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Isolation and Molecular Identification of Yeast Strains Causing Spoilage in Labneh Cheese

Özgür Kebabcı [1,a,*]

[1]*Department of Molecular Biology and Genetics, Faculty of Science, Sivas Cumhuriyet University, Sivas, Türkiye*

**Corresponding author*

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ABSTRACT

Labneh is among the dairy products with high nutritional value in the spreadable cheese category. It is consumed together with other dairy products in domestic food consumption. It has been observed that labneh cheese purchased for consumption spoils in the refrigerator after a while. Samples taken from spoiled labneh cheese were purified by single colony cultivation method (streak-plate technique) in PDA and then purified yeast strains were identified. EurX GeneMATRIX Plant & Fungi DNA isolation kit (Poland) was used for DNA isolation in the identification. The amount and purity of the isolated DNA were measured spectrophotometrically in Thermo Scientific Nanodrop 2000 (USA). For species determination, targeted gene regions were amplified by PCR with universal primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3'). A single-step PCR process was performed to amplify the region of approximately 700 bases. The amplification results obtained by PCR were electrophoresed in 1.5% agarose gel prepared with 1x TAE buffer at 100 volts for 90 minutes and images were taken under UV light using ethidium bromide dye. The results obtained with ITS1 and ITS4 primers were evaluated using the CAP contig assembly algorithm in BioEdit software to create a consensus sequence. Species determination of yeast isolates was determined according to the closest species in NCBI. One of the two yeast species isolated from spoiled labneh cheese was identified as *Yarrowia lipolytica* with a similarity rate of 99.06% and the other as *Rhodotorula mucilaginosa* with a similarity rate of 98.42%. Evolutionary analyses were performed in MEGA11 and the evolutionary distance between the two newly isolated species was shown.

Keywords: Labneh cheese, Spoilage, Molecular identification, *Rhodotorula mucilaginosa*, *Yarrowia lipolytica.*

[a] *ozgurkebabci@cumhuriyet.edu.tr* *https://orcid.org/0000-0002-9404-747X*

Introduction

The relationship between microorganisms and humans is complex. On the one hand, they provide benefits by being involved in the production of some industrial products, while on the other hand, they cause negative situations such as causing diseases in humans and spoiling food. Food spoilage causes serious economic losses worldwide. It can be said that more than 30% of the food produced every year is lost or wasted, which leads to a loss of billions of dollars [1]. It is possible for food to spoil from production to consumption. During the consumption phase, foods are stored in cooling environments such as refrigerators to prevent spoilage. However, there are many microorganisms that cause food spoilage even in the refrigerator. These microorganisms are one of the most effective causes of food spoilage. In brief, the main cause of food spoilage is contamination of food with bacteria, yeast and molds. Various factors play a role in microbial food spoilage and these factors are interconnected. First of all, the factors specific to food are internal factors and these are the nutritional composition of the food, the natural antimicrobial factors in the food and the chemical, physical and biochemical properties such as pH, aw and Eh or oxidation-reduction potential of the food. Another factor, process factors, are the factors that are effective during food processing, which are the factors that affect microbial colonization, survival and growth during food processing. Factors such as heating or irradiation and even contamination from equipment can be added to these process factors. The last factors that are effective in microbial food spoilage are external factors [2]. These are environmental factors and are factors such as storage temperature or atmosphere. If process factors are left aside, it can be said that the factors that are effective in food products for domestic consumption are internal and external factors.

Food poisoning may come first to mind in food spoilage, but it is known that not every spoiled food causes poisoning. Food spoilage is defined as a visual, odor, taste and tactile sensory change in food, and food spoilage is expressed as a metabolic process that makes food unsuitable for human consumption due to these sensory changes [3, 4]. Although this metabolic process is sometimes chemical, it is usually carried out by microorganisms. Bacteria and fungi play an important role in food spoilage. Fungi, in particular, are involved in spoilage with their resistance. It is known that some fungal species survive even under the most extreme physico-chemical conditions and thermal processing regimes used in commercial food production [5] . Although low temperature conditions seem to prevent food spoilage in

domestic consumption, many fungal species can multiply at these low temperatures and cause food spoilage [6]. Storing foods in cold conditions can deter mesophiles but allow uncontrolled growth of psychrophiles [7]. There are also microorganisms known as psychrotrophs that can tolerate cold conditions, which are microorganisms that can grow at temperatures similar to those of mesophiles but have adapted to tolerate cold conditions. Psychrotrophs' optimum growth temperature are between +25 and +30 °C, while their minimum growth temperature is (-5)-(+7) °C, while their minimum growth temperature is (-5)-(+7) °C [8]. Both psychrophiles and psychrotrophs (facultative psychrophiles) can cause food spoilage in cold storage conditions such as refrigerators. In cultured dairy products, yeasts are the primary cause of spoilage. This is because yeasts can grow at low temperatures (such as refrigeration temperatures) and pH values. [9]. One of these dairy products is labneh. Labneh is a food product obtained by combining pasteurized milk and yogurt culture, which is in the spreadable cheese category. This product, which contains 17 g fat, 4.5 g carbohydrates, 4.9 g protein and 120 mg calcium per 100 grams, provides the nutrients necessary for the growth of microorganisms. Yeast spoilage usually manifests itself with the growth on the surface of products such as cheese and meat and the fermentation of sugars in liquid and semi-liquid products [10].

In the past, studies conducted to identify yeast species in spoiled foods were conducted using phenotypic methods that included morphological, physiological and biochemical methods [11]. Phenotypic methods, although not frequently, can give incorrect results in identifying yeasts that cause food spoilage. Liu et al. reported that some *Yarrowia* species cannot be identified based on their phenotypic characteristics alone and recommended sequencing the D1/D2 LSU rRNA gene and ITS regions in the identification of these species [12]. Nagy et al. reported that they could not observe any difference between the phenotypic characteristics of this new species and the phenotypic characteristics of *Y. deformans* and *Y. lipolytica* in their study on the new species, and reported that the new species could be identified only by the sequences of the ITS and nuclear LSU rRNA gene D1/D2 regions [13]. For these and similar reasons, the use of DNA-based genotypic methods in the identification of yeasts has become inevitable [14, 15]. Various genotypic methods come to the fore in the identification of yeast isolates isolated and purified from spoiled foods. These are; PCR-RFLP (PCR-restriction fragment length polymorphisms), rep-PCR (repetitive sequence based polymerase chain reaction), AFLPs (Amplified Fragment Length Polymorphisms), RAPD (Random Amplified Polymorphic DNA), PCR using species-specific primers, sequence analysis of the 26S rRNA gene D1/D2 region and ITS (Internal Transcribed Spacer) sequence analysis [16-21]. The last two are powerful techniques used for the accurate identification of yeast species [11].

In this study, species identification of yeast strains isolated and purified from spoiled labneh cheese was performed and after identification, the evolutionary relationship of the yeast strains was shown as a phylogenetic tree.

Materials and Methods

Isolation of Yeasts

It has been observed that labneh cheese purchased for consumption spoiled after a while in the refrigerator. It has been observed that labneh cheese purchased for consumption spoiled after a while in the refrigerator. The samples inoculated on petri plates were incubated at 30°C for 48 hours. After incubation, samples thought to be yeast from the microorganisms that grew as a mixed culture were inoculated again on PDA with the streak-plate technique and incubated at 30°C for 48 hours. After a few passages, yeast isolates were purified.

DNA Isolation and PCR

DNA isolation from yeast isolates was performed using the EurX GeneMATRIX Plant & Fungi DNA isolation kit (Poland), designed for DNA isolation from different plant organs and tissues (leaves, seeds, fruits) as well as from fungi, algae and lichens [22]. In order to control the amount and purity of the DNA obtained after DNA isolation, spectrophotometric measurement was performed on the Thermo Scientific Nanodrop 2000 (USA) device. Then, PCR study of the DNA, the amount and purity of which were confirmed, was performed. For species identification of yeast isolates, targeted gene regions, nuclear ribosomal DNA 5.8S region, were amplified by PCR with universal primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') [23-25]. PCR was performed in a single step, using Solis Biodyne (Estonia) FIREPol® DNA Polymerase Taq polymerase enzyme to amplify a region of approximately 700 bases (Table 1.). After PCR, a single band was obtained on the agarose gel, indicating that the PCR process was successful. During the purification step of the PCR product, the obtained single-band samples were purified using the MAGBIO "HighPrep™ PCR Cleanup System" (AC-60005) purification kit according to the kit's procedures. For Sanger sequencing, the ABI 3730XL Sanger sequencing device (Applied Biosystems, Foster City, CA, USA) and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) were used in the Macrogen Netherlands laboratory. To create a consensus sequence, the results obtained with the ITS1 and ITS4 primers were turned into contigs using the CAP contig assembly algorithm in the BioEdit software [22]. In order to obtain the images of the amplification results obtained with PCR (kyratec thermocycler), electrophoresis was first carried out for 90 minutes at 100 volts in a 1.5% agarose gel prepared with 1x TAE buffer. Afterwards, the image was taken under UV light using ethidium bromide dye.

Table 1. PCR Conditions

Component	Concentration of Stock	Concentration of React.
PCR Buffer	10 x	1 x
$MgCl_2$	25 mM	1.5 mM
DNTP mix	20 mM	0.2 mM
Forward Primer	10 µM	0.3 µM
Reverse Primer	10 µM	0.3 µM
Taq DNA polymerase	5 U/µM	2 U/µM
DNA template	3 µM	
Makeup to 35 µL with PCR-grade water		
*	95 °C, 5 min (initial denaturation)	
** 40 cycles	95 °C for 45 s (denaturation)	
	57 °C for 45 s (annealing)	
	72 °C for 60 s (extension)	
***	72 °C for 5 min (final extension)	
The temperature is reduced to 4 °C, and the PCR is completed		

Phylogenetic Study with Maximum Likelihood Method

The evolutionary history of R. mucilaginosa and Y. lipolytica, which were isolated from spoiled labneh cheese and identified as species, was determined using the Maximum Likelihood method and the Tamura-Nei model [26]. The phylogenetic tree with the highest log probability (-3569.00) is shown (Figure 1). The initial tree(s) for the heuristic search were obtained automatically. Neighbor-Join and BioNJ algorithms were applied to a matrix of pairwise distances estimated using the Tamura-Nei model, and then the topology with the superior log probability value was selected. This analysis included 31 nucleotide sequences and there were a total of 1081 positions in the final dataset. Evolutionary analyses were performed in MEGA11 [27].

Results and Discussion

The identification of pure yeast strains isolated from spoiled labneh cheese was made according to the closest species in NCBI and the first yeast isolate was identified as *Rhodotorula mucilaginosa* with a similarity rate of 98.42% and the second yeast isolate was identified as *Yarrowia lipolytica* with a similarity rate of 99.06% (Table 2).

Table 2. Identification results of yeast strains isolated from spoiled labneh cheese

Yeasts	Sequence Analysis	Results
Lbn1	ACAGGGTTAGAAAGTGAGAGTTCGGACTCCAAGTTAAGTTGGACGTCCTATGT TCACTAATGATCCTTCCGTAGGTGAACCTGCGGAAGGATCATTAGTGAACATAG GACGTCCAACTTAACTTGGAGTCCGAACTCTCACTTTCTAACCCTGTGCATTTGT TTGGGATAGTAACTCTCGCAAGAGAGCGAACTCCTATTCACTTATAAACACAAA GTCTATGAATGTATTTAATTTTATAACAAAATAAAACTTTCAACAACGGATCTCT TGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTG CAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCATGGTATTC CGTGGAGCATGCCTGTTTGAGTGTCATGAATACTTCAACCCTCCTCTTTCTTAAT GATTGAAGAGGTGTTTGGATTCTGAGCGCTGCTGGCCTTACGGTCTAGCTCGTT CGTAATGCATTAGCATCCGCAATCGAACTTCGGATTGACTTGGCGTAATAGACT ATTCGCTGAGGAATTCTAATCTTCGGATT	*Rhodotorula mucilaginosa* 98.42%
Lbn2	TTCTGTGGATTTCTGTGTCTTATTACAGCGTCATTTTATCTCAATTATAACTATCA ACAACGGATCTCTTGGCTCTCACATCGATGAAGAACGCAGCGAACCGCGATATT TTTTGTGACTTGCAGATGTGAATCATCAATCTTTGAACGCACATTGCGCGGTAT GGCATTCCGTACCGCACGGATGGAGGAGCGTGTTCCCTCTGGGATCGCATTGC TTTCTTGAAATGGATTTTTTAAACTCTCAATTATTACGTCATTTCACCTCCTTCAT CCGAGATTACCCGCTGAACTTAAGCATATCAATAAGGCGGAGGAAGT	*Yarrowia lipolytica* 99.06%

After species identification, the phylogenetic tree was determined using the Maximum Likelihood method and the Tamura-Nei model [26]. Evolutionary analyses were studied in MEGA11 (Figure 1.) [27]. The numbers in the figure show the evolutionary distance of newly isolated *R. mucilaginosa* and *Y. lipolytica* strains between other *R. mucilaginosa* and *Y. lipolytica* strains and the evolutionary distance between two newly isolated species.

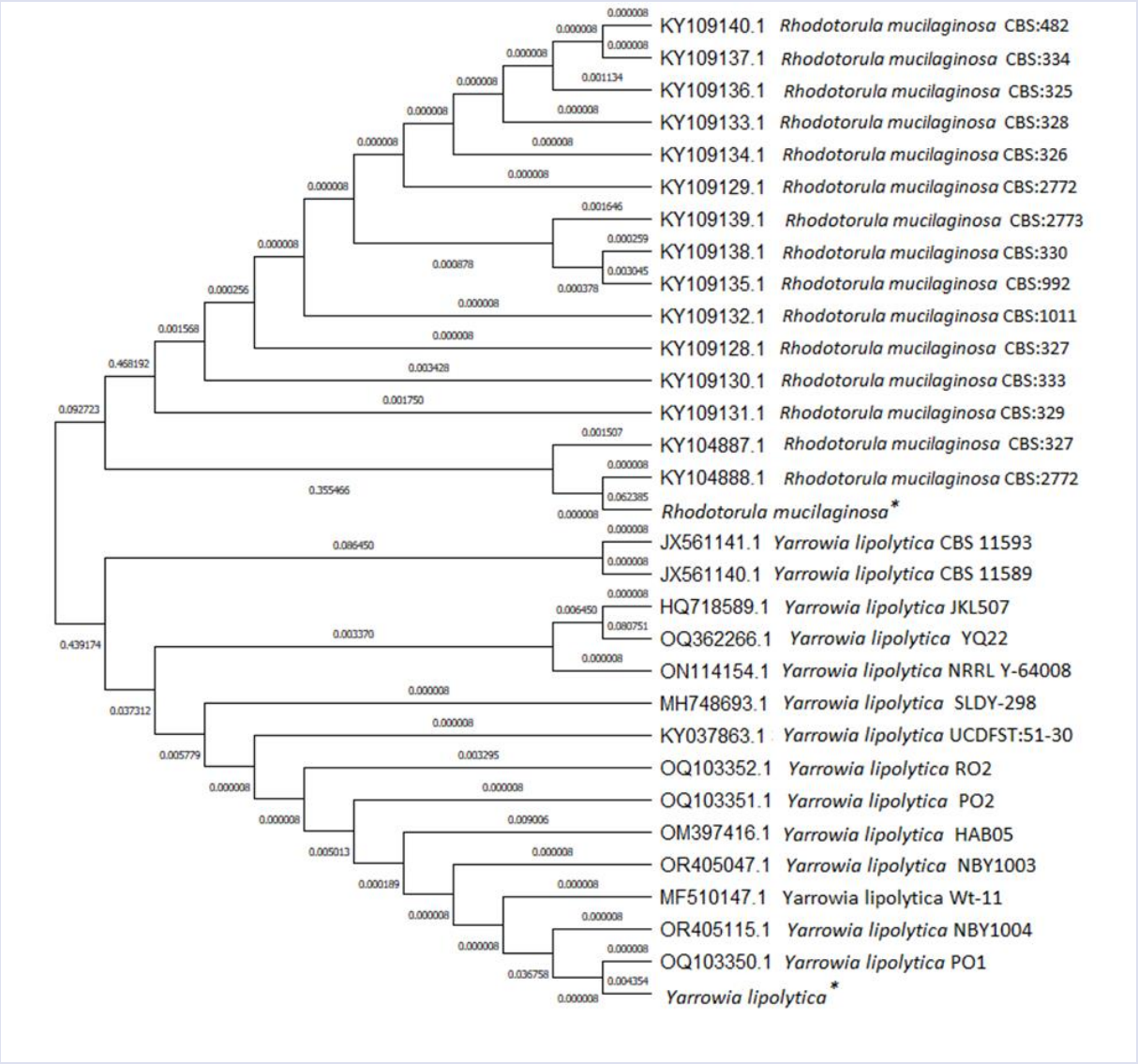

Figure 1. Phylogenetic relationships of *R. mucilaginosa* and *Y. lipolytica* to other closely related *Rhodotorula* sp and *Yarrowia* sp. (NCBI Accession number of *R. mucilaginosa* is PQ661383 and *Y. lipolytica* is PQ682298).

As a result of genetic analysis of yeast strains isolated and purified from spoiled labneh cheese, primarily named Lbn1 and Lbn2, Lbn1 was identified as *Rhodotorula mucilaginosa* with a similarity rate of 98.42%, and Lbn2 as *Yarrowia lipolytica* with a similarity rate of 99.06%. Labneh cheese is a highly nutritious food product in the spreadable cheese class. However, its nutritiousness for humans also makes this cheese type attractive to microorganisms. Yeasts are the main cause of spoilage in cultured dairy products due to their ability to grow at refrigeration temperatures and low pH values [28, 29]. Debaryomyces, *Yarrowia* and *Rhodotorula* species are frequently isolated from spoiled meat and cheese [10]. In a study, 86 samples were collected and analyzed. As a result of the analysis, 1032 yeast isolates were identified by the sequence analysis method targeting the transcript spacer region (ITS) of ribosomal DNA. It was determined that 9 of the identified yeasts were *R. mucilaginosa* and 7 were Y. lipolytica [11]. In another study, 85 yeasts were isolated from different cheese samples and the results obtained showed *Y. lipolytica* and *R. mucilaginosa* strains [30]. In a study investigating the yeast content in spoiled, decorated soft cheese packaged in modified atmosphere from two different dairies, both *Y. lipolytica* and *Rhodotorula* sp. were identified. *R. mucilaginosa* was detected in the results obtained from swab samples taken from production and packaging areas [31]. Tokak et al. obtained 20 yeast isolates from dairy products such as yogurt, cream, butter, curd cheese and Antep cheese and identified one as *Yarrowia lipolytica* [32]. Budak et al. isolated and identified one *Y. lipolytica* strain from outside the samples on the 60th day and six *Y. lipolytica* strains

from outside the samples on the 120th day during the ripening of traditional Turkish Divle Cave cheese [33]. There are many other studies in the literature on *R. mucilaginosa* and *Y. lipolytica*, which cause spoilage in cheese products. However, what draws attention in the publications is where these yeasts contaminate foods before causing spoilage. Fröhlich-Wyder et al. reported that *Y. lipolytica* is among the yeast species more frequently seen in raw milk [34]. Potential contamination sources can be encountered at all stages of the cheese production process and include raw milk, equipment surfaces, and the processing environment. Both *R. mucilaginosa* and *Y. lipolytica* can be the main spoilage yeast strains not only in cheese but also in other dairy products. Milanović et al. performed molecular identification of 74 spoilage yeasts isolated from 3 different buffalo milk yogurt batches throughout their shelf life and identified *Y. lipolytica* and *Rhodotorula* sp. strains [35]. However, there are also studies that isolated *R. mucilaginosa* or *Y. lipolytica* from other foods. In a study, *R. mucilaginosa* was identified in orange fruit and juice [36]. In another study, *R. mucilaginosa* was among the yeast strains isolated and identified from apples and citrus fruits [37]. In another study on carotenoid production, *R. mucilaginosa* strains were isolated and identified from Jerusalem artichoke, tomato juice, sugar cane and agricultural soil [38]. Studies show that cheese or any other food product can be spoiled by *R. mucilaginosa* and/or *Y. lipolytica*. There are various publications in the literature on the production of labneh cheese [39, 40]. However, no publication was found on the isolation of yeast from spoiled labneh cheese. Therefore, we believe that our study will contribute to the literature.

Conflicts of interest

The authors declare that they have no conflicts of interest in the publication.

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