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Isolation of Traditional Yogurt Yeast *Kluyveromyces marxianus* and Investigation of Major Probiotic Properties

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ABSTRACT

The aim of this study was to evaluate the probiotic potential of *Kluyveromyces marxianus* K3 yeast strain isolated from cow yogurt. *Kluyveromyces marxianus* yeast strain was isolated from yogurt and identified based on ITS sequences. The isolated strain showed high tolerance to low pH conditions. This feature can be associated with the ability of the strain to survive in acidic environments such as stomach acid. In addition, the strain showed tolerance to simulated human gastric and intestinal fluids, indicating that it can move healthily in the digestive system. It was determined that the isolate was resistant to antibiotics, indicating its potential to help protect the intestinal flora. It was determined that the strain grew well at human body temperature, exhibited hydrophobic properties and had deconjugation ability against bile salts. The research findings indicate that *Kluyveromyces marxianus* strain obtained from yogurt has the potential to be used as a probiotic in different food products. This species is an important candidate that can be included in food supplements or new probiotic formulations. In conclusion, this study reveals that yogurt is a rich source of probiotic yeast species and *Kluyveromyces marxianus* K3 strain may play an important role in this field.

Keywords: Immunity, Isolation, Yeast, Probiotic, Yoghurt.

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Introduction

The term probiotic is derived from the Greek word “pro bios” meaning “for life” [1]. Probiotics are defined as live microorganisms that are generally found in the intestines and benefit the host organism. These microorganisms can aid nutrient absorption and support the immune system by balancing the digestive system [2]. Additionally, they can have positive effects on health by preventing the establishment of pathogenic microorganisms. In recent years, beneficial fungal communities among probiotics have also attracted attention and new products are being developed in this field [3].

Probiotic mushrooms have attracted the attention of researchers and industries. Yeasts are widely found in various ecological niches, plants, water, airborne particles, and traditional fermented and unfermented food products [4, 5]. These creatures are also important as normal components of the gastrointestinal flora. Yeasts play important roles in food processing and fermentation technologies. In these processes, they contribute to increasing and preserving the flavor and nutritional value of foods. Since ancient times, various fungal communities have provided various benefits to human societies. It has had impacts on food processing, medical practices, and even ecological balance. Nowadays, probiotic mushrooms are the subject of intensive research and development studies, especially due to their health benefits [6, 7].

The unique cell architecture of fungi may make them a better class of probiotics than commercially available probiotic bacteria. In particular, the cell membrane of yeasts consists of two layers: an outer layer made of mannan (phosphopetidomannan or phospholipomannan) and an inner layer containing chitin and 1,3- and 1,6-β-glucan [8, 9]. This structure ensures safe passage through the gastrointestinal environment, which is an important factor in ensuring the probiotic effect. Additionally, many fungal species can grow optimally at different temperatures, indicating their ability to adapt to various environmental conditions. The antagonistic properties of fungi are also notable; Thanks to these properties, they can prevent the growth of pathogenic bacteria in the intestine. Therefore, the use of mushrooms as probiotics has the potential to provide positive effects on digestive health and the overall immune system.

Members of a group of fungal genera are novel probiotics: *Candida humilis*, *Debaryomyces hansenii*, *Debaryomyces occidentalis*, *Kluyveromyces lactis*, *Kluyveromyces lodderae*, *Kluyveromyces marxiamus*, *Saccharomyces cerevisiae var. boulardii*, *Pichia kluyveri*, *Issatchenkia orientalis*, *Pichia kudriavzevii*, *Candida tropicalis*, *Meyerozyma caribbica*, *Candida saitoana*, *Candida pintolopesii*, *Cryptococcus albidus* and *Torulaspora delbrueckii* [10, 11]. The best studied yeast is *Saccharomyces boulardii*.

Current reports suggest that the use of yeast strains alone or in combination with other probiotics has digestive health-promoting effects. These effects can occur by increasing good bacterial populations and at the

same time reducing pathogenic microorganisms through competition for space and food [12, 13]. This may play an important role in maintaining and improving gastrointestinal function. Various findings indicate that fungal-based probiotics affect the host organism in various aspects. These effects may include positive effects such as supporting digestive system health, strengthening the immune system and maintaining metabolic balance. In addition, the potential benefits of yeast strains can be better understood with more research and their use in probiotic therapeutic applications may increase.

The antimicrobial activity of probiotic yeasts plays an important protective role in the gastrointestinal tract. This activity ensures the integrity of the intestinal barrier by helping to maintain tight junctions between epithelial cells, particularly through E-cadherin recycling. In this way, it can contribute to reducing the population of pathogenic bacteria and potentially preventing intestinal infections [14, 15]. In addition, probiotic yeasts can also affect the intestinal microflora balance thanks to their metabolic activities. In particular, by increasing the synthesis of metabolites such as short-chain fatty acids, they can promote the production of compounds such as butyrate, which are important for intestinal health [16, 17]. These fatty acids are used as an energy source for intestinal cells and also have inflammation-reducing effects. These mechanisms of probiotic yeasts offer a potentially effective strategy to maintain and support digestive health. Therefore, the use of these yeast strains is increasingly being investigated in the treatment and prevention of gastrointestinal diseases.

The aim of the study is to identify the yeasts found in cow yoghurt, one of the traditional fermentation products, at the species level, to determine their biotechnological properties and to determine the probiotic properties of these organisms so that they can be used in industrial-scale fermentation processes.

Materials and Methods

Isolation of Yeast Isolates

Yeast was isolated from a traditional yoghurt sample (Budaklı, Sivas, Türkiye). Serial dilutions were made from homemade yoghurt and incubated on YEPD agar (20 g/L peptone, 20g/L glucose, 10g/L yeast extract, pH 6.3) at 25 °C for 3-5 days. Colonies exhibiting different morphologies were inoculated onto YEPD agar plates. Colonies were purified by the smear method on clean agar plates and gram staining was used for morphological identification under a light microscope. Purified isolates were then stored in 20% glycerol at − 80°C [18].

Molecular Identification of the Kluyveromyces marxianus

Genomic DNA was extracted [(EurX GeneMATRIX Plant & Fungi DNA isolation kit (Poland)] (https://eurx.com.pl/docs/manuals/en/e3595.pdf). The DNA was quantified (Thermo Scientific Nanodrop 2000, USA). Internally transcribed spacer (ITS) DNA was copied using ITS1 and ITS4 oligomers (5' TCCGTAGGTGAACCTGCGG 3' and 5' TCCTCCGCTTATTGATATGC 3', respectively, and 0.3µM each). Amplification of DNA (35µL, final volume) was started with a denaturation step (5 min, 95°C), and continued with 40 recycles of extension (denaturation, 45s, 95°C; annealing, 45s, 57°C; extension, 60s, 72°C; and an extension step, 5min, 72°C) (Kyratec, Australia), using DNA Polymerase, 2u, (Solis Biodyne FIREPol Estonia) in the presence of 1.5mM $MgCl_2$, and 0.2µM dNTPs. Amplification product was resolved (1.5% agarose gel). The band of interest was cut out and eluted (HighPrep™ PCR Clean-up System, MAGBIO, AC-60005, MD, USA). Nucleotide data generated by Sanger method (ABI 3730XL, Applied Biosystems, Foster City, CA, USA), showed 99.73% with *Kluyveromyces marxianus* (BLAST, ncbi.nlm.nih.gov). The nucleotide sequence information was deposited in GenBank (Accession No.: PP998224) and a dendrogram was produced for it (Figure 1).

Potential Probiotic Experiments

Growth at human body temperature

Yeast isolates were placed in 10 ml tubes in K3 YEPD broth pH 6.3 at 25°C, inoculated from stock strains and incubated for 24 hours. 100 µl samples were taken from activated liquid cultures, streaked onto solid media and incubated for 10 days [19].

Tolerance to low pH conditions

The active culture was inoculated into YEPD broth medium (pH 2.0, 3.0, 4.0 and 6.3) and incubated at 37°C for 3 hours. After incubation, 100 µl sample was taken from the active culture and planted on YEPD agar medium using the pour-plate method. It was incubated for 24 hours [20].

Tolerance to simulated human gastric juice

10 ml of active culture, incubated for 24 hours, was centrifuged at 2500 g for 10 minutes. Supernatant was removed. Cells were suspended in 1 ml of 0.85% sterile saline solution. 1 ml pepsin solution (pH 3) was added and incubated at 37°C for 3 hours. 50 µl of the prepared cell pepsin solution mixture was streaked onto YEPD agar medium. Cell mixture without pepsin was used as a control. It was incubated for 24 hours [19]. Tolerance was evaluated by the cast plate method.

Tolerance to simulated human ıntestinal juice

Trypsin (1 g/L) solution was prepared in PBS (pH 8). 9 ml of the prepared trypsin solution was taken into tubes and 1 ml of active culture was added. Line seeding was done on 100 µl YEPD agar plates. It was incubated at 37°C for 24 hours. As a control, pure cells were seeded in their own medium. It was checked for 24 hours [21].

Hydrophobicity

10 ml of activated yeast cultures were centrifuged at 5000 g for 5 minutes for 24 hours. The cell pellet was washed with Ringer's solution (6% NaCl, 0.0075% KCl, 0.01% $CaCl_2$, 0.01% Na_2CO_3). It was resuspended by adding 10 ml of Ringer's solution. 4 ml of sample was separated and optical density was measured using a 600 nm

spectrophotometer. The remaining 6 ml sample was divided into three equal volumes. The same volume (2 ml) of n-hexadecane, n-hexane, xlenes was added and mixed. Phase separation was allowed to occur by vortexing for 2 minutes. At the end of 30 minutes, the upper phase was removed and the aqueous phase optical density of the solution was measured using a spectrophotometer at 600 nm. Percent hydrophobicity was calculated as the decrease in OD_{600nm} using the following formula:

$$\% \text{ hidrofobisite} = \frac{OD0-OD}{OD} X\ 100$$

Here, OD_0 and OD are OD_{600nm} before and after extraction with n-hexadecane, n-hexane, xylenes, respectively [21].

Auto-aggregation

The culture was activated for 24 hours and mixed by vortex for 15 seconds. The optical density of the cells was measured using a spectrophotometer at 660 nm. It was incubated at 37°C for 4 hours. 10 ml of active culture was centrifuged at 5000 g for 15 minutes. Cells were suspended in 1 ml of PBS (pH 7.4). 400 µl of the sample was taken and inoculated into 20 ml of medium. It was incubated at 37°C for 24 hours. The optical density of the cells was measured every hour using a spectrophotometer at 660 nm.

$$\% \text{ Otoagregasyon} = [\ 1 - (\frac{ODt}{D0})\ ]\ X\ 100$$

Here OD is OD_{600} nm before incubation and OD_t is OD_{600nm} after incubation [19].

Antibiotic resistance screening

A smear of active culture (100 µl) was inoculated onto YEPD agar plates. Antibiotic discs were placed on the plates using sterile forceps. It was incubated at 37°C for 48 hours. Inhibition zones were determined [22].

Bile salt deconjugation test

The ability of the strains to deconjugate bile salts was examined [23]. YEPD agar medium was prepared with 0.5% sodium salts (sodiumglycocholate hydrate, sodium taurodeoxycholate, sodium taurocholic acid). The cells were dipped onto agar plates and incubated for 24 hours at 37°C [21]. Tolerance is indicated by the presence of precipitated bile acids (white opaque halo) around the colonies.

Results and Discussion

Isolation and Identification of Yeast Isolates

Morphological characterization of the yeast strain isolated from cow yoghurt was performed. The obtained sequences were scanned in the GenBank database and a new analysis data was created, including the closest species at the subfamily and genus level. Using this data, phylogenetic trees were constructed using maximum likelihood (ML) and neighbor joining (NJ) methods. On NJ trees, red numbers indicate branch lengths and black numbers indicate bootstrap support values. The resulting DNA sequence reads were used to create a phylogenetic tree. Sequences containing the ITS1 and ITS2 rRNA gene regions, created using forward and reverse raw chromatogram data, are used as DNA barcoding tools for yeasts. The ITS region provides sufficient data for the identification of the fungal sample. Sample K3 was identified and identified by DNA sequence as *Kluyveromyces marxianus* (Figure 1).

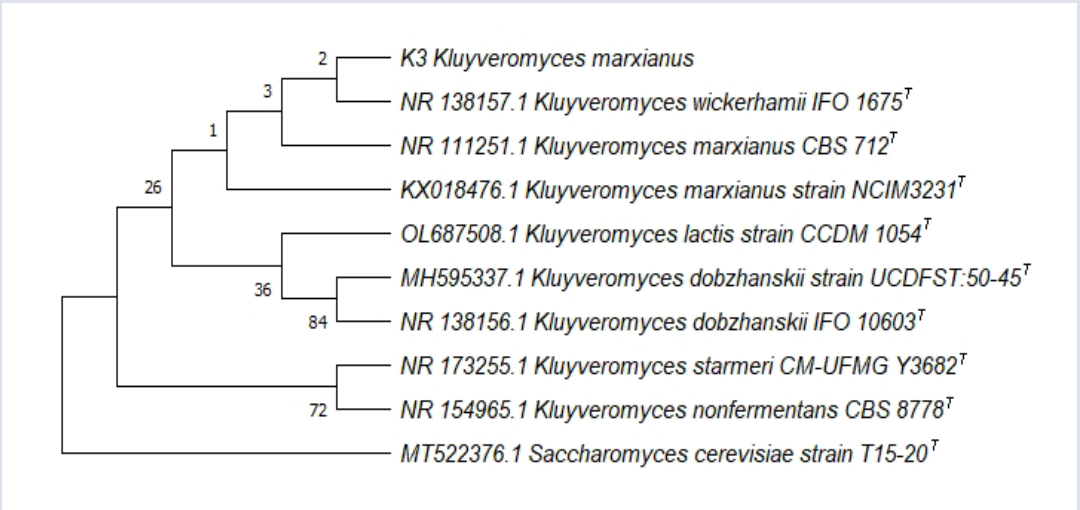

Figure 1. Dendrogram of *Kluyveromyces marxianus* including K3 isolate

Growth at Human Body Temperature

Isolate K3 was successfully grown on YEPD agar (Table 1). A single colony was obtained for each isolated strain. The yeast isolate grew successfully at 37°C. Its growth at human body temperature is important for yeasts to be evaluated as a potential probiotic. Strains were identified as yeast using macroscopic analysis based on morphological features. The *Kluyveromyces marxianus* isolate was observed to be smooth and white. It has been reported in the literature as smooth in strains isolated from kefir [18].

Table 1. Morphological and numerical characterization of yeast isolate K3

Yeast isolate	K3
Colony morphologies	Smooth-white
Cell morphologies	Global budding
Number of cells (Log 10 cfu/ml)	5.55

Low PH Tolerance and Bile Salt Resistance

Tolerance to low pH is one of the main selection criteria [24,25]. Because in order for probiotics to reach the small intestine, the stomach must have stressful conditions [26]. It was determined that *Kluyveromyces marxianus* K3 isolate could tolerate low pH values.

Microorganisms considered probiotics must have the ability to survive and grow under stressful conditions. In particular, the ability for bile salt hydrolysis is one of the desirable properties of a probiotic strain. This feature plays an important role in removing cholesterol from the human body [27]. Certain species of the native microflora of the human intestine develop deconjugated bile salts (amino acid residues of bile salts conjugated with glycine or taurine). This process depends on the presence of an enzyme called bile salt hydrolase (BSH). BSH activity occurs by hydrolyzing amino acid residues of bile salts and bile acid.

To detect the presence of BSH activity, YEPD agar plates containing 0.5% sodiumglycocholate hydrate, sodium taurodeoxycholate, and sodium taurocholic acid were used. 10-µl aliquots from overnight incubated cultures were spotted onto the plates. Strains that formed precipitation zones or opaque, granular white colonies were considered BSH positive. This method is a widely used technique to evaluate the bile salt hydrolysis ability of potential probiotic strains. BSH positive strains may have the potential to have positive effects on human health by hydrolyzing bile salts (Table 2).

Table 2. Degradation capacity for sodium salts

Sodium salts	Isolate K3
Sodium glycocholate hydrate	+
Sodium taurodeoxycholate	+
Sodium taurocholic acid	+

Tolerance to Simulated Human Gastric Juice

The isolated K3 isolate was exposed to pepsin for 3 hours and survival capacities were obtained by counting live cells (Table 3). In this study, it is stated that strain K3 was incubated in pepsin-PBS medium (pH 3.0) for 3 hours and the results showed that this medium did not completely inhibit the growth of isolate K3. Pepsin is a digestive enzyme active in low pH environments and is one of the main components of gastric juice. Therefore, the use of pepsin-PBS solution is intended to simulate the effect of gastric juice. However, according to the results of the study, the isolated K3 strain was able to maintain its ability to grow even in this low pH environment [19]. Probiotic microorganisms are generally consumed in the presence of nutrients such as milk protein and can exert protective effects depending on the initiators (prebiotics) to survive in the acidic environment of the digestive system. Thanks to these properties, probiotics can contribute to the healthy function of the digestive system. In conclusion, the ability of strain K3 to grow after 3 hours of incubation in pepsin-PBS solution (pH 3.0) suggests that this strain may potentially have the ability to survive even in challenging environments such as stomach acid. Such studies may help us understand the health benefits of probiotic microorganisms.

Tolerance to Simulated Human Intestinal Environment

High survival rates of yeast isolates were determined under simulated human intestinal environment conditions (Table 3). The K3 isolate exhibited a resistant profile and was determined to maintain its viability for 24 hours. Under these conditions, survival time of more than 24 hours is a very important criterion for probiotic selection.

Table 3. Probiotic properties of yeast isolate K3

Yeast Isolate	First log10 (cfu/ml)	GIT growth (log10 cfu/ml)	Growth at 37°C	Hydrophobicity %	Aggregation %	SIT growth
K3	5.55	8.1	++++++	n-hexadecane 2,39 n-hexane 10,19 xlenes 13,58	39.3	2.25

Hydrophobicity

Yogurt yeast isolates were analyzed for hydrophobicity (Table 3). Hydrophobicity can be defined as a nonspecific interaction between microbial and host cells. This interaction is mediated by cell surface proteins and lipoteichoic acids. In this study, K3 showed significantly higher hydrophobicity and was therefore capable of interacting with other cell bodies compared to other isolates. Hydrophobicity is crucial and species-specific for the adhesion of probiotic microorganisms to epithelial cells, where they can provide prophylactic and therapeutic benefits [19].

The cell surface hydrophobicity of the isolated strains was examined using two different hydrocarbons, n-hexadecane, n-hexane and xylene. The highest surface hydrophobicity percentage was determined in n-hexadecane and the lowest in n-hexane.

The study reported that the use of this test is limited due to the harmful effects of hexane on cell walls, triggering lysis [28]. However, since the use of n-hexadecane and xylene did not have a negative effect on bacterial cells, this organic solvent was recommended. This result was associated with cell surface hydrophobicity with xylene in our study.

Autoaggregation

Autoaggregation or automatic aggregation can be defined as cells of the same strain coming together to form clusters [29]. This property can vary depending on factors such as hydrophobicity on the surface of cells and is one of the important properties of probiotics. In particular, this property also determines the capacity to adhere to eukaryotic cells, especially the mucosal epithelium. The autoaggregation capacities of isolated strains are usually measured after 24 hours of incubation at 37 °C and are usually measured at a wavelength of 600 nm. The autoaggregation percentages of yogurt isolates in this study are shown in Table 3, and these results were found to be consistent when compared with previous studies. Automatic assembly of cells may provide protection against environmental stresses. For this reason, it has been stated that the autoaggregation abilities of yeasts are species-specific and different yeast strains may differ in this feature [19]. Such studies help us understand the physiological properties of probiotic yeast strains and evaluate the impact of these properties on health benefits.

Antibiotic Tests

Antibiotic resistance models were generated using the disk diffusion method [30]. An important feature of probiotic bacteria is that they do not carry any infectious antibiotic resistance genes. The results obtained from this study (Table 4) show that only K3 from three different isolates is sensitive to 7 different antibiotics. Vancomycin 30 µg/ml, Neomycin 30 µg/ml, Rifampin 5 µg/ml, Erythromycin 15 µg/ml, Penicillin 2 µg/ml, Gentamicin 30 µg/ml, Chlorophenicol 30 µg/ml and the relevant inhibition zone obtained is Table 3. is also shown. The appearance of the medium is also given in Figure 2. Antimicrobial activity can also be considered one of the most important selection criteria for probiotics. The fact that only one of the isolates was sensitive may be due to the fact that the species are from different sources and the antibiotic tests are species-specific [22].

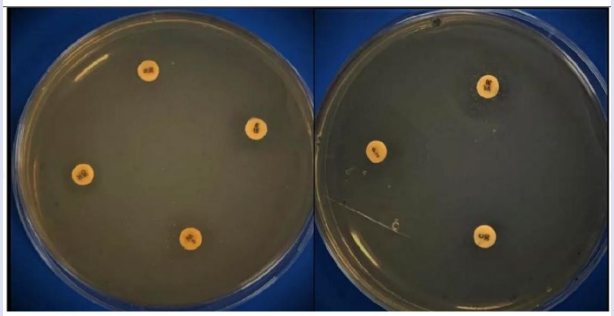

Figure 2. Antibiotic inhibition zones of isolate K3

Table 4. Antibiogram results of isolate K3

Antibiotic	Shortening	Concentration (µg/ml)	Zones of Inhibition (cm)
Vancomycin	VA	30 µg/ml	1.9
Neomycin	N	30 µg/ml	0.8
Rifampicin	RA	5 µg/ml	1.1
Erythromycin	E	15 µg/ml	1.2
Penicillin	P	2 µg/ml	2.7
Gentamicin	CN	30 µg/ml	1.0
Chlorophenicol	C	30 µg/ml	---

Conclusion

This study shows that *Kluyveromyces marxianus* K3 strain can be considered as a potential probiotic. This isolated strain has the ability to survive in acidic stomach and intestinal pH conditions and has a good growth performance at human body temperature. It was also found to be sensitive to antibiotics, suggesting that these strains may be potential candidates for new probiotic formulations or food supplements. However, further research is required to clearly determine whether these strains are truly effective probiotics. In particular, further studies on their potential to adhere to intestinal mucosa and clinical analyses are important. Such studies will help us understand the potential benefits of strains on human health and optimize the use of probiotic yeast. In conclusion, increasing the knowledge about potential probiotic yeast strains such as *Kluyveromyces marxianus* K3 is of great importance in terms of improving the effectiveness and areas of use of probiotics.

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Conflicts of interest

There are no conflicts of interest in this work.

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