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ORIGINAL ARTICLE

Molecular identification and phylogenetic analysis of yeast strains isolated from dairy products in Isfahan, Iran

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ABSTRACT

Background and Objectives: Yeasts play a dual role in dairy processing, serving as beneficial fermentative agents that enhance product quality through flavor, texture, and probiotic properties, while also posing spoilage risks if uncontrolled. This study aimed to characterize yeast isolates from industrial and traditional dairy products in Isfahan using PCR-sequencing and phylogenetic analysis.
Materials and Methods: A total of 155 dairy samples (fresh/stored, traditional/industrial) were collected. Yeasts were cultured and identified via PCR amplification and sequencing of the ITS1-5.8S rDNA-ITS2 region.
Results: Analysis of ITS sequence data identified 28 yeast strains representing eleven species across seven genera, including *Saccharomyces cerevisiae* (n=8) *Kluyveromyces marxianus* (n=6), *Pichia kudriavzevii* (n=4), *Candida orthopsilosis* (n=2), *Pichia membranifaciens* (n=2), *Pichia cactophila* (n=1), *Pichia fermentans* (n=1), *Galactomyces candidum* (n=1), *Torulaspora delbrueckii* (n=1), *Debaryomyces hansenii* (n=1), and *Kluyveromyces lactis* (n=1). Phylogenetic analysis grouped isolates into two clusters. Industrial cheese and both industrial/traditional yogurts showed the highest yeast diversity and counts. Notably, *C. orthopsilosis* was found only in industrial milk and cheese, suggesting potential processing-related contamination.
Conclusion: This study highlights the diversity of yeast microbiota in dairy products and underscores the efficacy of ITS sequencing for accurate yeast identification in the dairy industry, aiding quality control and spoilage prevention.

Keywords: Yeast; Dairy products; Polymerase chain reaction; Sequence analysis; Iran

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INTRODUCTION

Yeasts have traditionally been utilized in food fermentation processes such as beer brewing and bread-making. In the dairy industry, specific yeast strains are also recognized for their probiotic properties (1). Their frequent occurrence in dairy products reflects their ability to thrive in substrates rich in proteins, lipids, carbohydrates, and organic acids (2). Some yeast species contribute positively to flavor development in cheese and are intentionally used as starter cultures (1). However, the rapid growth of certain strains can lead to significant spoilage of dairy products. Spoilage yeasts are commonly associated with undesirable outcomes, including gas formation, off-flavors, discoloration, and textural defects (3).

Yeasts belonging to the genera *Cryptococcus, Rhodotorula*, *Debaryomyces*, *Kluyveromyces*, *Trichosporon*, and *Yarrowia* are commonly found in dairy products (4). These yeasts are capable of causing spoilage at various stages of production, from the farm to the final product, and often persist as natural contaminants within dairy environments, frequently colonizing raw milk and processing equipment. Effective control of contamination in dairy products requires a well-structured strategy that includes the implementation of enhanced hygiene protocols, identification of critical control points along the production line, and thorough tracing of contamination sources (2). Applying these measures is essential for minimizing or eliminating spoilage-causing microorganisms (5).

In recent years, yeasts have garnered increasing global attention due to their dual role as both beneficial and spoilage microorganisms in dairy products (4, 6). Despite this, limited data are available on the yeast microbiota in dairy products from Isfahan, Iran. Therefore, the present study aimed to investigate the diversity and composition of yeasts in dairy products available in Isfahan markets. Accurate identification of yeast isolates is essential for comprehensive analysis of the yeast microbiota (7). Traditionally, yeast identification has relied on phenotypic methods, including the assessment of morphological, physiological, and biochemical characteristics. However, these approaches are often labor-intensive, exhibit low discriminatory power, and frequently result in misidentification (8). Recent advances in molecular biology have revolutionized yeast taxonomy by enabling comprehensive genomic characterization (7, 9). The development of sequencing techniques targeting conserved genetic markers-particularly 18S/26S rRNA genes and internal transcribed spacer (ITS) regions-has significantly improved the accuracy of yeast identification and classification (7, 10). Among these, ribosomal DNA (rDNA) sequencing has proven especially effective and is now widely used for precise identification of yeast isolates from dairy products (11). In this study, a polyphasic approach was employed to characterize yeast isolates obtained from various dairy products-including industrial and traditional milk, dough, cheese, and yogurt-commercially available in Isfahan. The primary objective was to evaluate yeast biodiversity through PCR-based sequencing of the complete rDNA-ITS region. Additionally, phylogenetic analyses were performed to differentiate yeast strains based on their origin and to explore potential correlations between yeast sources and dairy product types.

MATERIALS AND METHODS

Sampling and yeast isolation. Yeasts were isolated from a variety of industrial and traditional dairy products collected from markets in Isfahan, Iran. A total of 155 samples were analyzed, including milk (n=30; 1kg), industrial dough (n=25; 1kg), traditional dough (n=25; 260g), industrial cheese (n=25; 200g), traditional cheese (n=24; 200g), industrial yogurt (n=13; 1kg), and traditional yogurt (n=13; 1kg). Sampling was conducted randomly by the Institute of Standards and Industrial Research of Iran (ISIRI) by reference numbers 164, 326, and 8923-5 (12, 13). Spoiled products were excluded based on comprehensive quality assessment criteria, including the presence of off-odors (e.g., sour, rancid smell), abnormal textures (e.g., sliminess, curdling), visible discoloration or mold growth, deviations in taste (e.g., bitterness, excessive sourness), swollen packaging indicative of microbial gas production, unacceptable pH levels (milk<4.5, yogurt>4.6), expired use-by dates, and storage temperatures exceeding 4°C (40°F). For microbiological analysis, 10 g of each sample were homogenized in a 2% sodium citrate solution and plated onto yeast extract dextrose chloramphenicol agar (YEDCA) (LAB M, UK). Plates were incubated at 25°C for 3-5 days (14). The isolated yeast colonies were subsequently stored at 4°C for further molecular identification.

DNA extraction. Genomic DNA was extracted using Whatman® FTA Elute MicroCards (Whatman Inc., Clifton, NJ, USA), following the manufacturer's protocol with minor modifications in the volumes of distilled water and cell suspension (15). Briefly, a fresh single yeast colony was suspended in 90-100 µL of distilled water, and 6 µL of the suspension was applied to a 4-mm disc punched from an FTA card. The disc was incubated at 25°C for a minimum of 5 hours to ensure proper drying. After incubation, the disc was eluted by immersing it in 400 µL of sterile water for 10 seconds. It was then transferred to a new microcentrifuge tube containing 40 µL of distilled water and incubated at 95°C for 15 minutes. Following incubation, the disc was removed, and the resulting DNA-containing eluate was stored at -20°C for subsequent PCR analysis (15).

PCR and sequencing. The internal transcribed spacer region (ITS1–5.8S rDNA–ITS2) was amplified using a PCR mixture containing 5 µl of 10× reaction buffer, 0.4 mM of dNTP, 1.5 mM of MgCl2, 2.5 U of Taq polymerase, 30 pmol of each primer-ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and 2 µl of extracted DNA in a final reaction volume of 50 µL. PCR amplification was carried out under the following conditions: initial denaturation at 94°C for 5 minutes; followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 1 minute, with a final extension step at 72°C for 7 minutes. A negative control containing distilled water (DW) was included in each PCR run. The amplified products were resolved by electrophoresis on a 1.5% agarose gel and visualized under UV light using a gel documentation system (16). Sequencing of the PCR products was performed using the ITS forward primer by Bioneer (Daejeon, Korea). Raw sequencing chromatograms were analyzed using Chromas 2.3 software (http://chromas.software.informer.com/2.4/), and nucleotide sequences were identified by comparison against the NCBI GenBank database using the Basic Local Alignment Search Tool (BLAST). Fig. 1 illustrates the methodology employed for identifying yeast strains isolated from dairy products.

Phylogenetic analyses. The phylogenetic relationships of the sequenced yeast strains were analyzed using MEGA 7.0 (Molecular Evolutionary Genetics Analysis) software (17). The ITS1-5.8S-ITS2 sequences were aligned using ClustalW with default settings. A maximum likelihood (ML) phylogenetic tree was inferred under the General Time Reversible (GTR) substitution model, which was identified as the best-fit evolutionary model through likelihood-based statistical testing. Branch support was evaluated using 1000 bootstrap replicates, and only bootstrap values ≥50% are reported to ensure robust phylogenetic interpretation.

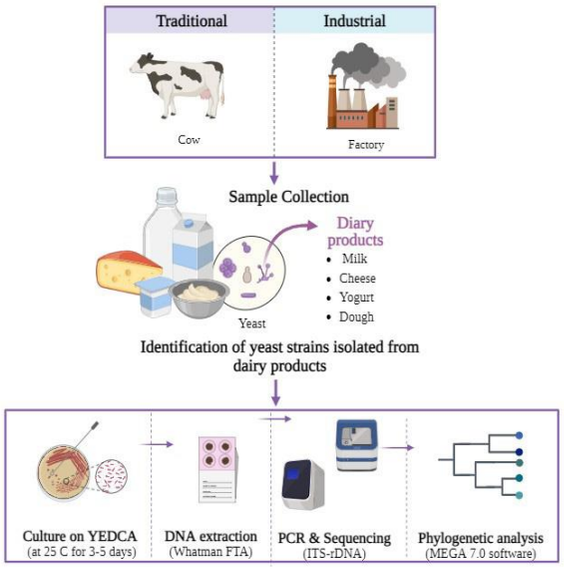

Fig. 1. Schematic overview of the identification process for yeast strains isolated from dairy products.

Statistical analyses. Statistical analyses were performed using SPSS software (version 22; SPSS Inc., Chicago, IL, USA), and Chi-square (χ^2) tests and analysis of variance (ANOVA) were applied to evaluate the data.

RESULTS

Morphological characterization of yeasts isolated from dairy products. A total of 155 dairy product samples were analyzed, including milk (n=30; 19.3%), industrial and traditional dough (each n=25; 16.1%), industrial cheese (n=25; 16.1%), traditional cheese (n=24; 15.4%), and industrial and traditional yogurt (each n=13, 8.3%). Morphological characterization revealed seven distinct yeast genera: *Saccharomyces* (n=8) and *Pichia* (n=8) were the most fre-

quently isolated, followed by *Kluyveromyces* (n=7), Candida (n=2), *Debaryomyces* (n=1), *Torulaspora* (n=1), and *Galactomyces* (n=1).

Molecular identification and phylogenetic analysis. Out of 155 dairy product samples, a total of 28 yeast strains (18.06%) were isolated. The highest recovery rate was observed in cheese (9 strains; 32.1%), followed by yogurt (8 strains; 28.5%), dough (6 strains; 21.4%), and milk (5 strains; 17.8%). Molecular identification revealed eleven yeast species: *S. cerevisiae* (28.5%, n=8), *K. marxianus* (21.4%, n=6), *P. kudriavzevii* (14.2%, n=4), *C. orthopsilosis* (7.1%, n=2), *P. membranifaciens* (7.1%, n=2), *P. cactophila* (3.5%, n=1), *P. fermentans* (3.5%, n=1), *G. candidum* (3.5%, n=1), *T. delbrueckii* (3.5%, n=1), *D. hansenii* (3.5%, n=1), and *K. lactis* (3.5%, n=1). The most frequently identified species were *S. cerevisiae*, *K. marxianus*, and *P. kudriavzevii*, with cheese and yogurt samples being the primary sources (Table 1). The obtained sequences have been deposited in GenBank under the following accession numbers: OP763777.1, OP763778.1, OP765325.1-OP765328.1, OP764022.1, OP764023.1, OP764055.1-OP764062.1, OP778185.1, OP764003.1-OP764008.1, OP763797.1, OP763998.1, OP764031.1, OP763995.1, OP764017.1, and OP764009.1.

Phylogenetic analysis results. Phylogenetic reconstruction of the yeast isolates was performed in MEGA 7 using maximum likelihood analysis of the aligned ITS1-5.8S rDNA-ITS2 sequences (aligned with ClustalW). The resulting tree, supported by 1000 bootstrap replicates, revealed non-monophyletic clustering, forming two distinct groups. Cluster 1 contained *C. orthopsilosis* (OP763777.1, OP763778.1; closely related to strains from three different countries), *D. hansenii* (most similar to US isolate), *K. marxianus* (grouping with Chinese MN853731.1 strain), and *K. lactis* (OP763998; showing highest similarity to MT993347.1). Cluster 2 comprised several *Pichia* species, including *P. cactophila* (OP764009; clustered with reference strains), phylogenetically distinct *P. kudriavzevii* (OP765327), divergent Iranian *P. membranifaciens* (OP764022.1-OP764023.1; separated from North American strains), and *P. fermentans* (OP764017; showing limited similarity to isolates from cheese in France and orange juice in Greece). The phylogenetic relationships between strains isolated from dairy products and reference strains-analyzed using MEGA7-are critical for accurate microbial taxonomy, tracking evolutionary adaptation, and evaluating functional traits. Researchers construct phylogenetic trees using methods such as Neighbor-Joining or Maximum Likelihood to assess genetic relatedness, trace potential contamination sources, and classify novel isolates based on known reference sequences. This analytical approach uncovers evolutionary dynamics, such as horizontal gene transfers and niche-specific adaptations, thereby enhancing our understanding of microbial behavior in dairy ecosystems. Fig. 2 shows the phylogenetic tree obtained from the ClustalW alignment using MEGA7 software for ten of the novel sequences (as indicated

Table 1. Distribution and characteristics of yeast isolates from industrial and traditional dairy products in Isfahan, Iran (n=155 samples).

No.	Dairy Products	Industrial/ Traditional	Yeast isolates
1	Milk	Industrial	*C. orthopsilosis*
2	Milk	Traditional	*S. cerevisiae*
3	Milk	Industrial	*S. cerevisiae*
4	Milk	Industrial	*S. cerevisiae*
5	Milk	Traditional	*K. marxianus*
6	Yogurt	Industrial	*P. kudriavzevii*
7	Yogurt	Traditional	*G. candidum*
8	Yogurt	Industrial	*S. cerevisiae*
9	Yogurt	Traditional	*K. marxianus*
10	Yogurt	Traditional	*P. fermentans*
11	Yogurt	Industrial	*P. cactophila*
12	Yogurt	Industrial	*K. marxianus*
13	Yogurt	Traditional	*K. marxianus*
14	Cheese	Industrial	*K. lactis*
15	Cheese	Industrial	*C. orthopsilosis*
16	Cheese	Industrial	*P. membranifaciens*
17	Cheese	Industrial	*P. kudriavzevii*
18	Cheese	Traditional	*D. hansenii*
19	Cheese	Traditional	*P. membranifaciens*
20	Cheese	Industrial	*T. delbrueckii*
21	Cheese	Industrial	*S. cerevisiae*
22	Cheese	Industrial	*P. kudriavzevii*
23	Dough	Industrial	*S. cerevisiae*
24	Dough	Traditional	*S. cerevisiae*
25	Dough	Industrial	*K. marxianus*
26	Dough	Traditional	*K. marxianus*
27	Dough	Traditional	*S. cerevisiae*
28	Dough	Industrial	*P. kudriavzevii*

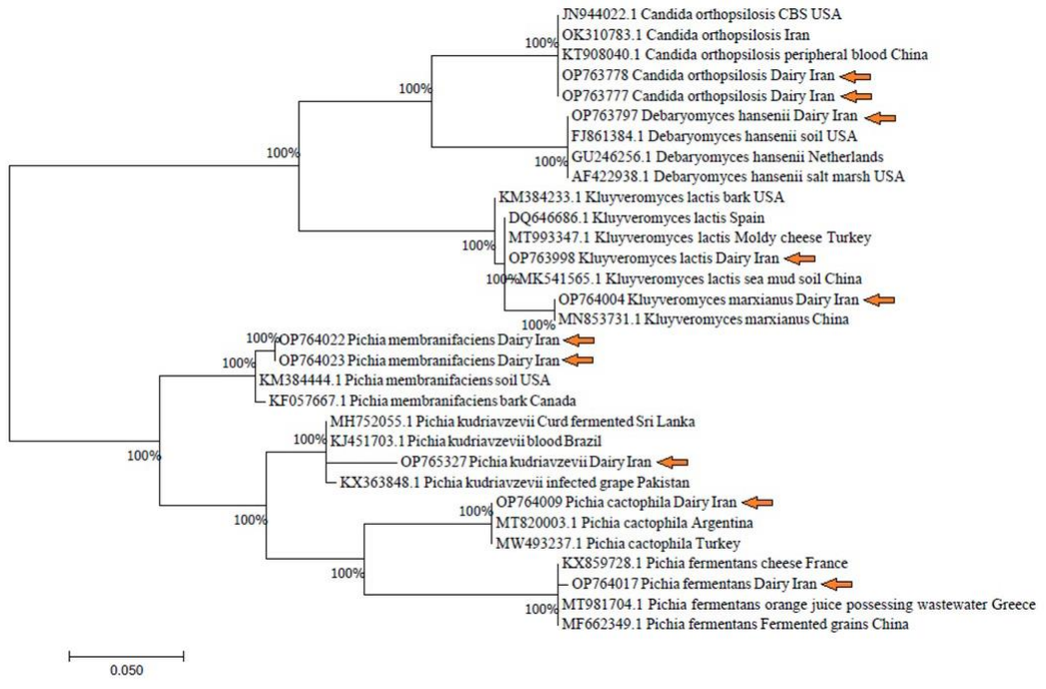

Fig. 2. Maximum likelihood phylogenetic tree constructed in MEGA 7 based on ClustalW-aligned ITS1-5.8S-ITS2 rDNA sequences, showing evolutionary relationships among yeast strains: *C. orthopsilosis*, *D. hansenii*, *K. marxianus*, *K. lactis*, *P. cactophila*, *P. kudriavzevii*, *P. membranifaciens*, and *P. fermentans* strains. Orange arrows indicate themes identified in this study (GenBank accession numbers OP763777.1, OP763778.1, OP763797.1, OP764004.1, OP763998.1, OP764009.1, OP765327.1, OP764022.1, OP764023.1, and OP764017.1).

by orange arrows) and representative lines from most of the previously described species (as indicated by GenBank accession numbers).

DISCUSSION

Dairy products, whether traditional or industrial, are vulnerable to yeast and fungal contamination through various pathways. Traditional methods are particularly vulnerable due to inadequate milking hygiene, the use of unpasteurized milk, and exposure to environmental fungal spores from air, soil, or improperly sanitized equipment (18). In contrast, contamination in industrial settings often results from biofilm formation in processing equipment, insufficient thermal treatment, and post-processing exposure during storage and packaging-especially in cheeses and fermented dairy products (18). Fungal contaminants such as *Aspergillus* and *Penicillium* are of particular concern due to their ability to produce mycotoxins, including aflatoxin M1, and their role in accelerating spoilage (19). Favorable conditions for fungal proliferation, such as high moisture content and inadequate refrigeration, further compromise product safety and shelf life (20). Such microbial contamination leads to considerable economic losses, with microbial spoilage accounting for approximately 20% of global dairy waste, while mycotoxin-related regulatory compliance imposes additional financial burdens (21).

Yeasts have a dual role in the dairy industry, contributing beneficially to fermentation processes in products like cheese, yogurt, and kefir by enhancing flavor, texture, and probiotic potential (22). However, their uncontrolled proliferation can result in spoilage, off-flavors, and undesirable gas production, negatively impacting product quality and shelf life (23). In the present study, *S. cerevisiae* and *K. marxianus* were the most prevalent yeast species isolated from the analyzed dairy products. *S. cerevisiae* is a well-characterized industrial microorganism known for its exceptional biochemical production capacity and broad pH tolerance. Under specific conditions, it acts as a functional component of the fermentation process rather than a contaminant (6). *Kluyveromy-*

ces, an ascomycetous yeast genus within the family Saccharomycetaceae, belongs alongside *Saccharomyces* to the "*Saccharomyces* complex," a subclade within *Saccharomycetes* (24). *K. marxianus*, the teleomorph of *Candida pseudotropicalis*, is commonly associated with dairy environments (25). In the current study, *K. marxianus* was detected in various dairy products, except cheese. Notably, this species exhibits both industrial significance, such as in bioethanol production, and clinical relevance, having been occasionally isolated from human specimens (24, 26).

Within the Saccharomycetaceae family, the genus *Pichia* encompasses the teleomorphic states of several *Candida* species (27). This genus comprises more than 100 species described, among which *P. anomala* is frequently found in cheese and raw milk. Studies conducted in India by Chakrabarti et al. (2001) and in Brazil by Paula et al. (2006) identified *P. anomala* as an etiological agent of pediatric fungemia (28, 29). Yildiz et al. (2021) applied RAPD-PCR with an M13 primer to profile yeast species in traditional Turkish blue cheese. Their analysis revealed six predominant species: *Yarrowia lipolytica, K. marxianus, P. membranifaciens, Debaryomyces hansenii, P. fermentans,* and *Candida zeylanoides.* Among these, *P. membranifaciens* was one of the most frequently isolated species. The researchers suggested that yeast diversity in mouldy Civil cheese may contribute significantly to the development of characteristic blue cheese flavors. Furthermore, they proposed that selected yeast strains could serve as adjunct cultures to enhance final product quality (30). Our results align with existing literature, confirming *P. membranifaciens* colonization in traditional cheeses.

In the present study, *Torulaspora delbrueckii* was isolated from traditional cheese samples. This yeast species is known for its functional roles in food biotechnology, including its use in the preparation of frozen dough (31) and its involvement in various fermentation processes such as those used in red wine (32) and dairy production (33). Despite these beneficial applications, *T. delbrueckii* is also recognized for its capacity to spoil certain food items, notably soft drinks and dairy products, under specific conditions (33, 34). While *G. candidum* has been previously documented in cheese, milk, and alcoholic beverages (35, 36), the current study represents its first reported isolation from traditional yogurt. This yeast-like fungus significantly contributes to food product flavor and aroma profiles (37), aligning with consumer preferences. Notably, *G. candidum* demonstrates remarkable adaptability to the challenging conditions of cheese ripening, thriving in environments characterized by high salinity, low water activity, and acidic pH (38). Additionally, *D. hansenii* was recovered from traditional cheese in this study. Although generally considered non-pathogenic, this yeast produces mycocins, a class of mycotoxins that exhibit killer activity against competing yeast species. Research by Banjara et al. (2016) highlighted its potential in inhibiting pathogenic species such as *Candida albicans* and *C. tropicalis*, underscoring its relevance in competitive microbial ecosystems (39).

This study identified *C. orthopsilosis* as a contaminant in industrial milk and cheese samples. *C. orthopsilosis* is a pathogenic yeast belonging to the *C. parapsilosis* species complex. In 2005, the *C. parapsilosis* complex was reclassified into three distinct species: Group I (*C. parapsilosis*), Group II (*C. orthopsilosis*), and Group III (*C. metapsilosis*) (40). Although *C. orthopsilosis* and *C. metapsilosis* are rarely isolated, their prevalence is likely underestimated, as commercial identification systems often misidentify them as *C. parapsilosis* (41). In a prior investigation on microbial contamination in dairy products from Isfahan, Iran, the genus *Penicillium* was isolated in 33.5% of cases, while *C. orthopsilosis* was found in only 1.2%, marking the first documented detection of this species in regional dairy products (42). In the present study, *C. orthopsilosis* was again identified, this time as the exclusive fungal contaminant in industrial milk and cheese. Its presence points to possible environmental contamination or lapses in hygiene during production and handling. This constitutes the second recorded occurrence of *C. orthopsilosis* in dairy products from Isfahan, emphasizing the critical need for rigorous sanitation practices throughout the dairy supply chain to prevent fungal contamination and safeguard public health (42).

To mitigate contamination, dairy processors must implement strict hygiene protocols and controlled fermentation conditions (23). Effective mitigation strategies include pasteurization, strict temperature control, modified-atmosphere packaging, and the use of bioprotective cultures (e.g., *Lactobacillus* spp.) (43, 44). Advanced detection methods (e.g., PCR, flow cytometry) enable early microbial monitoring, preventing large-scale spoilage (45).

The phylogenetic assessment conducted in this

study revealed that *C. orthopsilosis* isolates grouped closely with strains known to be clinically relevant, including those obtained from bloodstream infections. These findings suggest a potential public health concern and emphasize the necessity for strengthened surveillance of fungal contamination in dairy products. Additionally, the majority of yeast sequences showed high similarity to species previously documented in dairy products and environmental samples. These findings reinforce the effectiveness of ITS-rDNA sequencing as a dependable method for identifying yeast species associated with dairy. The ITS-rDNA region's sequence variability has been established as a robust molecular marker for fungal classification (17), despite being somewhat labor-intensive to analyze. Recent advances in molecular techniques have significantly enhanced the resolution of fungal identification, offering greater sensitivity, efficiency, and cost-effectiveness compared to traditional culture-based methods (8). As a result, culture-independent approaches are now favored for detecting fungi in food products, including dairy (46). These methods have also revealed the previously underestimated pathogenic potential of certain yeast isolates, alongside their beneficial roles in fermentation. Given these findings, molecular techniques are increasingly emphasized for fungal detection in dairy products. In this study, *C. orthopsilosis* was identified as a contaminant in milk and cheese, highlighting the need for further research on pathogenic fungi in dairy.

This study is subject to certain limitations. Notably, it did not investigate the presence of key pathogenic microorganisms such as *Staphylococcus aureus* and *Salmonella* spp., which are critical indicators of food safety. Additionally, the analysis did not account for the potential presence of chemical preservatives in the dairy products, which could impact both microbial growth and overall product quality. These unexamined variables may have influenced the findings and should be addressed in future research to provide a more comprehensive assessment of dairy safety.

CONCLUSION

This investigation demonstrates the reliability of ITS-rDNA PCR sequencing combined with phylogenetic analysis for the precise identification of yeast species in dairy products collected from Isfahan, Iran. The detection of opportunistic pathogenic fungi, particularly in industrial milk and cheese, suggests possible lapses in processing or environmental sanitation. As dairy products may act as carriers for fungal pathogens, these findings reinforce the critical importance of rigorous quality assurance protocols, enhanced sanitation measures, and routine microbiological screening throughout the production chain. Future studies should expand on these results by evaluating the distribution, virulence, and toxin-producing capabilities of fungal contaminants across larger and more diverse dairy networks.

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