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Original article

# Molecular identification and probiotic characterization of isolated yeasts from Iranian traditional dairies

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**Summary.** Yeasts as natural live microorganisms with probiotic properties are important in human nutrition and improving health due to their ability to ferment food production. The traditional fermented dairy foods as a rich source of wild native strains of yeasts can introduce new strains with probiotic properties. The aim of this study is to isolate and identify yeasts from Iranian traditional dairy products and characterize their probiotic properties. Fifty-five fresh stored yogurt and cheese were purchased from rural areas and the native yeasts of them were characterized using the intragenic transcribed spacer-PCR (ITS1 and ITS2) regions through specific primers and the amplified genes were sequenced. Also, some probiotic assessment tests including; resistant to acid and bile, antimicrobial activity and antifungal susceptibility were performed. Seventeen yeast species belonged to 4 genera were identified as *Pichia, Kluyveromyces, Saccharomyces*, and *Candida*. The difference between survival rates after treatment with acid (pH 2.0) and bile salts (0.3%) for 3h were not significant for 24 strains. Finally, after antimicrobial activity and antifungal tests 5 yeast strains indicated potentially probiotic properties. Our findings showed that among the isolated yeasts from home-made cheese and yogurt, *Pichia fermentans* and *Pichia kudriavzevii* were the predominant strains. Meanwhile, 10 strains revealed antifungal susceptibility but only 5 strains were susceptible to all antifungals, so we can consider these strains as a native probiotic for implicating in food industries, however extra examinations required for introduction into food products.

**Key words:** probiotics, yeast, functional food, intragenic transcribed spacer

## Introduction

Nowadays, there are significant attentions to the design of substantial functional foods including probiotics that are responsible for host wellness through improvement of intestinal microflora which enhance the body health (1). These live microorganisms are increasingly incorporated into foods as dietary adjuvant to maintain a healthy microbial gastrointestinal (GI) balance (2). According to recent Food and Agriculture Organization (FAO) and World Health Organization (WHO) guidelines, probiotics are expected to be safe and effective, non-pathogenic and non-toxic, resistant to low pH and to bile salts, survive in the gastrointestinal tract and also must be able to proliferate and colonize in the digestive tract (3). Therefore, probiotic characterization must be carried out through standard *in vitro* experiments (4). Probiotics should be able to tolerate harsh conditions similar to gastrointestinal tract (i.e., high bile salts [0.3% (w/v)] and low pH [pH 2.0 to pH 3.0]) for a minimum of 90 min (5, 6). Yeasts as probiotics adapted themselves with rich pro-

tein, lipid, sugar and organic acids media. Besides, the wide distribution of yeasts is a result of their proteolytic and lipolytic activities, their ability to ferment/assimilate lactose and utilize citric, lactic and succinic acids. Moreover, yeasts are able to grow in harsh media like environments with high salt concentration, low temperature, and low pH. Their natural ability to adapt with complex substrates, capacitate them as an important component in ripening processes, fermentation and fortification of dairy products. Dairy products are suitable environments for the growth and detection of different yeast species (2, 7). There are a lot of yeast species in dairy products that show a good adaptation to rich protein, lipid, sugar and organic acids media. However, high diversity of yeast species like *Cryptococcus*, *Rhodotorula*, *Debaryomyces*, *Kluyveromyces*, *Trichosporon*, *Yarrowia*, *Candida*, and *Saccharomyces* naturally grow in high number in several types of dairy products (8, 9).

In addition, due to the proteolytic and lipolytic activity of yeasts, they can be introduced as good candidates for the production of starter cultures (10, 11). Recent studies have shown that yeasts as part of starter cultures, may contribute to the enhancing flavor development and ripening of cheese. In this way, *Debaryomyces hansenii* and *Yarrowia lipolytica* are good candidates for the production of starter cultures due to their proteolytic and lipolytic activities and positive attributes to cheese ripening (12). Besides, spoilage, gas production, some off-flavors, inhibition of the starter cultures growth and dairy production changes both in texture and color are some detrimental effects of dairy's yeasts (8, 13). In addition, yeasts increase the pH value via lactic acid utilizing properties and favoring the growth of less acid-tolerant microorganisms such as *micrococci* and *coryneform* bacteria (14). On the other hand, some yeast species exhibit the inhibitory effects on undesired bacteria, such as spore-forming bacteria of the genus *Clostridium* like *C. butyricum* and *C. tyrobutyricum* (15). However, the extensive usage of benign yeasts as beneficial or spoilage microorganisms in the dairy products and distribution in different products have been attracting considerable interests of food microbiologists to develop reliable methods for characterization and identification of yeasts from dairy products. In this way, progress in the molecular biology in the last decade has opened up possibilities of characterizing yeasts at the genomic level (8). According to this issue, accurate identification methods are essential for analysis of isolated yeasts microbiota. Traditional identification methods by focusing on phenotypic properties of microorganisms and based on morphological, physiological and biochemical characteristics are inaccurate and require remarkable experiences and skills (16, 17). Recently, some molecular methods like, mitochondrial DNA (mt-DNA) restriction analysis, Polymerase chain reaction (PCR) based techniques have been successfully applied to yeast strain typing and identification (18, 19). As well, some established methods based on fragment analysis, such as pulsed filed gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) have been already performed as reliable tools for the rapid identification of foodborne yeasts (18, 20-22). The sequencing of the genes coding for 18S, 26S ribosomal RNA (rRNA), and ITS regions, has brought about many changes in the identification and classification of yeasts (23). The evidences showed that the 18-28S rDNA targeted PCR evaluation can be appropriate as reliable identification tool because these sequences explain a high degree of sequence and length variation at the genus and species levels, so analysis of ITS-PCR-amplified 18S-28S rDNA has been highly efficient and accurate for new species identification (24).

Researchers are able to isolate and characterize new yeast species from different natural origins by using these accurate and reliable methods and also can investigate their possible health beneficial effects (25-28). There are different traditional dairy products in Iran such as cheese, yogurt, curd, shiraz, and tarkhineh that prepare by traditional fermentation methods and contain various native strains of probiotic yeasts and bacteria (29, 30). However, some traditional dairy products like cheese and yogurt are good resources for identification of new native yeast strains with probiotic properties that make unknown useful health benefits (31). In the present study we employed PCR based technique for identification of native dairy yeasts and different probiotic assessment tests were carried out for evaluation of probiotic properties of identified yeasts.

## Materials and methods

*Sampling and isolation of yeasts*

Fifty-five samples either yogurt and cheeses were randomly collected from several different rural areas of Kurdistan province of Iran where, dairy products are produced in the traditional way, from January to April 2016. After sample collection and registration of their names and locations, samples were immediately placed in an ice box and sent to the laboratory to further analysis as quickly as possible. Five grams of the samples were homogenized with 90 ml of 2% sodium citrate solution and serially diluted in sterile 0.1% (w/v) peptone solution and were plated onto YMB (Yeast Malt Broth) agar medium (10 $g.L^{-1}$ yeast extract, 10 $g.L^{-1}$ glucose, 0.1 $g.L^{-1}$, 15 $g.L^{-1}$ agar) with chloramphenicol (DRBC, Merck, Darmstadt, Germany) for the selective isolation (32). After incubation for 4 days at 25°C, the colony forming units (cfu) with different color, shape and morphology were selected, assuming that colonies with the same morphology belonged to the same genus or species. The single colonies were inoculated on YMB medium and kept at 4°C until they were identified.

*Molecular identification of isolates*

Each isolated single colony yeast cells were grown over nightly in 25 ml of YMB medium at 37°C in aerobic condition. Two ml of overnight culture were transferred into the sterile 2 ml micro tube and were centrifuged at 3500 rpm for 15 min at 4°C then the supernatants were removed carefully. The achieved cell plates were subjected to 500 µl Harju lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH 8.0). The cell lysates were used to isolate the genomic DNA according to Harju S et al method (19). The quality and quantity of the extracted DNA were checked and evaluated by 1% agarose gel electrophoresis (Biometra, Gottingen, Germany) and spectrophotometric method, respectively.

The PCR amplification was conducted using a thermal cycler PTC 200 (MJC research, Waltham, USA) on extracted genomic DNA of each yeast by applying the amplification sets of primers Forward: 5´-CGATGCGAGAGCCAAGAGAT-3´ and Reverse: 5´-GTCGTGCTGGGGATAGAGCATT-3´ for amplifying the ribosomal DNA fragments including; the internal transcribed spacer 1 (ITS 1), 5.8S rDNA, and internal transcribed spacer 2 (ITS 2) (Fig. 1). The PCR amplification was performed under the following condition: denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 40 sec, extension at 72 °C for 1 min, and a final extension step at 72°C for 10 min. The PCR products were again checked and visualized via 1% agarose gel electrophoresis and staining with ethidium bromide, respectively. The gel monitoring apparatus (Biometra, Gottingen, Germany) was used to evaluate the quality of the PCR products and genomic DNA (Fig. 2) (33). The gel recovered PCR products were sequenced by a Korean sequencing company (Macrogene). The sequences were analyzed using the BLAST program on NCBI (http://www.ncbi.nlm.nih.gov/BLAST). Then, the isolates were identified and discriminated by comparing the obtained sequences with the sequences deposited in NCBI and GenBank. Strains with at least 98% homology were considered to the same species.

*Acid and Bile tolerance*

The isolated yeasts with $10^7$-$10^8$ cfu/ml in YMB broth were centrifuged for 10 min at 6000 ×*g*. The cell pellets were suspended in phosphate buffer saline (PBS, 1M HCl, and pH 2.0) and the viable cells were subsequently resuspended in bile salts (0.3% Oxgall, Sigma) by incubating at 37°C for 3h. The harvested viable cells

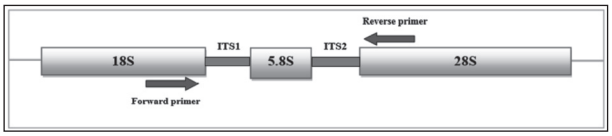


**Figure 1**. Map of rDNA primer. rDNA: ribosomal DNA, 18S: 18S rDNA, 5.8S: 5.8S rDNA, 28S: 28S rDNA, ITS: Internal transcribed spacer.

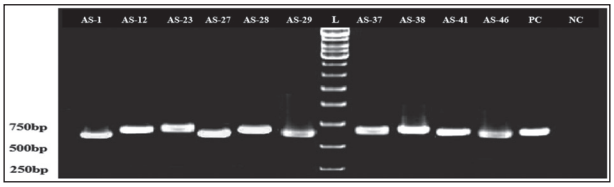


**Figure 2**. Agarose gel electrophoresis of ITS-PCR products of the isolated yeast species from different samples of cheeses and yoghurts. L: DNA Ladder 1 kb, PC: Saccharomyces cerevisiae (PTCC: 5052) as Positive control, NC: Negative control

were inoculated into 10 ml YMB broth medium and a given amount of each dilution (100 µl) was spread-plated onto YMB agar and incubated in the aerobic condition at 37˚C for 72 h. The resistant rates were calculated by comparing treated/untreated cell survivals (34).

*Antimicrobial activity assay*

The antimicrobial activity of the each isolated yeast was assayed against a wide variety of important human pathogens such as, *Bacillus cereus subsp. Kenyae* (Persian Type Culture Collection, Tehran, Iran. [PTCC 1539]), *Candida albicans* (PTCC 5027), native isolate of *E. coli* (026), *E. coli 0157* (PTCC 1276), *Enterococcus faecalis* (PTCC 1394), *Klebsiella pneumonia* (PTCC 1053), *Listeria monocytogenes* (PTCC 1163), *Pseudomonas aeruginosa* (PTCC 11811), *Serratia marcescens* (PTCC 1187), *Staphylococcus saprophyticus* (PTCC 1440), *Salmonella typhimurium* (American Type Culture Collection, Virginia, USA [ATCC 14028]), *Shigella flexneri* (PTCC 1234), *Streptococcus mutans* (PTCC 1683) and *Staphylococcus aureus* (ATCC 25923). Briefly, the isolated yeasts were cultured in YMB broth overnightly at 37˚C and cell free supernatants were prepared by centrifuging the culture broth at 8000×g for 15 min. The supernatant's pH was adjusted to pH 6.5 to avoid pH effects on bacterial growth then was filtered through 0.22 µm membrane (Millipore Co., Bedford, MA, USA) filtration, then, 50 µl of each filtrate was added to 7 mm diameter wells were cut into Mueller-Hinton agar plates (Difco Laboratories, Detroit, MI, USA) incubated overnight by indicator pathogens at 37 ˚C (35). After overnight incubation, the antimicrobial activity was assayed based on the diameter of the clear zones around the each well (inhibition zone). A clear zone more than 1 mm around wells was accepted as positive antibacterial effect (36).

*Antifungal susceptibility test*

The antibiotic susceptibility of isolated yeasts was determined using the disc diffusion method (37, 38). The Neo-Sensitabs tablet assay was accomplished according to the manufacturer's instructions (Neo-Sensitabs user's guide; Rosco Diagnostica, Taastrup, Denmark) and M44-A2 guidelines {Gionchetti, 2002 #14}. The antifungal disks including Clotrimazole (10 µg), Miconazole (10 µg), Fluconazole (25 µg), Flucytosine (1 µg), Itraconazole (10 µg), Ketoconazole (15 µg), Amphotericin B (10 µg), Griseofulvin (25 µg), Terbinafine (30 µg), Nystatin (50 µg) and Voriconazole (1 µg) were purchased from Rosco Diagnostica, Taastrup, Denmark. Briefly, the overnightly cultured yeasts in YMB broth (10 ml) were suspended in 5 ml of phosphate buffered solutions (PBS) and were gently vortexed to achieve a homogenized suspension. The optical density (OD) of the suspensions were adjusted to 0.08 to 0.1 at a wavelength of 625 nm to yield turbidity equal to 0.5 McFarland criteria (39). Agar plates (80 mm diameter) containing Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg/ml methylene blue (MH-MB) were inoculated by using a swab dipped in a cell suspension that previously adjusted to the turbidity of a 0.5 McFarland standard. To determine the antifungal susceptibility patterns of the isolates, a Neo-Sensitabs disk of each mentioned antifungals were placed onto the surface of the inoculated plates and the plates were incubated at 37˚C (40). The inhibition zones around the disks were measured by digital caliper after incubation of the plates for 24 h and the susceptibility was expressed in terms susceptible (S), intermediate (I) and resistant (R) (Table. 4).

## Results

*Molecular identification of isolates*

Forty six yeast strains were isolated from 55 samples of fresh and stored traditional dairy products including yogurt and cheese. Four isolated yeasts genera sequenced on the basis of the ITS1-ITS2 PCR products analysis were classified into 17 species. The isolated yeasts identified in *Pichia*, *Saccharomycea, Candida* and *Kluyveromyces* genera. As well, among all genera and species, *Pichia kudriavzevii*, were frequently isolated species from 44 types of cheese and yoghurt. In contrast, all other isolated and identified species were found in limited types of samples (Table 1).

*Acid and Bile Tolerance*

Twenty four yeast strains were resistant to pH 2.0 and 0.3% bile salt after 3h incubation in harsh condition, thus they were selected for antimicrobial and antifungal susceptibility examinations. Among these

**Table 1.** Criteria of susceptibility and resistance of antifungal disks (72).

| Antifungal drugs | Potency | Zone diameter in mm | | |
|---|---|---|---|---|
| | | Sensitive | Intermediate | Resistance |
| Clotrimazole | 10μg | ≥20 | 19-12 | ≤11 |
| Miconazole | 10μg | ≥20 | 19-12 | ≤11 |
| Fluconazole | 25μg | ≥19 | 18-15 | ≤14 |
| Flucytosine | 1μg | ≥20 | 19-12 | ≤11 |
| Itraconazole | 10μg | ≥23 | 22-14 | <13 |
| Ketoconazole | 15μg | ≥28 | 27-21 | ≤20 |
| Amphotericin B | 10μg | ≥15 | 14-10 | <10 |
| Griseofulvin | 25μg | ≥10 | - | No zone |
| Terbinafine | 30 μg | ≥20 | 19-12 | ≤11 |
| Nystatin | 50 μg | ≥15 | 14-10 | No zone |
| Voriconazole | 1 μg | ≥17 | 16-14 | ≤13 |

strains, 6 species of *Pichia,* 1 species of *Saccharomyces*, 5 species of *Candida* and 2 species of *Kluyveromyces* were resistant to acid/bile. The highest and lowest resistance against low pH belong to *Saccharomyces cerevisiae AS28* (95%) and *Pichia deserticola AS20* (38%). However, *Saccharomyces cerevisiae AS27* (93%) and *Candida pseudolambica AS30* (53%) had the highest and lowest resistance against 0.3% bile salt, respectively (Table 2).

*Antimicrobial activity*

Fifteen yeast isolates showed strong to weak inhibitory activities against the indicator bacteria, while the extents of inhibitory effects were variable. The highest inhibitory activities were found against the *E. coli (026), Ecoli (0157)* and *Listeria monocytogenes* pathogens and all 15 isolates had remarkable inhibitory effects against them. Furthermore, the most extended inhibition zones around all indicator bacteria observed in *Pichia kudriavzevii AS8*, *Saccharomyces cerevisiae AS27*, *Saccharomyces cerevisiae AS28* and *Kluyveromyces marxianus AS41* (Table 3).

*Antifungal susceptibility*

As shown in table 4, the antifungal susceptibility of 15 isolated yeasts possessing antimicrobial activity against the important antifungals were assessed using extent the diameter of inhibition zone. All ten strains (AS-1, AS-12, AS-23, AS-27, AS-28, AS-29, AS-37, AS-38, AS-41 and AS-46) were sensitive for Clotrimazole, Ketoconazole, and Itraconazole. In addition, AS-12, AS-23, AS-27, AS-28 and AS-41 were sensitive to all antifungals whereas AS-29 and AS-37 were resistant to Fluconazole, Flucytosine, Griseofulvin, Terbinafine, and Voriconazole. Other yeast strains were semi sensitive to investigated antifungal drugs (Table 5).

## Discussion

Dairy products as good resources of native microorganisms attracted much attention due to their beneficial effects on human health, wellness and life span (41, 42). These live microorganisms with probiotic properties not only promote food safety but also improve digestion and nutrient absorption (43, 44). Probiotic regular consumption, in suitable amounts of viable cells per gram of food (45), can be an effective and low-cost method at the household level to improve dietary quality in regions where food insecurity,

| | | | |
|---|---|---|---|
| | *fermentans* | AS-1 | Cheese, yogurt |
| *Pichia* | *kudriavzevii (Issatchenkia orientalis)* | AS-2 | Cheese |
| | | AS-3 | |
| | | AS-4 | |
| | | AS-5 | |
| | | AS-6 | |
| | | AS-7 | |
| | | AS-8 | |
| | | AS-9 | Cheese, yogurt |
| | | AS-10 | |
| | | AS-11 | |
| | | AS-12 | |
| | | AS-13 | |
| | | AS-14 | |
| | | AS-15 | |
| | | AS-16 | |
| | | AS-17 | |
| | *jaroonii* | AS-18 | Cheese |
| | | AS-19 | |
| | *deserticola* | AS-20 | |
| | *membranifaciens* | AS-21 | |
| | | AS-22 | |
| | | AS-23 | Cheese |
| | | AS-24 | yogurt |
| | *manshurica* | AS-25 | Cheese |
| | | AS-26 | |
| *Saccharomyces* | *cerevisiae* | AS-27 | yogurt |
| | | AS-28 | |
| *Candida* | *krusei* | AS-29 | Cheese, yogurt |
| | *pseudolambica* | AS-30 | |
| | *ethanolica* | AS-31 | |
| | *tropicalis* | AS-32 | |
| | *xylopsoci* | AS-33 | |
| | | AS-34 | |
| | *albicans* | AS-35 | |
| | *dubliniensis* | AS-36 | |
| | *thaimueangensis* | AS-37 | |
| *Kluyveromyces* | *marxianus (lactis)* | AS-38 | Cheese |
| | | AS-39 | |
| | | AS-40 | |
| | | AS-41 | |
| | | AS-42 | |
| | | AS-43 | |
| | | AS-44 | |
| | | AS-45 | |
| | *dobzhanskii* | AS-46 | |

**Table 3.** Acid and bile tolerance of the yeast isolates after 3h of incubation at pH 2.0 or 0.3% bile salt

| Isolate name | | Isolation origin | pH 2.0 (log cfu/ml) | | | | 0.3% bile salt (log cfu/ml) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 h | 1 h | 2 h | 3 h | SR* | 0 h | 1 h | 2 h | 3 h | SR* |
| AS-1 | *Pichia fermentans* | cheese, yogurt | 7.56 | 7.34 | 6.94 | 6.75 | 89% | 7.32 | 7.14 | 7.03 | 6.34 | 86% |
| AS-2 | *Pichia kudriavzevii* | cheese | 8.21 | 8.11 | 7.94 | 7.66 | 93% | 8.72 | 8.61 | 8.32 | 7.98 | 91% |
| AS-5 | *Pichia kudriavzevii* | cheese | 8.46 | 7.84 | 7.62 | 7.04 | 83% | 8.54 | 7.98 | 7.24 | 7.01 | 82% |
| AS-8 | *Pichia kudriavzevii* | cheese | 8.45 | 6.85 | 5.10 | 4.55 | 51% | 8.65 | 7.23 | 6.87 | 5.23 | 78% |
| AS-9 | *Pichia kudriavzevii* | cheese, yogurt | 8.35 | 8.12 | 7.98 | 7.61 | 91% | 7.38 | 7.22 | 7.00 | 6.82 | 92% |
| AS-12 | *Pichia kudriavzevii* | cheese, yogurt | 8.47 | 8.24 | 8.03 | 7.58 | 89% | 7.72 | 7.21 | 6.68 | 6.03 | 78% |
| AS-16 | *Pichia kudriavzevii* | cheese, yogurt | 8.43 | 8.12 | 7.92 | 7.54 | 89% | 8.82 | 8.14 | 7.91 | 7.75 | 87% |
| AS-17 | *Pichia kudriavzevii* | cheese, yogurt | 6.97 | 6.78 | 6.51 | 6.38 | 91% | 6.73 | 6.43 | 6.05 | 5.94 | 88% |
| AS-18 | *Pichia jaroonii* | cheese | 8.26 | 7.49 | 6.01 | 5.19 | 62% | 8.74 | 7.16 | 6.18 | 5.01 | 57% |
| AS-19 | *Pichia jaroonii* | cheese | 8.66 | 7.91 | 7.22 | 6.04 | 69% | 8.35 | 8.74 | 7.94 | 6.28 | 75% |
| AS-20 | *Pichia deserticola* | cheese | 7.99 | 6.80 | 4.05 | 3.06 | 38% | 7.47 | 6.24 | 5.04 | 4.87 | 65% |
| AS-23 | *Pichia membranifaciens* | cheese | 8.87 | 8.29 | 7.83 | 7.43 | 83% | 8.73 | 8.24 | 8.01 | 7.60 | 87% |
| AS-24 | *Pichia membranifaciens* | yogurt | 7.68 | 7.24 | 7.11 | 5.97 | 77% | 7.57 | 6.34 | 5.22 | 4.71 | 62% |
| AS-26 | *Pichia manshurica* | cheese | 7.59 | 6.64 | 5.43 | 4.22 | 56% | 6.98 | 6.54 | 5.88 | 5.12 | 73% |
| AS-27 | *Saccharomyces cerevisiae* | yogurt | 8.64 | 8.57 | 8.49 | 8.43 | 93% | 8.87 | 8.66 | 8.39 | 8.17 | 93% |
| AS-28 | *Saccharomyces cerevisiae* | yogurt | 8.41 | 8.38 | 8.31 | 8.03 | 95% | 8.35 | 8.04 | 7.65 | 7.44 | 89% |
| AS-29 | *Candida krusei* | cheese, yogurt | 8.91 | 8.58 | 8.17 | 7.98 | 89% | 7.78 | 7.10 | 6.26 | 5.76 | 74% |
| AS-30 | *Candida pseudolambica* | cheese, yogurt | 7.44 | 6.14 | 6.01 | 5.23 | 70% | 8.69 | 6.35 | 5.03 | 4.61 | 53% |
| AS-32 | *Candida tropicalis* | cheese, yogurt | 7.68 | 7.35 | 7.14 | 6.86 | 89% | 7.61 | 7.36 | 7.19 | 6.93 | 91% |
| AS-36 | *Candida dubliniensis* | cheese, yogurt | 8.86 | 8.32 | 8.08 | 7.71 | 69% | 8.15 | 7.85 | 7.21 | 6.28 | 77% |
| AS-37 | *Candida thaimueangensis* | cheese, yogurt | 8.92 | 8.75 | 8.54 | 6.22 | 83% | 8.67 | 8.33 | 7.97 | 7.56 | 87% |
| AS-38 | *Kluyveromyces marxianus* | cheese | 8.52 | 6.23 | 5.65 | 5.11 | 59% | 7.74 | 6.48 | 5.31 | 4.89 | 63% |
| AS-41 | *Kluyveromyces marxianus* | cheese | 8.98 | 7.80 | 7.05 | 6.41 | 71% | 7.87 | 7.33 | 6.94 | 6.54 | 83% |
| AS-46 | *Kluyveromyces dobzhanskii* | cheese | 7.36 | 5.67 | 4.14 | 3.48 | 47% | 7.56 | 6.91 | 5.27 | 4.16 | 55% |

** Survival rate (%) = (log cfu N1/log cfu N0) ×100*

*N1= Total viable counts of yeast isolates in the YMB agar after being treated with extra bile salts or in low acidic conditions, N0 = Total viable counts of isolates before incubation in harsh conditions.*

malnutrition and immunosuppressive disease are highly prevalent (42, 46). Moreover, their consumption are so advantageous in varied health-related issues such as inflammation, infections, allergy, antibiotic-related diarrhea, gastroenteritis, constipation, lactose intolerance and some type of malignancies (47).

According to recent FAO and WHO guidelines (48), probiotics are expected to be safe and effective,

**Table 4.** Antimicrobial activity assay of isolated yeasts (mean ± standard error)

| Pathogens | Origin | Diameter of inhibition zone (mm) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **AS-1** | **AS-2** | **AS-8** | **AS-12** | **AS-19** | **AS-23** | **AS-26** | **AS-27** | **AS-28** | **AS-29** | **AS-32** | **AS-37** | **AS-38** | **AS-41** | **AS-46** |
| *B. cereus* | PTCC 1539 (ATCC 11778) | 21.0±0.8 | 19.0±0.0 | 19.2±0.9 | 12.0±0.2 | 8.6±0.7 | 6.1±0.8 | 3.3±1.3 | 20.6±0.7 | 23.2±0.9 | 6.9±0.6 | 6.3±0.1 | 3.6±0.5 | 15.0±0.0 | 24.6±0.9 | 6.1±0.8 |
| *C. albicans* | PTCC 5027 (ATCC 10231) | 2.9±0.2 | 4.8±2.0 | 22.2±0.1 | 4.8±0.9 | 6.1±0.8 | 3.8±0.3 | 9.5±0.2 | 18.7±0.7 | 17.2±0.6 | 3.9±0.2 | 2.8±0.1 | 4.0±0.7 | 13.5±0.4 | 12.2±0.3 | 3.5±0.2 |
| *E. coli (026)* | Native strain | 19.9±0.7 | 18.3±1.3 | 21.7±1.9 | 23.3±0.5 | 19.4±0.9 | 25.7±0.1 | 17.6±0.8 | 25.8±0.7 | 24.5±0.4 | 23.4±0.7 | 14.4±0.4 | 16.9±1.2 | 22.1±1.8 | 21.5±0.9 | 22.6±1.4 |
| *E. coli (0157)* | PTCC 1276 | 18.4±0.8 | 22.3±0.7 | 27.5±0.4 | 20.3±0.1 | 15.3±0.3 | 24.3±0.1 | 19.5±0.5 | 27.0±0.2 | 24.6±1.9 | 20.7±0.6 | 12.9±0.9 | 14.0±0.6 | 24.0±0.7 | 19.0±0.5 | 18.8±0.5 |
| *E. faecalis* | PTCC 1394 | 9.0±0.0 | 6.0±0.9 | 24.6±0.7 | 10.6±0.3 | 8.6±0.1 | 12.2±0.6 | 7.3±0.5 | 20.2±1.0 | 22.3±0.9 | 9.0±0.8 | 8.9±0.8 | 10.1±0.8 | 14.8±0.2 | 20.2±1.1 | 18.1±0.7 |
| *K. pneumoniae* | PTCC 1053 (ATCC 10031) | 8.6±0.7 | 4.0±0.0 | 20.0±0.2 | 7.8±0.1 | 6.0±0.3 | 10.9±0.1 | 3.1±0.9 | 21.0±0.6 | 27.8±0.2 | 7.9±0.1 | 4.0±1.0 | 12.0±0.8 | 15.0±0.3 | 15.3±0.4 | 17.6±0.3 |
| *L. monocytogenes* | PTCC 1163 | 22.8±1.2 | 21.0±1.0 | 23.5±0.8 | 17.7±0.6 | 20.7±0.7 | 22.8±1.3 | 22.6±0.9 | 28.3±1.1 | 29.4±0.7 | 22.2±0.8 | 21.9±0.5 | 20.5±0.6 | 22.6±0.8 | 19.6±1.4 | 21.7±0.7 |
| *P. aeruginosa* | PTCC 1181 | 11.3±0.8 | 8.5±0.6 | 19.0±0.0 | 7.0±0.0 | 10.3±0.5 | 5.0±0.8 | 10.3±0.7 | 21.8±1.2 | 24.0±0.0 | 6.0±0.0 | 0.0±00 | 8.5±0.6 | 18.0±0.0 | 19.0±0.8 | 20.3±0.5 |
| *S. marcescens* | PTCC 1187 (Native strain) | 10.5±0.5 | 11.2±0.8 | 17.7±0.5 | 9.4±0.6 | 6.0±0.3 | 5.6±0.6 | 6.3±0.5 | 20.8±0.7 | 19.8±0.6 | 12.5±0.9 | 9.5±0.5 | 11.2±0.8 | 19.7±0.5 | 22.4±0.6 | 12.0±0.0 |
| *S. saprophyticus* | PTCC 1440 (CIP76.125) | 9.5±0.2 | 14.2±0.6 | 21.5±1.1 | 14.4±0.9 | 16.2±1.2 | 4.0±0.5 | 11.3±0.9 | 25.6±0.7 | 21.2±0.8 | 9.8±0.9 | 12.3±0.7 | 5.0±0.4 | 11.5±1.1 | 24.4±0.9 | 19.2±1.2 |
| *S. typhimurium* | ATCC 14028 | 5.3±0.2 | 5.4±0.2 | 24.1±0.6 | 4.6±0.3 | 4.9±0.4 | 4.8±0.2 | 5.3±0.7 | 21.1±0.6 | 23.1±0.9 | 4.4±0.5 | 2.0±0.0 | 4.2±0.6 | 15.9±0.2 | 18.4±0.3 | 13.8±0.1 |
| *S. flexneri* | PTCC 1234 (NCTC 8516) | 8.8±0.7 | 9.0±0.0 | 20.5±0.8 | 11.5±0.9 | 7.5±0.5 | 4.0±0.3 | 7.9±0.6 | 24.0±04 | 28.0±0.2 | 4.5±0.8 | 8.8±0.7 | 7.9±0.6 | 10.5±0.8 | 19.5±0.9 | 7.5±0.5 |
| *S. mutans* | PTCC 1683 (ATCC 35668) | 9.9±0.6 | 8.4±0.9 | 19.8±0.4 | 3.0±0.0 | 7.6±0.7 | 9.3±0.9 | 5.0±0.0 | 21.6±0.5 | 22.0±0.3 | 6.4±0.9 | 3.0±0.0 | 8.4±0.9 | 12.8±0.4 | 21.3±0.8 | 7.6±0.7 |
| *S. aureus* | ATCC 25923 | 13.4±0.3 | 15.7±0.5 | 20.6±0.2 | 4.8±0.1 | 5.3±0.5 | 7.5±0. 9 | 4.1±0.2 | 19.6±0.7 | 23.0±0.6 | 5.1±0.6 | 6.0±0.3 | 4.1±0.3 | 10.3±0.5 | 14.7±0.2 | 8.0±0.0 |

*Values are mean ± standard error. S (strong, r ≥ 20 mm), M (moderate, r < 20 mm and >10 mm), and W (weak, r ≤ 10 mm). CIP: Collection of Bacteries de l'Institute Pasteur, Paris, France. ATCC: American Type Culture Collection, Virginia, USA. NCTC: National Collection of Type Cultures, London, UK. PTCC: Persian Type Culture Collection, Tehran, Iran.*

**Table 5:** The Susceptibility of isolated yeasts by disc diffusion method.

| Antifungals | AS-1 | AS-12 | AS-23 | AS-27 | AS-28 | AS-29 | AS-37 | AS-38 | AS-41 | AS-46 |
|---|---|---|---|---|---|---|---|---|---|---|
| Clotrimazole | S | S | S | S | S | S | S | S | S | S |
| Fluconazole | I | S | S | S | S | R | I | I | S | S |
| Griseofulvin | S | S | S | S | S | S | R | S | S | I |
| Ketoconazole | S | S | S | S | S | S | S | S | S | S |
| Miconazole | I | S | S | S | S | I | S | S | S | I |
| Terbinafine | I | S | S | S | S | S | R | I | S | I |
| Amphotericin B | S | S | S | S | S | I | S | S | S | S |
| Voriconazole | S | S | S | S | S | I | R | S | S | S |
| Nystatin | I | S | S | S | S | I | I | S | S | R |
| Flucytosine | I | S | S | S | S | R | S | I | S | I |
| Itraconazole | S | S | S | S | S | S | S | S | S | S |

*S: Sensitive; I: Intermediate; R: Resistant. The criteria of the susceptibility determination mentioned in Table 1.*

non-pathogenic and non-toxic, resistant to low pH and to bile salts, to survive in the gastrointestinal tract and also must be able to proliferate and colonize in the digestive tract (49). Some yeasts and bacteria as probiotic microorganisms are able to colonize in the lower intestine and improve gastrointestinal health and so enhance immune function (50, 51). To date, well-known probiotic yeasts and bacteria are exerted to beverages and dairy products. However, due to the important role of probiotics in food industry, the identification and application of new microorganisms with novel probiotic properties is a necessity. Precise recognition of yeast isolates is a major part of detecting novel yeast species. Traditionally, identification and classification of yeasts are performing through imprecise phenotypic methods by assessing morphological, physiological and biochemical characteristics. Furthermore, many experiences and proficiency are required in the performance and assessment of yeast strains (52). There are different new identification methods such as commercially identification kits, simplified identification method (SIM) (53), pulsed-field gel electrophoresis (PFGE, karyotyping) (54), restriction enzyme analysis (RFLP) (17), PCR-based techniques (Ribotyping, Randomly Amplified Polymorphic DNA (RAPD) analysis) (55) and sequencing of ribosomal RNAs (56). Among the various methods the intragenic transcribed spacer-PCR (ITS-PCR) was used in this study for molecular identification of isolated yeasts. This method is based on the PCR amplification of coding sequence located between conserved genes encoding the 18S and 28S rRNA using two specific PCR primer sets (57). Because of high degree of sequence and length variation of ITS-PCR-amplified 18S-28S rDNA sequences at the genus and species levels, the 18-28S rDNA targeted PCR assessment can be considered as a reliable identification tool for new species identification (24).

In our study yeasts in the Iranian dairy products showed notable diversity, including; 16 strains of *P. kudriavzevii*, 2 strains of *P. jaroonii*, 1 strain of *P. deserticola*, 4 strains of *P. membranifaciens,* 2 strains of *P. manshurica*, 2 strains of *S. cerevisiae*, 2 strains of *C. xylopsoci*, 1 strain of *C. krusei*, *C. pseudolambica*, *C.*

*ethanolica*, *C. tropicalis*, *C. albicans*, *C. dubliniensis*, *C. thaimueangensis,* 8 strains of *K. marxianus* and 1 strain of *K. dobzhanskii*. Also, Senses-Ergul *et al.* isolated 22 yeast strains from foods and characterized them by traditional and molecular techniques. They showed that isolated yeast strains that grouped in 12 species belonging to 11 genera as follows: *C. parapsilosis*, *R. mucilaginosa*, *D. hansenii*, *C. humicolus*, *C. albidus*, *Aureobasidium spp*., *H. valbyensis*, *M. pulcherrima*, *L. thermotolerans*, *P. anomala*, *G. candidum* and *Y. lipolytica* (16). In another study, Kumura *et al.* isolated 95 yeast strains from 4 types of commercial blue cheese and classified into 5 species. They revealed that *D. hansenii* was frequent species in 3 types of cheese and other species were found only in one type of cheese (9). Also, EL-Sharoud *et al.* studied the diversity and ecology of yeasts associated with traditional Egyptian dairy products by using molecular techniques in yeast identification such as RFLPs of the ITS-5.8S rDNA region. The yeast isolates were identified as *I. orientalis* (13 isolates), *C. albicans* (4 isolates), *C. lusitaniae* (*C. lusitaniae*) (9 isolates), *K. ohmeri* (*P. ohmeri*) (1 isolate), *K. marxianus* (6 isolates), and *C. catenulate* (7 isolates) (58).

Furthermore, numerous studies showed that more than 23 different yeast species existed in kefir grains and fermented beverages and the major species were *S. cerevisiae*, *S. unisporus*, *C. kefyr*, and *K. marxianus ssp. marxianus* (59, 60). Our results suggested that Iranian dairy products including cheese and yoghurt possess great biodiversity of yeasts that may be considered as potential probiotics.

Moreover, in the current study, 24 isolates showed high acid and bile resistance phenotypes and were appropriate for further probiotic assessments. Fifteen yeast strains selected from previous test represent moderate to remarkable inhibitory role against the indicator bacteria (Table 3).

Likewise, Živković *et al.* showed that dairy yeast isolates exhibit strain-specific probiotic properties. As well two strains of *K. lactis* ZIM 2408 and ZIM 2453 grew appropriately in the complex colonic medium during 24 h of cultivation, while *T. delbrueckii* ZIM 2460 was the most resistant isolate in chemically-simulated conditions of gastric juice and upper intestinal tract (61). Moreover, Psomas *et al.* showed that most of the yeast strains such as *I. orientalis, C. parapsilosis* and *C. albicans*, two strains of *D. hansenii*, one strain of *K. marxianus* and one strain of *K. lactis* survived satisfactory at pH 2.0 and Oxgall 0.3% after 72 h (34). In another study investigators isolated 34 yeast strains from kefir that were able to grow in the presence of 0.5 % (w/v) bile salts in the culture medium and exhibited survivals between 50% and 90% after 3 h incubation at pH 2.5 (62).

Additionally, in our study after antibacterial screening of selected isolates, 5 yeast strains including AS-12, AS-23, AS-27, AS-28 and AS-41 passed all tests and presented suitable probiotic properties (Table 5), thus we can optimistically classify these benign yeast strains as probiotic microorganisms.

Based on numerous studies findings, different yeast species such as *S. cerevisiae*, *S. boulardii*, *D. hansenii*, *T. delbrueckii*, *K. lactis*, *K. marxianus*, *P. fermentase*, *C. krusei* (*I. orientalis*) have shown the tolerance to acid and bile similar to the gastrointestinal tract condition, antimicrobial activity against enteropathogens and antifungal susceptibility (9, 63). Unlikely, some opportunistic yeast species such as *Candida* and *Cryptococcus* have been reported to cause yeast infections when the excretion of these yeast populations in the feces increased than $10^6$ cfu/g and causes diarrhea and other gastrointestinal diseases in humans (45, 64-66). Oftentimes, abovementioned inconveniences are associated with individuals who are receiving antibiotics, or who have immunodeficiency problems, or some other inherent disorders. Generally, consumption of foods with yeast contamination merely was not the initiating factor in the gastrointestinal disorders (45). However, recent evidences showed that, in the pleasant circumstances, some yeasts can colonize in the intestinal tract, and contribute to gastroenteritis and some infections (67, 68). It may be concluded from the overall evidences, yeasts demonstrated low risk to consumers as foodborne microorganisms in the gastrointestinal infections and epidemiological statistics on foodborne microbial disease are notable for their absence of any data about yeasts (69, 70). There are well-known yeast strains such as, *S. cerevisiae*, *S. boulardii* and some strains of *Candida*, *Pichia*, and *Kluyveromyces* that used in food industry as beneficial probiotics due to their health benefits (71). In accordance with different stud-

ies, opportunistic yeasts accepted as safe agents in the production of fermented foods but the more investigations about identification and screening of new species in natural edible resources are required.

## Conclusions

Nowadays, there are many interests to the design of functional foods that contain probiotic microbial strains responsible for health benefits in the host. Most of the probiotic microorganisms belong to the genera of lactic acid producing bacteria, but some yeast strains that exist in dairy and fermented products are classified as probiotics. Asides, probiotic effects are strain and species specific and accurate screening is required for selection of truly probiotic yeasts. Hence, numerous investigators are trying to find new strain of yeasts from natural sources with probiotic properties that possess better and useful activities than former probiotics. In this regard, it seems that traditional fermented dairy foods such as various natural cheese and yogurt are good and original resources for finding novel probiotics in particular the safe and beneficial yeasts. Although recent studies proved the health-promoting properties of probiotic yeasts, but it is needed the several investigations for finding the new probiotic yeast strains as new nutraceutical.

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