Determination of yeast profile in cheese using Vitek2 and molecular method

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Abstract

In our study, the VITEK2 system and a molecular method (PCR) were used to determine the yeast profile in cheeses produced traditionally from raw milk. The samples were cultured on Rose Bengal Chloramphenicol (RBC) agar, and suspicious colonies were examined under a microscope and identified using the VITEK2 system and the ITS sequencing method. The species *Cutaneotrichosporon curvatum*, *Debaryomyces fabryi*, *D. prosopidis*, and *Tausonia pamirica*, identified by PCR, were not recognized by the VITEK2 system. In addition, the species *Debaryomyces hansenii*, *Kluyveromyces marxianus*, *Pichia kudriavzevii*, *Torulaspora delbrueckii*, and *Yarrowia lipolytica*, identified by the molecular method, were subsequently identified as *Candida famata*, *Candida kefyr*, *Candida krusei*, *Candida colliculosa*, and *Candida lipolytica* using the VITEK2 system. The yeast species *Candida zeylanoides*, *Candida parapsilosis*, *Geotrichum silvicola*, and *Trichosporon inkin* were identified using both methods in the same manner. Upon reviewing the results, some yeasts could not be identified by the VITEK2 system. It was also found that there may be differences in the results of the two methods for yeast identification, VITEK2 and PCR. These differences may be due to telemorphic and anamorphic multiplication in yeasts, and biochemical tests may not be sufficient to distinguish closely related species. Furthermore, identifying foodborne yeasts with the VITEK2 Compact system is limited by the database.

Keywords: ITS sequencing; PCR; VITEK2 system; Yeast

Introduction

Due to their tolerance of low pH and water activity, high salt concentrations, and low storage temperatures, as well as certain chemicals such as sanitizers and cleaning agents, yeasts are a crucial component of the cheese microbiota (Banjara *et al*., 2015; Ozmen Togay *et al*., 2020). Yeasts can easily be transmitted from various sources during cheese production, such as brine, personnel, equipment surfaces, and raw milk. Some yeasts can consume lactose, proteins, fats, and some organic acids. They may also be used to mature cheeses and other dairy products by producing specific taste components and altering their texture (Ferreira and Viljoen, 2003; Awasti and Anand, 2020; Zheng *et al*., 2021). Yeasts can utilize lactic acid, a primary fermentation product produced by starter lactic acid bacteria (LAB). This raises the pH at the surface and within the cheese, supporting the growth and enzyme activity of starter LAB, which are responsible for more effective protein degradation by acid-sensitive

secondary flora such as Brevibacterium linens (Wyder and Puhan, 1999). Additionally, some yeast species produce proteolytic and lipolytic enzymes that contribute to the characteristic flavor and structure of cheese. Yeast enzymes break down cheese proteins, such as casein, into peptides and amino acids, releasing free amino acids that contribute to cheese aroma. These enzymes convert amino acids into volatile aroma compounds, such as sulfur compounds, aldehydes, and alcohols, via reactions including decarboxylation, transamination, and deamination (Suzzi *et al*., 2001). Through their lipase activity, yeasts contribute to the development of aroma by breaking down fats into free fatty acids. These free fatty acids can then be converted into esters and methyl ketones, which contribute to the cheese's fruity notes (Collins *et al*., 2003).

However, some yeast strains can spoil dairy products by producing gas, imparting flavors, and altering texture and color (Abdel-Aziz *et al*., 2016; Garnier *et al*., 2017). *Debaryomyces, Yarrowia, Candida, Zygosaccharomyces, Cryptococcus, Geotrichum, Kluyveromyces, Trichosporon, Rhodotorula, Torulaspora,* and *Saccharomyces* are the yeast genera that are most frequently isolated from dairy products. Dairy samples are typically used to exhibit *Kluyveromyces marxianus*, *Debaryomyces hansenii*, and *Saccharomyces cerevisiae* species (Geronikou *et al*., 2020; Bintsis, 2021; Guneser *et al*., 2022). Furthermore, *D. hansenii* and *Yarrowia lipolytica* can be utilized as supplementary cultures to enhance flavor during cheese ripening (Gottardi *et al*., 2023).

In recent years, studies on the probiotic potential of various yeast species, including Debaryomyces, Kluyveromyces, Yarrowia, Pichia, and Torulaspora, isolated from fermented foods, traditional beverages, human microbiota, and natural sources, have attracted attention (Staniszewski and Kordowska-Wiater, 2021; Tullio, 2024). The tolerance of Debaryomyces yeast strains isolated from fermented foods and natural sources to bile and stomach acid suggests they may support the gut microbiota (Homayouni-Rad *et al*., 2020; Staniszewski and Kordowska-Wiater, 2023; Astuti *et al*., 2023). Furthermore, *D. hansenii* has antifungal properties that can prevent mould growth and reduce aflatoxin contamination in foods through biotechnological applications (Kurtzman *et al*., 2011; Peromingo, 2019).

Yeasts have positive effects on the production and ripening of food, while some can cause food spoilage and others have potentially pathogenic properties (He *et al*., 2024). Due to these different effects in food, the correct identification of yeast flora is crucial for both improving product quality and ensuring consumer safety. Methods for yeast identification have diversified with technological advances, and research in this field has accelerated. Traditional culture methods, biochemical tests, and molecular biology techniques are commonly used to identify microorganisms. Automatic biochemical identification systems, such as the VITEK2 Compact system, are widely used, especially in clinical laboratories, because they offer the advantages of standardization and rapid results (Pincus, 2006). In food microbiology, the VITEK2 Compact system has been found to be limited in the detection of foodborne pathogens (Hassan *et al*., 2018; Wally, 2022; Sulaiman *et al*., 2023; Hashhash, 2023). Polymerase chain reaction (PCR) is considered a gold standard method in food microbiology testing due to its high sensitivity and specificity. Many studies have emphasized that PCR provides reliable results, especially for rare and difficult-to-detect species (Schmidt *et al*., 2020; López-Mondéjar *et al*., 2021).

Automatic biochemical identification is performed with the VITEK2 system. This system provides satisfactory results, especially for bacteria and some yeast species (Garcia *et al*., 2018). However, the accuracy of the VITEK2 system in identifying yeast species compared to molecular methods remains controversial, and misidentification rates between species are substantial in the literature (Lee *et al*., 2019; Kord *et al*., 2020; Bokulich *et al*., 2021). Foods may contain rare yeast species of environmental or different ecological origins, depending on where they are produced, and these may not be found in the VITEK2 Compact system database. Furthermore, distinguishing species with closely related biochemical characteristics can be difficult (Odumeru *et al*., 1999; Bakirci and Kose, 2017). Given these findings, this study aimed to identify and characterize the yeasts isolated from cheese samples using molecular techniques and the VITEK2 system.

Materials and Methods

For yeast isolation, cheese samples produced by traditional methods from raw milk sold in the central markets of Erzurum were used. The cheese samples were collected from local businesses under aseptic conditions and transported to the laboratory under cold chain conditions. Twenty-five grams of each cheese sample were weighed into sterile bags and homogenized with 225 ml of sterile Ringer's lactate solution. Dilutions of the samples were prepared and inoculated onto RBC (Rose Bengal Chloramphenicol) agar. The inoculated Petri dishes were incubated at 20°C for 5 days. Yeasts grown on the agar were examined, and those with different morphologies were selected and observed under a microscope. The yeast strains chosen for the analyses were passaged three times, examined

under a microscope, and purified after complete purification. The selected yeast samples were purified and identified using the VITEK2 Compact system and ITS-PCR. Sequence analyses were then performed.

Identification of yeast species with the VITEK2 ID compact system

Yeast species were identified using the automated VITEK2 ID Card System (bioMérieux, France). Yeast species from a 24-hour culture, examined morphologically and microscopically, were inoculated into polystyrene tubes containing 3 ml of isotonic solution, and McFarland turbidity was adjusted to 1.8–2.2. A VITEK2 YST card (Catalog No: 21343) identification card was placed in each tube. The prepared samples were placed in the device and incubated at 35.5 ± 1.0 °C for 18 hours. The YST ID card contains various sugars, enzymes, and substrates such as L-lysine arylamidase, L-malate, leucine arylamidase, arginine GP, erythritol, glycerol, tyrosine arylamidase, β-N-acetyl-glucosaminidase, arbutin, amygdalin, D-gentibialactose, D-glucose, lactose, methyl-A-D-glucopyranoside, cellobiose, D-γ-glutamyltransferase, D-maltose, D-refinose, PNP-N-acetyl-BD-galactosaminidase-1, D-mannose, D-melibiose, D-melizitose, L-sorbose, L-rhamnose, xylitol, D-sorbitol, sucrose/sucrose, urease, α-glucosidase, D-turanose, D-trehalose, nitrate, L-arabinose, D-galacturonate, esculin, L-glutamate, D-xylose, DL-lactate, acetate, citrate (sodium), glucuronate, L-proline, 2-keto-D-gluconate, N-acetyl-glucosamine, and D-gluconate biochemical tests (Pincus, 2006). Classification of each yeast isolate by the VITEK2 Compact system as excellent, very good, good, acceptable, or low discrimination at the species level was considered an accurate identification.

Genotypic characterization of yeasts

Genomic DNA was extracted from purified yeast isolates using the Promega Wizard Genomic DNA Purification Kit (Promega, Southampton, UK), according to the manufacturer's instructions. The internal transcribed spacer (ITS) region of the ribosomal RNA gene was amplified using the universal primers ITS1 (5'-GTTTCCGTAGGTGAACTTGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Altun *et al*., 2020). PCR amplifications were carried out in a 25 µL reaction volume containing 1× PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer, 1.0 U Taq DNA polymerase, and 1–2 µL of template DNA (approximately 10–50 ng). Nuclease-free water was added to reach the final reaction volume. Amplifications were performed in a thermal cycler with an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s, with a final extension step at 72°C for 7 min. A no-template control was included in each PCR run to monitor potential contamination (Akbulut *et al*., 2022). PCR products were separated by electrophoresis on 1.5% (w/v) agarose gels prepared in 1× TAE buffer, run at 100 V for 35–45 min, using a 100 bp DNA ladder as a molecular size marker. Gels were stained with ethidium bromide (0.5 µg/mL) and visualized under UV transillumination using a gel documentation system. Amplicons showing a single, clear band were selected for sequencing.

Purified PCR products were sequenced by Macrogen Company (Amsterdam, the Netherlands). The obtained ITS sequences were edited and compared with reference sequences available in the NCBI GenBank database using the BLAST algorithm, and accession numbers were assigned to representative isolates.

Multiple sequence alignment of the ITS sequences was performed using the ClustalW algorithm in MEGA version 4.0. Phylogenetic relationships among the isolates were inferred using the neighbor-joining method based on Kimura 2-parameter (K2P) evolutionary distances. The robustness of the phylogenetic tree was evaluated by bootstrap analysis with 1000 replications, and bootstrap values were displayed at the corresponding nodes (Baltaci *et al*., 2017).

Results

A total of 32 yeasts were isolated from cheeses traditionally produced and sold in the Erzurum region and identified by the VITEK2 system and molecular methods. Using the VITEK2 system, nine were identified as *Candida famata*, six as *Candida zeylanoides*, two as *Candida colliculosa*, four as *Candida kefyr*, and one each as *Candida parapsilosis*, *Geotrichum silvicola*, *Candida krusei*, *Candida robusta*, *Candida lipolytica*, and *Trichosporon inkin*, while five yeast species could not be identified. According to the results, nine yeasts were identified as *Debaryomyces hansenii*, one as *Pichia kudriavzevii*, six as *Candida zeylanoides*, one as *Geotrichum silvicola*, two as *Debaryomyces prosopidis*, two as *Torulaspora delbrueckii*, four as *Kluyveromyces marxianus*, one as *Trichosporon inkin*, one as *Debaryomyces fabryi*, one as *Cutaneotrichosporon curvatum*, one as *Yarrowia lipolytica*, one as *Saccharomyces cerevisiae*, one as *Tausonia pamirica*, and one as *Candida parapsilosis* by the molecular method (Table 1).

Table 1. Related yeast species and similarity rates of isolates according to ITS-PCR sequence analysis.

Code	Related species	Similarity rate	Accession number
16d	*Debaryomyces hansenii*	99.90	PQ044636
14d	*Debaryomyces prosopidis*	99.90	PQ044637
14c-2	*Pichia kudriavzevii*	98.90	PQ044643
b4-3	*Candida zeylanoides*	99.90	PQ044647
k3-4	*Debaryomyces hansenii*	99.80	PQ044645
14c-4	*Geotrichum silvicola*	98.50	PQ044650
23y-2	*Candida zeylanoides*	99.80	PQ044655
2a	*Debaryomyces prosopidis*	99.90	PQ044656
5b-2	*Candida zeylanoides*	99.90	PQ044657
b4-1	*Torulaspora delbrueckii*	99.90	PQ044679
4a-2	*Debaryomyces hansenii*	99.80	PQ044680
24y-2	*Kluyveromyces marxianus*	99.85	PQ044684
18a	*Debaryomyces hansenii*	99.70	PQ044685
23y-4	*Kluyveromyces marxianus*	99.70	PQ044686
9b-2	*Candida zeylanoides*	99.90	PQ044687
8b-1	*Trichosporon inkin*	99.40	PQ044690
24y-3	*Kluyveromyces marxianus*	99.10	PQ044691
17c	*Debaryomyces hansenii*	99.80	PQ044692
5c	*Debaryomyces fabryi*	99.80	PQ045730
8b-3	*Cutaneotrichosporon curvatum*	99.80	PQ044700
b4-2	*Torulaspora delbrueckii*	99.70	PQ044702
k3-3	*Debaryomyces hansenii*	99.70	PQ044701
14c-3	*Candida zeylanoides*	99.80	PQ044703
14c-1	*Yarrowia lipolytica*	98.60	PQ047658
13a	*Debaryomyces hansenii*	99.70	PQ044705
17b-1	*Candida zeylanoides*	99.80	PQ044706
13c-1	*Saccharomyces cerevisiae*	97.90	PQ044718
20y-1	*Kluyveromyces marxianus*	99.40	PQ044719
17d	*Debaryomyces hansenii*	99.70	PQ044721
8b-4	*Tausonia pamirica*	86.00	PQ044811
23y-3	*Candida parapsilosis*	99.60	PQ044720
14c	*Debaryomyces hansenii*	99.70	PQ044807

The identification results showed that naming conventions for yeasts can differ depending on the identification method used (Table 2). The yeast species *Candida zeylanoides* was identified by both the VITEK2 system and PCR. The yeast species identified as *Candida famata* by the VITEK2 system was named *Debaryomyces hansenii* by the molecular method. The yeast identified by PCR as *Kluyveromyces marxianus* was identified by the VITEK2 system as *Candida kefyr*. Yeasts identified by molecular methods as *Cutaneotrichosporon curvatum*, *Debaryomyces fabryi*, *Debaryomyces prosopidis*, and *Tausonia pamirica* could not be identified by the VITEK2 system.

To further validate the yeast characterization, a phylogenetic tree was constructed using a neighbor-joining method based on ITS sequences (Figure 1).

According to results, *Debaryomyces hansenii* (28%) was the dominant yeast in the cheese samples, followed by *Candida zeylanoides* (18%), *Kluyveromyces marxianus* (12.5%), *Torulaspora delbrueckii* (6.25%), *Trichosporon inkin* (3%), *Debaryomyces fabryi* (3%), *Cutaneotrichosporon curvatum* (3%), *Yarrowia lipolytica* (3%), *Saccharomyces cerevisiae* (3%), *Tausonia pamirica* (3%), and *Candida parapsilosis* (3%).

Table 2. Yeast species identified using PCR and VITEK2 Compact.

Yeast Species identified by PCR	Yeast Species identified by VITEK2	Number isolated n (%)
Candida zeylanoides	*Candida zeylanoides*	6 (18)
Candida parapsilosis	*Candida parapsilosis*	1 (3)
Cutaneotrichosporon curvatum	*ND*	1 (3)
Debaryomyces fabryi	*ND*	1 (3)
Debaryomyces hansenii	*Candida famata*	9 (28)
Debaryomyces prosopidis	*ND*	2 (6)
Geotrichum silvicola	*Geotrichum silvicola*	1 (3)
Kluyveromyces marxianus	*Candida kefyr*	4 (12.5)
Pichia kudriavzevii	*Candida krusei*	1 (3)
Saccharomyces cerevisiae	*Candida robusta*	1 (3)
Tausonia pamirica	*ND*	1 (3)
Torulaspora delbrueckii	*Candida colliculosa*	2 (6.25)
Trichosporon inkin	*Trichosporon inkin*	1 (3)
Yarrowia lipolytica	*Candida lipolytica*	1 (3)

ND: Non-Detection.

Discussion

The VITEK2 Compact system and PCR (Polymerase Chain Reaction) are two fundamental, complementary approaches for yeast identification. The VITEK2 Compact system provides rapid, automated identification based on biochemical reactions, such as carbohydrate assimilation, while PCR offers high accuracy and precise species-level identification by targeting specific gene regions. Yeasts are found in the microflora of many types of cheese. The proteolytic and lipolytic properties of certain yeast species suggest that they can contribute to the development of sensory qualities during cheese production and ripening and can be used as auxiliary starter cultures. However, identifying yeasts by morphological and biochemical methods is complex and challenging. In addition, phenotypic analyses may result in some yeast species being poorly, incompletely, or incorrectly identified (Abu-Mejdad *et al*., 2020). Yeasts can be given different names depending on their telemorphic and anamorphic reproduction. To ensure the accuracy of these different nomenclatures, molecular identification is necessary (Alsohaili and Bani-Hasan, 2018). Our study also found that the yeasts identified in the VITEK2 system and in PCR analyses had different names. Yeast species identified by the molecular method as *Debaryomyces hansenii*, *K. marxianus*, *Pichia kudriavzevii*, *Torulaspora delbrueckii*, and *Yarrowia lipolytica* were identified by the VITEK2 system as *Candida famata*, *Candida kefyr*, *Candida krusei*, *Candida colliculosa*, and *Candida lipolytica*. In our study, similar to the existing literature, differences were observed in the identification of yeast isolates. The species *Cutaneotrichosporon curvatum*, *Debaryomyces fabryi*, *D. prosopidis*, and *Tausonia pamirica*, identified by molecular methods, were not recognized by the VITEK2 system. The presence of unidentified yeast species may be due to the limited database of the VITEK2 Compact system. The match rate for *Candida zeylanoides*, *Candida parapsilosis*, *Cutaneotrichosporon curvatum*, *Debaryomyces fabryi*, *Debaryomyces hansenii*, *Debaryomyces prosopidis*, *Geotrichum silvicola*, *Kluyveromyces marxianus*, *Pichia kudriavzevii*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Trichosporon inkin*, and *Yarrowia lipolytica* species identified by the ITS-PCR method was found to be higher than 98%, while this rate was 86% for *Tausonia pamirica*. Although most yeast isolates showed high ITS sequence similarity (>98%), one isolate exhibited a comparatively low similarity (86%) to *Tausonia pamirica*. This isolate was therefore interpreted as the closest related taxon based on ITS sequence alignment and phylogenetic clustering rather than as a definitive species-level identification. Previous studies have demonstrated that the ITS region may provide limited taxonomic resolution for specific basidiomycetous yeasts, due to high interspecific variability and overlap between closely related taxa (Li *et al*., 2022). In addition, the taxonomic placement of rare or recently described yeast species may be affected by the limited representation of reference sequences in public databases, leading to lower similarity values despite correct phylogenetic affiliation (Jiang *et al*., 2024). Therefore, ITS-based identifications

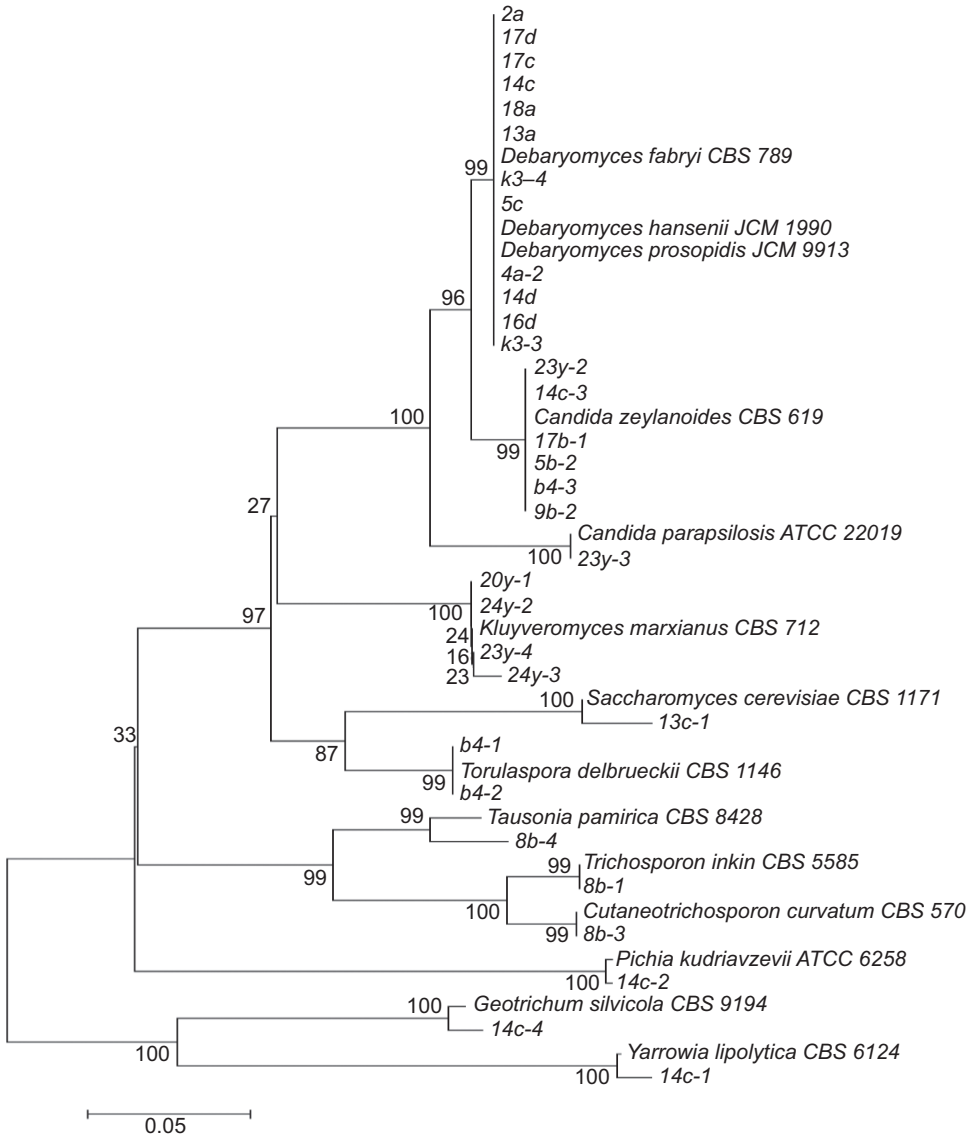

Figure 1. Phylogenetic relationships of isolates based on ITS sequence analyses. The tree was constructed using a neighbor-joining method. Bootstrap values based on 1000 replications are listed as percentages at branching points.

showing low similarity values should be interpreted with caution, and complementary molecular markers or multilocus approaches are recommended for more precise species-level resolution.

The yeast species *Debaryomyces hansenii* (28%) (anamorphic form of *Candida famata*) isolated in our study was frequently detected in various cheeses (Roostita and Fleet, 1996; Vasdinyei and Deak, 2003). *Debaryomyces hansenii*, often isolated from dairy products, contributes positively to cheese ripening and limits the development of bacteria that cause spoilage (Cholet *et al*., 2007; Urcar Gelen and Ceylan, 2021). *K. marxianus* (12.5%) (anamorphic form of *Candida kefyr*) is commonly associated with traditional dairy products such as fermented milk, kefir, yogurt, and cheese (Coloretti *et al*., 2017). It has been reported that the sources of yeast contamination in dairy products can typically be attributed to production facilities, raw materials, brine, air, and personnel (Atanassova *et al*., 2016; Banjara *et al*., 2015; Fröhlich-Wyder *et al*., 2019). The species isolated in our study, *C. zeylanoides*, *C. parapsilosis*, and *P. kudriavzevii* (anamorphic form of *Candida krusei*), are considered opportunistic pathogens that infect immunocompromised individuals (Johansen and Jespersen, 2017; Tofalo *et al*., 2019; Riesute *et al*., 2021; Urcar Gelen and Ceylan, 2021).

However, no outbreaks or adverse effects on human health have been reported in connection with the consumption of dairy products contaminated with these yeast species (Jacques and Casaregola, 2008; Geronikou *et al*., 2023).

To provide a precise and accurate characterization of yeasts, numerous molecular techniques based on comparative DNA studies have been developed in recent years (Chen *et al*., 2010). One of the most crucial regions by which yeasts can be identified is the ITS region, whose sequences are publicly available via the NCBI, EMBL, or DDBJ sequence databases for all known yeasts. In this study, ITS gene region–based identification of yeasts isolated from cheese samples was performed. According to the results, *Debaryomyces hansenii* (28%) was the dominant yeast in the cheese samples. According to published research, *Debaryomyces hansenii* is the most frequently found species in nearly all types of cheese (Karasu-Yalcin *et al*., 2017; Ozmen Togay *et al*., 2020). In another study, *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Debaryomyces prosopidis*, *Yarrowia lipolytica*, and *Pichia kudriavzevii* were isolated from homemade fermented cow's milk products. These results are compatible with our work. However, in the same study, the species *Kazachstania exigua*, *Meyerozyma guilliermondii*, and *Pichia bruneiensis* were also isolated. These species were not detected in the current study (Lama and Tamang 2022).

In subsequent research, the same amplicon-based NGS approach was used to analyze 137 cheese samples from 10 countries, including both mold- and smear-matured cheeses, focusing on the ITS region. This NGS investigation verified the high frequency and abundance of the *Debaryomyces* and *Geotrichum* genera. However, lower frequencies were observed for the *Yarrowia, Trichosporon*, and *Kluyveromyces* genera at the subdominant level. These results are also largely compatible with our work (Wolfe *et al*. 2014).

Conclusion

The VITEK2 Compact system provides rapid results, while PCR serves as a definitive confirmation tool. Using both methods together ensures speed and reliability. This study primarily focused on isolating yeasts from raw-milk cheeses produced using traditional methods. A total of 32 yeast strains belonging to the genera *Kluyveromyces, Debaryomyces, Pichia, Geotrichum, Torulaspora, Yarrowia,* and *Candida* were identified. Yeast identification was performed using both biochemical and molecular analyses to determine the natural yeast flora of the collected cheese samples. More comprehensive studies are needed to determine the biotechnological properties of these identified yeasts, their potential as probiotics, and their possible use in cheese production. Furthermore, given the limited database of the VITEK2 Compact system, it is considered appropriate to use molecular methods for the identification of yeast species whose biotechnological properties are planned to be determined.

Author Contributions

All authors designed and projected the study. SUG and MOB performed the laboratory work. SUG and GCA wrote the original version. MOB prepared Figure 1. MA and AA drew tables 1 and 2. All authors participated in the revision and editing process for all versions of this manuscript.

Conflicts of Interest

The authors declare no competing interests.

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