium in the isolation of *Lactobacillus* spp. is a critical step, and the medium can be used as an effective selective medium for the isolation of *Lactobacillus* spp. Similar to the yeast, *Lactobacillus* spp. has white mucoid colonies in MRS agar. The microscopic appearance of lactobacilli is Gram-positive, rods with straight form. However, they can be in a spiral or coccobacillary form under certain conditions. They are often found in pairs or chains of varying lengths (Altermann *et al.*, 2005). On the other hand, yeasts can have circular, elliptical, triangular, bottle-shaped appearance (de Becze, 1956). Therefore, the phenotypic identification is often unreliable, and molecular identification of *Lactobacillus* spp. is required.

In recent years, PCR is utilized as a rapid and powerful technique for *in vitro* amplification of DNA (especially 16S rDNA genes) to identify bacterial genus and species (Goldstein *et al.*, 2015). Moreover, in the last few years, the quantification of 16srRNA gene by quantitative RT-PCR, has become one of the most useful methods (Schefe *et al.*, 2006). However, in addition to the operational advantages, RT-PCR (TaqMan5' Nuclease) is more sensitive and reproducible. Therefore it has recently

replaced traditional PCR in diagnostic studies (Gibson *et al.*, 1996). In a study conducted by Haarman and Knol (2006), all *Lactobacillus* spp. from the samples were correctly detected by RT-PCR, while closely related *Enterococcus* spp. and *Propionibacterium* spp. strains did not yield any amplification.

The Minor Groove Binder (MGB) probe was used in this study, which consisted of two sections: 5' reporter dye and 3' Non-Fluorescent Quencher (NFQ). The advantage of NFQ is to provide a lower background signal, which results in better sensitivity in the quantification. On the other hand, the MGB fraction balances the hybridization of the probe with single-stranded DNA targets by increasing the melting temperature and thereby reducing the length requirements of the oligodeoxynucleotides. In practice, the specificity of an MGB probe on a traditional TaqMan probe should be increased (Yao *et al.*, 2006). Due to the stability of the generated DNA duplexes, it is possible to use shorter probes by MGB probes, with higher sensitivity to single base mismatches, providing additional sequence specificity when the mismatch is below the MGB site. To benefit from these properties, fluorogenic 3′-MGB probes were prepared and studied in 5'-nuclease PCR analysis. MGB probes are shorter length probes with better sequence specificity and lower fluorescence background than none-MGB probes (Kutyavin *et al.*, 2000). The sensitivity of the TaqMan probe technique and the specificity of the probes and primers are found to be 1,000 to 10,000 times more sensitive than conventional PCR analysis.

In this research, DNA was extracted from 40 samples of LAB with similar morphology after incubation in MRS broth media and then, visualized by nano-spectroscopy and agarose gel. All of the DNA extracts were detected to be between 55-200 ng/µL at 260-280 nm wavelength. Twenty-five of 40 isolates obtained by culture method were identified as *L. plantarum* by RT- PCR assay using a gene-specific TaqMan probe and primer pairs specific to the 16S-23S intergenic region (Figure 1).

PCR analysis performed by CRISPR primers showed amplification in 14 of 25 *L. plantarum* strains. A typical CRISPR sequence (Repeat-Spacer) was found in only one of the 14 isolates (Figure 2). In the other 13 isolates, no locus was found on the CRISPRFinder database. However, each of the 14 isolates was identified to have CRISPR-associated proteins in the NCBI database. The spacer sequences, which were detected in an isolate (designated as the L3), were also examined. The length of the CRISPR region was found to be 440 base pairs (bp) and the repeat region was 36 bp (Table 5).

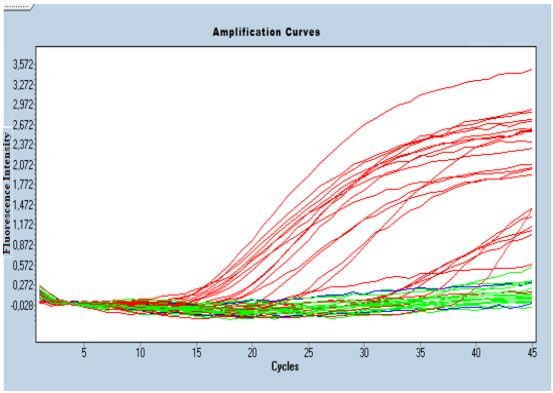

Figure 1: RT-PCR amplification curve.

The red lines running exponentially in the amplification curve shown in the figure indicate the species identified as *L. plantarum.*

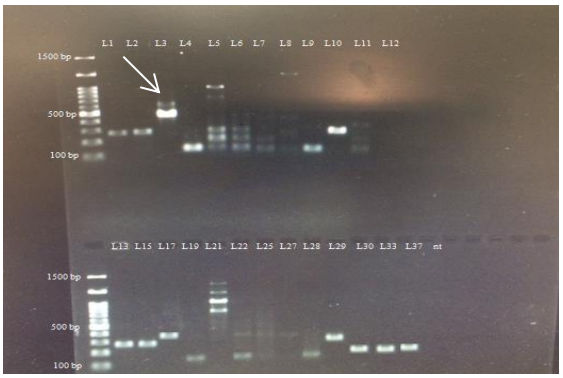

Figure 2: Agarose gel electrophoresis of CRISPR gene regions in *L. plantarum* isolates.
A complete CRISPR sequence approximately of 500 bp long was detected in L3.

According to studies on LAB, eight CRISPR families are generally represented. Eight CRISPR families were identified by a comparative analysis of repeat and Cas 1 gene sequences. Members of CRISPR families called Sthe3, Sthe1, Efam1, Lsal1, Blon1, Lhel1, Sthe2 and Ldbu1 consist of various LAB genera and species. It was found that the repeat length which was determined to be 36 bp was preserved in the first 5 families (Horvath *et al.*, 2008). As a result of this study, the repeat sequences detected in the isolate of L3 were also found to be 36 bp.

Table 5. CRISPR array results of L3 in CRISPRFinder database.

Repeat sequences	Spacer sequences
ATTCTACAACTGCTTAAAATGACCACTGTCC GAGAC	CCGGATGACAGGTCGAAACGATCCT
GTTCTAAACCTGTTTGGTATGACTACTATTC AAGAC	CGATTTGGATGGCTCTTTTTTTTGAATT
GTTCTAAACCTGTTTGGTATGACTACTATTC AAGAC	GACCTAGCTAAAACGTCTGAGAACGTCTTA
GTTCTAAACCTGTTTGGTATGACTACTATTC AAGAC	TAGCTACGCAAACTCATGGCAAAATGACTA
GTTCTAAACCTGTTTGGTATGACTACTATTC AAGAC	CGGCATTCTCACATAATCCACTCATTAATTCA AATCACGCCATNTN
GTTCTAAACCTGTTTGGTATGACTACTATTC AAGAC	GCCCACGATTCAAGGATTACACGGGCGGCG
GTTCTAAACCTGTTTGGTATGACTACTATTC AAGAC	

The samples, in which CRISPR sequences could not be detected in CRISPRFinder database, were also analyzed using NCBI-Blast. Analyzes were first performed based on nucleotide similarities. Later, nucleotides were used "blastx" to examine their homology with proteins. The sequences of the isolates encoded as L29, L30, L10, L17, L5, L1 and L2 showed homology with the DEAD / DEAH helicase family whereas the sequences of those designated as L9, L13, L15, L21, L37 and L3 showed homology with ATP-dependent helicase. In a study conducted by Crawley

et al., (2018), CRISPR typing and activities of *cas* genes were studied in *Lactobacillus* spp. CRISPR type I, II, and III vary in *Lactobacillus* spp. Type II CRISPR loci is detected mainly in *L. plantarum* strains and type I is rare. Type III CRISPR locus was not found in *L. plantarum* strains (Crawley *et al.,* 2018). The results of this study are similar to the type I CRISPR loci. Because the DEAD / DEAH helicase family, one of the domains of the key Cas3 protein in type I CRISPR systems, shows high homology with the sequences of the samples analyzed in this study. Sinkunas *et al.,* (2011), revealed the structural analysis of Cas3 protein in *Streptococcus thermophilus* DGCC7710 strain, which is a LAB species. The Cas3 protein consists of three domains in the strain. One of these domains is defined as DExD helicase. Cas 3 shows a nuclease and helicase function that cleaves single-stranded DNA. While nuclease activity is carried out by the HD domain, helicase activity is due to the function of the DEAD / DEAH domain (Sinkunas *et al.*, 2011). Moreover, in Type I-F systems, fusion of Cas3 and DEAD / DEAH family exonuclease with a different Type IE system version in several genomes, and various conserved Cas2 fusions in Firmicutes, was detected (Makarova and Koonin, 2013). In our study, predominantly detection of the DEAD / DEAH helicase family in *L. plantarum* strains indicates the presence of CRISPR loci in these species. However, CRISPR-Cas systems are not always naturally active in the microorganisms that have *cas* protein domains. For example, *L. paracollinoides* strains always contain the *cas* gene but do not contain repeat sequences (Crawley *et al.,* 2018).

In this study, strains without repeat-spacer sequences were determined. To determine whether the sequences scanned in fasta format in the analyses are present in databases such as CRISPRFinder, the sequence must have at least 2 repeat and 1 spacer regions. Therefore, it is not possible to detect CRISPR sequences with the CRISPRFinder database in strains that have not been exposed to phage or in strains that have lost their function over time and are inactive.

One of the important findings of the present study is the ability of the TaqMan5 'Nuclease test to detect bacteria with the same morphology as well as bacteria that have close genetic similarity with high sensitivity at a species-specific level.

L. plantarum isolates, analyzed in this study, were isolated from home-type pickled olive waters collected from Aydın and Izmir. The formation of CRISPR sequences usually occurs by exposure of the microorganism to phage and plasmids or by horizontal gene transfer. Of the 14 strains of *L. plantarum* isolated from domestic fresh

brine, a typical CRISPR sequence was found only in one isolate (L3). For the isolates in which CRISPR sequences were not encountered but the presence of CRISPR-related proteins was detected, further studies have to be performed using transcriptomic approaches in detail to determine whether these proteins are active or not. However, CRISPR loci may show polymorphism in microorganisms. Therefore, the primers used in CRISPR locus analysis may not always give results since spacer contents may change and new sequences can be formed in the locus with horizontal gene transfers. Whole-genome analysis with the approach of next-generation sequencing will contribute to the field for the analysis of gene regions that are constantly dynamic such as CRISPR.

From recent studies on LAB phages, it is clear that a two-pronged approach is required to fully understand phage biology. It is necessary to understand the genetics and biology of both phage and host to define the phage requirements for infection and to determine how phage and host evolved together to adapt to the threat posed by the other. It does not seem possible to eliminate the problem of phage contamination in LAB used in various food fermentations. However, the primary approach to gain insights into phage and host interactions should be the development of tools and strategies to control and prevent phage infection during food fermentations.

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