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Diversity of Yeast Microbiota in Spoiled Iranian Doogh and Effects of *Kluyveromyces marxianus* on its Protein Profiles

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Abstract

Background and Objective: Microbial contamination can cause undesirable changes in food products. In addition to consumer's health, these changes can challenge the product market. Doogh as one of the traditional dairy may be contaminated by a wide range of microbiota. Awareness of the contaminant types can be an accurate strategy to eliminate contaminations. Regarding importance of food safety as well as industry demand, the present study was an attempt to better understanding of the yeast microbiota diversity in spoiled Doogh. Also the effect of predominant yeast metabolites on proteins and peptides profiling of Doogh were investigated. Results demonstrated purposeful industrial strategies to secure quality parameters and boost the shelf life of Doogh.

Material and Methods: Swollen packages of pasteurized Doogh samples were collected from a local dairy plant within one year. Identification was carried out using morphological, biochemical and molecular techniques. Then, protein and peptide profiles of the inoculated Doogh samples with high proteolytic activities of the isolated yeasts were compared with control using SDS-PAGE and MALDI-TOF-MS techniques.

Results and Conclusion: Totally, 13 isolates belonged to two genera of *Kluyveromyces* and *Candida* were isolated and identified. Based on 18S rRNA gene sequencing, eight isolates were identified as *Kluyveromyces marxianus*; four isolates were matched with *Kluyveromyces lactis* and one isolate was identified as *Candida kefyr*. Furthermore, o-phthaldialdehyde assay showed that *Kluyveromyces marxianus* contain the highest proteolytic activity, compared to other identified isolates. Results showed that the activity of *Kluyveromyces marxianus* linked the protein profiles to β-lactoglubolin and α-lactalbumin isoforms and several peptides with molecular weight less than 10 kDa. Small changes in organoleptic properties can directly affect the product marketing and jeopardizes survival of the industries. By increasing necessary measures on the critical points in the production lines, adoption of right strategies to decrease or eliminate contaminants and hence improve the quality of the products is possible.

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1. Introduction

Doogh is a Iranian traditional yoghurt drink, made by mixing of yoghurt with drinking water, salt and herbal essence such as mint (Figure 1) [1]. "Ayran" in Turkey, "Than" in Armenia and "Lassi" in Southern Asia are similar drinks to Doogh; however, they may differ in dilution ratio, rheological characteristics, fat contents and sensory characteristics [2]. Doogh includes health benefits such as improving lactose degradation in people with lactose intolerance, decreasing serum cholesterol levels and inducing the immune system. These advantages have led Doogh to be produced on a large scale in small and large dairy units and highly consumed in Iran [3].

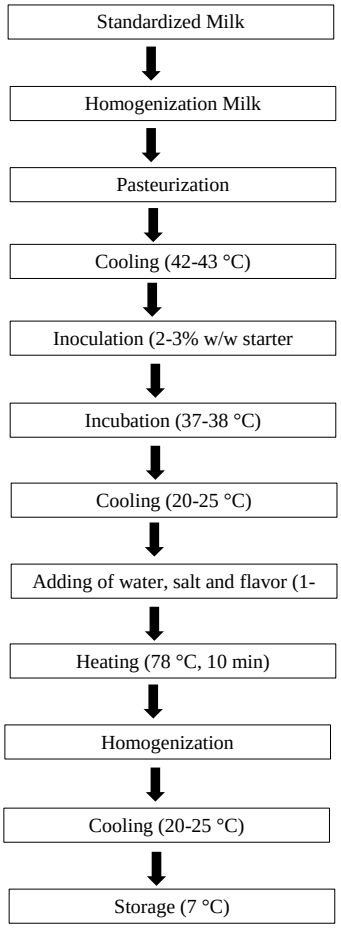


Figure 1. Flowchart of Doogh production flowchart

Types of spoilage can differ widely based on the production practice, formulation, processing, packaging, storage, distribution and refrigeration [4-6]. Dairy products are appropriate environments for the growth and activity of yeasts. Yeasts are essential parts of microbiota in dairy fermented products such as cheese, kurut and koumiss due to the production of desirable aromatic components [7]. Yeasts may produce undesirable flavors and odors, bitterness and swelling in spoiled dairy products as a result of their proteolytic and lipolytic activities [8]. The most important yeast genera causing spoilage in dairies include *Candida*, *Saccharomyces*, *Kluyveromyces* and *Galactomyces* [9,10]. Since processing of dairy products does not completely inactivate the proteases from residual yeasts and milk, milk proteins are broken down into small proteins and peptides and hence the peptide profile changes during the shelf life [11]. Literatures have provided evidence in the field of diversity of bacterial and fungal spoilages in dairy products [12-14]. However, there is a little evidence on the identification and characterization of spoilage microorganisms in Doogh. The present study was the first study on the identification of yeasts isolated from pasteurized Doogh with blowing signs in package as well as using proteomics techniques for analyzing effects of yeast proteases on the peptide profiles of Doogh. Therefore, the aim of this study was to isolate, characterize and identify yeasts that caused package swelling in pasteurized Doogh samples collected from a local dairy plant, Mashhad, Iran, using morphological, biochemical and molecular techniques. Furthermore, proteolytic activity of the isolates was assessed and effects of yeasts with the highest protease activity on Doogh protein profiles were investigated.

2. Materials and Methods

2.1 Doogh sampling

A total of ten pasteurized Doogh packages with swelling condition were collected from a local dairy plant in Mashhad, Iran, from September 2015 to August 2016 within 30-day intervals. To stop changes during transportation, Doogh samples were immediately cooled down to 4 °C, transferred to the laboratory under appropriate conditions using isotherm containers and analyzed rapidly.

2.2 Isolation and primary characterization of yeasts

Yeasts were isolated using yeast extract glucose chloramphenicol (YGC) agar. Briefly, 1 ml of Doogh sample was inoculated into the culture media and incubated at 25 ºC for 3-7 days [15]. Isolated colonies were stained with lacto phenol-cotton blue and investigated microscopically to determine the morphological characteristics such as shape of the cells and budding (mono, bi and multipolar) or fission multiplication. Moreover, growth structures in potato dextrose broth (mucoid sediment, ring or pellicle of yeasts) were assessed [15].

2.3 Physiological and biochemical characteristics of the yeast isolates

Ability of the yeast isolates to grow aerobically and utilize carbon or nitrogen as a sole source of energy were studied. In this study, several carbon sources (glucose, galactose, lactose, maltose, sucrose, raffinose, soluble starch, cellobiose, trehalose, xylose, glycerol, mannitol, ethanol and methanol) were used. The nitrogen sources included nitrate and L-lysine. Assimilation assessments were carried out using plate method in two replications, where a set of plates containing various carbon or nitrogen sources in carbon or nitrogen basal agar media were inoculated by the yeast isolates [15]. Moreover, fermentation abilities of the yeast isolates (anaerobic assimilation) for some carbohydrates (glucose, lactose, maltose, raffinose, galactose, sucrose, mannitol, cellobiose and xylose) were investigated using Durham glass tubes. Quantity of the tested carbohydrates was 2%. Fermentation abilities were investigated at the frequent intervals within three weeks for accumulated gases in Durham tubes [15]. Moreover, other physiological characterizations were carried out to examine the osmotic pressures of the yeast isolates such as growth abilities on 50, 60% w v^{-1} glucose, 10% NaCl and 5% glucose. In addition,

growth ability at 30 and 37 °C, hydrolysis of urea, starch formation and tolerance to 1% acetic acid were assessed [15].

2.4 Molecular characterization of the isolates

2.4.1 Genomic DNA extraction of the yeasts

DNA was extracted from fresh colonies via the CTAB (cetyl trimethyl ammonium bromide) method as published previously [16]. Purity of the DNA was quantified at 260 nm using UV-visible double beam spectrophotometry (Shimadzo UV 1800, Japan) and the DNA was stored at -20 °C until use [17].

2.4.2 Identification of the yeast isolates using PCR amplification and sequencing methods

For the identification of the yeast isolates at species level, amplification of 18S rRNA fragments of the fungi (603 bases) was carried out using the following primers of forward NS3 (5′-GCAAGTCTGGTGCCAGCAGCC-3′) and reverse NS4 (5′-CTTCCGTCAATTCCTTTAAG-3′). Each PCR reaction (20 µl) included 1 µl of 50-ng genomic DNA as the template, 0.5 µl of 10-pmol primers (Dena Zist Asia, Iran), 8 µl of RNase-free water and 10 µl of master mix red solution (PCR reaction buffer, $MgCl_2$, dNTP and Taq DNA polymerase) (Ampliqon, Denmark). The PCR program was set in thermal cycler as 180 s of initial denaturation at 94°C, followed by 15 amplification cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s with decreasing 0.5 °C per cycle and elongation at 72 °C for 60 s. Then, 15 amplification cycles were carried out as denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and elongation at 72 °C for 60 s. The final elongation was set as 72 °C for 5 min. Amplicons were separated on 1% agarose gel using electrophoresis method, stained with DNA Gold Viewer Dye and visualized under UV light using gel documentation device [18]. For purifying and sequencing the amplified DNA fragments, amplicons were loaded on 1.5% agarose gels with 100-bp DNA ladder. Then, DNA bands were cut off and extracted using gel extraction kit before sequencing to investigate the closest homologous species. Nucleotide sequences of 18S rRNA fragments were searched in GenBank (National Centre for Biotechnology Information, Rockville Pike, Bethesda, USA) using BLAST similarity search of sequences in the database [19]. Nucleotide sequences were aligned and compared with each other and the phylogenetic tree was generated using neighbor-joining method with the maximum composite likelihood model and MEGA Software v.7.0 (Biodesign Institute, Tempe, USA) [20].

2.5 Assessment of the yeast proteolytic activity

Proteolytic activity of the yeasts was investigated in duplicate by assessing total contents of the released peptides and amino acids using o-phthaldialdehyde (OPA) to quantify free-NH_3 group due to the measurement of the absorbance at 340 nm [21].

2.6 Precipitation of water-soluble proteins of samples

First, yeast with the highest proteolytic activity index was selected for further analysis. Then, the yeast was inoculated to the pasteurized Doogh sample followed by the incubation at 30 °C for 48 h. Inoculated samples and non-inoculated samples (controls) were centrifuged at 18,000× g for 18 min at 4 °C. To precipitate the proteins, samples were mixed with 70% ammonium sulfate solution and agitated at 4 °C overnight. Then, samples were centrifuged (Heraeus Biofuge Pico, England) at 10000× g for 20 min at 4 °C. Pellets were dissolved at 50% of the initial volume in phosphate buffer (pH 7) (Merck, Darmstadt, Germany) and dried using freezer dryer. These were stored at -20 °C until use [11].

2.7 Purification and concentration of the peptides using centrifugal ultrafilters

To remove inhibitory substances of majorly salts and to concentrate peptides smaller than 30 kDa, protein sample was used in Amicon centrifugal ultrafilter units (Sigma-Aldrich, Darmstadt, Germany) with cutoff ranged 3-30 kDa. For this purpose, 10 mg of the protein sample were dissolved in 1 ml of Milli Q water [11]. Based on the protocol of centrifugal ultrafilters, protein solution was injected into the ultrafilters and then centrifuged at 5000× g for 45 min at 10 °C.

2.8 Separation of the proteins using SDS- PAGE method

Protein samples were analyzed based on their molecular weights using SDS-PAGE and 16% tricine gel, anode buffer (1 M tris base, pH 8.9) and cathode buffer (1 M tris base, pH 8.3, 1 M tricine, 1% SDS) to separate proteins. Samples with protein concentrations of 5 mg ml^{-1} were diluted using sample buffer (0.5 M tris-HCl, pH 6.8, 10% glycerol, 10% SDS, 5% 2-β mercaptoathanol and 1% bromophenol blue) in the ratio of 4:1 (buffer:sample) and incubated at 95 °C for 5 min. Aliquots of 15 µl of each sample were injected into each well of the gel. Electrophoresis was carried out using constant voltage of 30 V for the first 20 min and then 100 V for 90 min. Proteins were stained with Coomassie brilliant blue R-250 for 1 h and decolorization was carried out with two types of solutions. The first solution included 90 ml of 50% methanol and 10 ml of acetic acid and the second solution included 90 ml of 7% methanol and 10 ml of acetic acid. The gel was stored in each solution for 1 h [22]. The most intense bands were cut and hydrolyzed with trypsin. Then, hydrolyzed proteins were analyzed using MALDI-TOF-MS (AB SCIEX TOF/MS5800, Milan, Italy).

2.9 Evaluation of the protein and peptide profiles using MALDI-TOF-MS method

For MALDI-TOF-MS (matrix-assisted laser desorption/ionization-time of flight- mass spectrometry) analysis,

digested samples with C18 Zip-Tip (Merck, Darmstadt, Germany) that were preconditioned with CAN of water (50%) and TFA (1%) (LC-MS grade; Sigma-Aldrich, Darmstadt, Germany) were spotted on MALDI plate mixed with α-cyano-4-hydroxy-cinnamic acid (CHCA) (Sigma-Aldrich, Darmstadt, Germany) in 50% ACN containing 0.1% of TFA (1:2 ratio) as matrix solution and then air dried. The MALDI-TOF-MS analysis was carried out using mass spectrometer and nitrogen light in a form of reflector positive and postponed extraction (140 ns). Speed voltage was adjusted to 20 kV. For each sample, 1000 singular spectra were spontaneously produced from various spot statuses in a mass area of 800-3000 Da and then summed up [23].

3. Results and Discussion

3.1 Morphological identification of the yeast isolates

In this study, a total of 13 yeast isolates were used based on their differences in colony structures, cell shapes and growth morphologies. All the isolates were biochemically characterized. Morphological characteristics of the isolates are summarized in Table 1. Almost in all isolates, colonies appeared small and creamy to pale creamy that were somewhat further grew in solid media. Margins were smoothing glabrous to filamentous and cell shapes were often spherical to elongate. To identify yeasts, biochemical characteristics of the isolates were compared to standard reference on taxonomy and identification of the yeasts [15]. Study of the microbial diversity in dairy products has traditionally relied on culture-based and physiological assays, which can lead to ambiguities. Sometimes, it has been reported that the activity of microorganisms is different in the laboratory than their activity in real habitats. It is noteworthy that the biochemical and physiological characteristics of yeasts can be used in assessing biotechnological characteristics of these microorganisms, especially fermenting species, in various industries [10,24-27].

3.2.1 Assimilation assessment

All the yeast isolates assimilated glucose, galactose, lactose, maltose, sucrose, raffinose, cellobiose, xylose and mannitol. None of the isolates utilized methanol as a sole carbon source. Furthermore, all of the isolates poorly assimilated glycerol, soluble starch and ethanol. Consumption of trehalose by the isolates of N39Y, N40Y and N41Y was negative (Table 2).

Table 1. Morphological characterization of the yeast isolates

Isolates	Colony on solid medium	Cells shape	Growth in broth media
N4Y, N5Y, N15Y, N17Y, N18Y, N19Y, N26Y, N39Y, N40Y, N41Y, N65Y	Smooth surface, raised, cream-color	Globose, ovoidal,	White precipitate
N55Y, N56Y, N58Y	Smooth surface, raised, pale cream-colored, fringed with filaments	Ovoidal to elongate	White precipitate and precipitate pellicles

Table 2. Assimilation of carbon sources using selected yeast isolates

Isolates	Glucose	Galactose	Maltose	Sucrose	d-xylose	D-mannitol	Cellobiose	Raffinose	Lactose	Trehalose	Glycerol	Soluble starch	Ethanol	Methanol
N4Y	+	+	S	+	W	+	+	+	+	+	W	W	W	-
N5Y	+	+	+	+	W	+	+	+	+	+	W	W	W	-
N15Y	+	S	S	S	W	S	S	+	S	+	W	W	W	-
N17Y	+	+	+	+	W	+	+	S	S	+	W	W	W	-
N18Y	+	+	+	+	W	+	+	S	S	+	W	W	W	-
N19Y	+	+	S	S	W	S	S	S	S	S	W	W	W	-
N26Y	+	+	W	+	W	+	+	+	S	+	W	W	W	-
N39Y	+	+	S	S	+	W	W	W	S	-	W	W	W	-
N40Y	+	+	S	S	+	W	W	W	S	-	W	W	W	-
N41Y	+	+	S	S	+	W	W	W	S	-	W	W	W	-
N55Y	+	+	+	+	+	+	+	+	+	+	W	W	W	-
N56Y	+	W	W	W	+	+	+	+	++	+	W	W	W	-
N65Y	+	+	+	+	+	+	+	+	+	+	W	W	W	-

Symbols: (+) positive; (-) Negative; (S) Strong positive; (W) Weak positive

3.2.2 Fermentation assessment

All of the isolates were able to ferment glucose, galactose and sucrose. Most of the isolates could ferment lactose and raffinose, Maltose was fermented only by the isolates of N19Y, N39Y, N41Y, N55Y and N65Y. None of the isolates fermented cellobiose, xylose and mannitol (Table 3).

3.2.3 Additional biochemical assessments

Based on Table 4, all of the isolates were able for grow at 30 and 37 °C. Isolates performed weakly for L-lysine assimilation, starch formation and growth in media containing 50% glucose. None of the isolates could assimilate nitrate, grow in media containing 10% NaCl and 5% glucose, hydrolyze urea and grow at media containing 1% acetic acid. Furthermore, only the isolates of N15, N17 and N39 grew up in 60% glucose poorly. The other isolates could not tolerate this media.

3.3 Molecular identification of the yeast isolates

Yeast isolates with various morphological and biochemical characteristics were identified using sequencing of the 18S rRNA gene. Amplicons with sizes of 603 bp were achieved for all of the isolates (Figure 2A). A total of 13 isolates were identified at the species level after comparing 18S rRNA gene sequences with the available sequences in databases of NCBI. Phylogenetic relationships between the species of the yeast isolates are shown in Figure 2B. Based on the sequencing results, eight isolates (N4Y, N5Y, N15Y, N17Y, N39Y, N41Y, N55Y and N65Y) were classified as *K. marxianus* out of 13 isolates. Four isolates (N19Y, N26Y, N40Y and N56Y) were detected as *K. lactis* and the last isolate of N18Y was identified as *C. kefyr* (Table 5). The *K. marxianus* is generally recognized as the GRAS and is abundant in fermented foods [28]. It includes a high growth rate and is able to utilize and ferment various carbon sources such as lactose. The microorganism has been separated from the native fermented dairy products such as kefyr, koumiss, suusac and gariss [28]. In this study, *K. marxianus* consumed lactose in various ways. Other studies have shown that subspecies of *K. marxianus* assimilate lactose changeable [29]. Findings of this study on the assimilation and fermentation of glucose, galactose, mannose, maltose, xylose, lactose, arabinose, glycerol and sucrose by *K. marxianus* were similar to those of Goshima et al. (2013) and Ghosh (2011) [27,30]. Another study showed that two species of *K. marxianus* and *K. lactis* could consume non-fermentable carbon sources such as ethanol, glycerol and lactic acid [31].

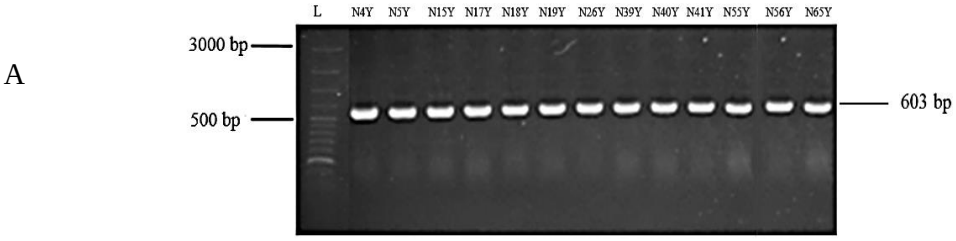
In this study, the two species consumed ethanol and glycerol weakly. The potent capability of *K. marxianus* and *K. lactis* to use lactose is considered as a key characteristic involved to their growth in milk products and these species likely play roles with lactic acid bacteria in initial fermentation of milks [27].

Table 3. Fermentation abilities of the selected yeast isolates

Isolates	Glucose	Galactose	Maltose	Sucrose	Dxylose	D-mannitol	Cellobiose	Raffinose	Lactose
N4Y	+	+	-	+	-	-	-	-	+
N5Y	+	+	-	+	-	-	-	-	+
N15Y	+	+	-	+	-	-	-	+	+
N17Y	+	+	-	+	-	-	-	+	-
N18Y	+	+	-	+	-	-	-	+	-
N19Y	+	+	+	+	-	-	-	+	-
N26Y	+	+	-	+	-	-	-	+	+
N39Y	+	+	+	+	-	-	-	-	+
N40Y	+	+	-	+	-	-	-	-	+
N41Y	+	+	+	+	-	-	-	+	+
N55Y	+	+	+	+	-	-	-	+	+
N56Y	+	+	-	+	-	-	-	-	+
N65Y	+	+	+	+	-	-	-	+	+

Table 4. Additional biochemical assessments using the yeast isolates

Isolates	Assimilation of L-lysine	Assimilation of Nitrate	Growth at 30°c	Growth at 37°c	Starch formation	Growth inGlucose 50%	Growth in Glucose 60%	Growth in NaCl 10%+ glucose 5%	Hydrolysis of urea	Growth at media contain Acetic acid 1%
N4Y	W	-	+	+	W	W	-	-	-	-
N5Y	W	-	+	+	W	W	-	-	-	-
N15Y	W	-	+	+	W	W	W	-	-	-
N17Y	W	-	+	+	W	W	W	-	-	-
N18Y	W	-	+	+	W	W	-	-	-	-
N19Y	W	-	+	+	W	W	-	-	-	-
N26Y	W	-	+	+	W	W	-	-	-	-
N39Y	W	-	+	+	W	W	W	-	-	-
N40Y	W	-	+	+	W	W	-	-	-	-
N41Y	W	-	+	+	W	W	-	-	-	-
N55Y	W	-	+	+	W	W	-	-	-	-
N56Y	W	-	+	+	W	W	-	-	-	-
N65Y	W	-	+	+	W	W	-	-	-	-



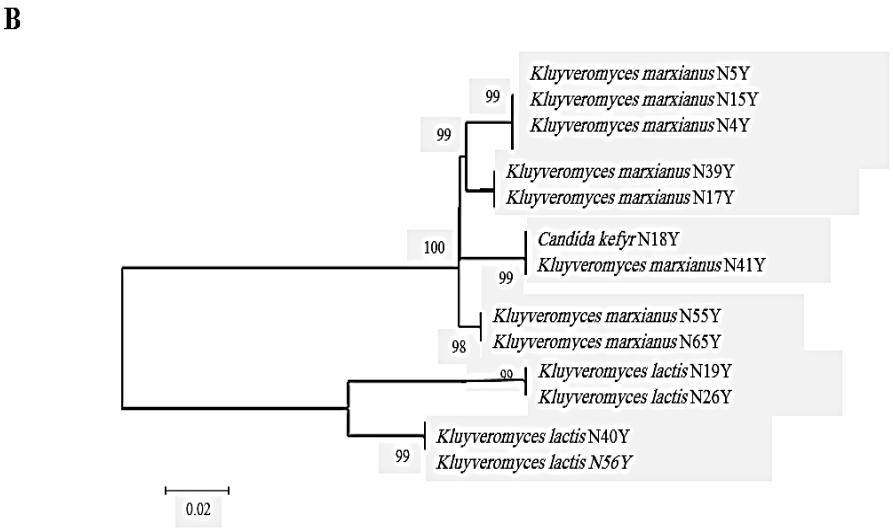


Figure 2. A) Amplification of 18S rRNA gene of the isolated yeasts from Doogh samples; L, molecular ladder; Lanes N4Y–N65Y, PCR products. B) Phylogenetic tree based on 18S rRNA sequence analysis of the isolated strains constructed by the neighbor-joining statistical method

Table 5. The identified yeast isolates using sequencing method

Isolates	Characteristics	Percentage similarity (%)	Accession number
N4Y	*Kluyveromyces marxianus* strain AS4	97	KM516767.1
N5Y	*Kluyveromyces marxianus* strain CCT7735	97	CP009307.1
N15Y	*Kluyveromyces marxianus* strain CBS 4857	97	CP015058.1
N17Y	*Kluyveromyces marxianus* DMKU3-1042	99	AP012217.1
N18Y	*Candida kefyr*	99	M60303.1
N19Y	*Kluyveromyces lactis* strain JCABKL22	99	KU058163.1
N26Y	*Kluyveromyces lactis* strain JCABKL7	98	KU058157.1
N39Y	*Kluyveromyces marxianus* strain Y9	95	KM516767.1
N40Y	*Kluyveromyces lactis* strain JCABKL6	98	KU058156.1
N41Y	*Kluyveromyces marxianus* strain Y12	99	JF715169.1
N55Y	*Kluyveromyces marxianus* strain PAZ	99	KF964550.1
N56Y	*Kluyveromyces lactis* strain JCABKL8	98	KU058158.1
N65Y	*Kluyveromyces marxianus* strain Y15	98	JF715170.1

Furthermore, it has been reported that *K. marxianus* and *K. lactis* are involved in formation of aroma and increase of the concentrations of odorous compounds by the production of ethanol and acetaldehyde [27]. In this study, dominance of *K. marxianus* and *K. lactis* was similar to that of other studies. Abdel Rahman et al. stated spreads of these two yeast species in the fermented camel milk [32]. Bai et al. showed the predominance of these species in fermented milks prepared in Tibet [33]. In the present study, the two identification methods were similar for all of the strains, except the isolate of N18Y that identified as *K. marxianus* using biochemical methods while identified as *C. kefyr* using molecular methods. Misidentification usually occurs due to several reasons; first, newly introduced yeast species may not currently be available in databases; second, variability of particular strains to specific biochemical assays may occur and third, yeast species sometimes include similar assimilation profiles [34]. Previous literatures have shown that *C. kefyr* is a yeast with teleomorph cycle similar to *K. marxianus*; therefore, these two yeasts include similar biochemical profiles. In previous studies, *C. kefyr* was isolated from traditional yoghurts, traditional fermented camel milks, Ethiopian naturally fermented buttermilks and Zimbabwean fermented milks [34-37]. However, biochemical assays alone are not reliable as identification techniques for all yeast species from various environments [37] but when combined with molecular methods, they allow accurate, reliable and rapid identification of microorganisms [38].

3.4 Analysis of proteolytic activity of the yeasts

To verify potential roles of the yeast species in protein proteolysis of Doogh, isolated strains were assessed for their abilities to hydrolyze milk proteins using o-phthaldialdehyde index. Because of high similarities between the 12 genera of *Kluyueromyces*, only one isolate from each species *(K. marxianus* strain CBS 4857 and *K. lactis* strain JCABKL22) and *Candida kefyr* M60303.1, were used for the investigation. Results showed that , the Doogh sample inoculated with *K. marxianus* included significantly high o-phthaldialdehyde index compared to other species ($p < 0.05$). Furthermore, *K. marxianus* strain CBS 4857 with 0.564 µg µl^{-1} concentration showed the highest concentration of free peptides. However, species of *K. lactis* and *C. kefyr* with free peptide concentrations of respectively 0.351 ±0.002 and 0.359 ±0.005 µg µl^{-1} showed more proteolytic activities than that the control did (0.175 µg µl^{-1}). Due to the highest proteolytic activity of *K. marxianus*, only Doogh samples inoculated with *K. marxianus* and control were subjected to peptide profiling.

3.5 Peptide profiling of the Doogh samples by SDS-PAGE and MALD-TOF-MS

Follow the ultrafiltration to remove proteins larger than 30 kDa, primary patterns of the peptides of control and inoculated doogh sample with *K. marxianus* were investigated using SDS-PAGE (Figure. 2). Figure 2 shows the peptide profiles of Doogh samples from two major zones of ~18 and ~14 kDa. Only in the sample inoculated with *K. marxianus*, weak bands in lower section of the gel (nearly 4-5 kDa) were seen. Presence of very weak bands less than 5 kDa for inoculated doogh samples verified further decomposition of the protein molecules and formation of short chain peptides with very low concentrations in this region [39]. Major differences between the peptide patterns of the sample inoculated with *K. marxianus* included formation of weaker bands with lower densities in regions of 18 and 14 kDa, compared to the control. This occurred due to the proteolytic activity of the sample containing yeasts (*K. marxianus*), which was able to partially decompose milk proteins [40]. In SDS-PAGE, milk proteins were divided into four main regions: caseins (19-25 kDa) and three main whey proteins, β-lactoglobulin (18 kDa), α-Lactoalbumin (14 kDa) and bovine serum albumin (66 kDa). In fermented dairy products such as yoghurt, a clear band of BSA protein is generally not detectable and whey protein accumulation is more evident due to the proteolytic activity of the starter cultures [41,42]. As shown in Figure 3, casein-associated bands were not visible on the polyacrylamide gel. This is possibly due to the proteolytic activity of lactic acid bacteria, which used caseins as the major substrate for nitrogen feeding [41].

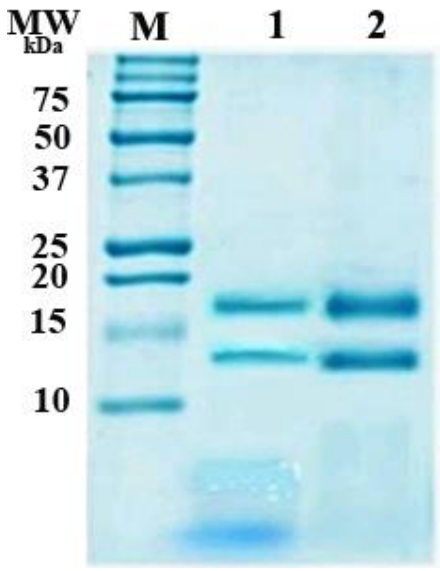


Figure 3. Comparison between the peptide profiles of concentrated fractions from inoculated samples with *K. marxianus* (1) and control sample (2) using marker (M) in trisin SDS-PAGE

In the present study, patterns of protein bands on gel electrophoresis were similar to the patterns presented by Gooding et al. (2014) and Farvin et al. (2010) [41,42]. Based on very low-density, small weak bands in lower portion of the lane in inoculated samples, those bands were not subjected for further analyses. To reveal the identity of two major peptide bands in the two samples, proteomics method was used. First, peptides digested with trypsin and analyzed using MALDI- TOF-MS ranged 800–3000 Da. Figure 4(A, B) demonstrates typical MALDI-TOF-MS spectra of the two bands in inoculated Doogh samples. Most of the signals were detected in a range of 800-2700 Da. In the two spectra of the 18 and 14 kDa bands (4A and 4B, respectively), the most signals were recorded in the range of 800-2700 (m z^{-1}) for spectrum 4(A) as well as 800-2000 (m z^{-1}) for spectrum 4B, which were in the beginning to the middle of the spectra. No detectable signal was observed on the right side of the spectrum. The predominant mass spectra in the peptide profile of 18 kDa band including 837.4 m z^{-1}, 832.3 and 270.2 m z^{-1}. For 14 kDa band, this included 832.3, 842.5 and 1232.6 m z^{-1} (Figure 4). Results from the product ion scans were subjected to MASCOT database search. Based on database search, two types of proteins were predicated, including β-lactoglobulin and α-lactalbumin (Table 6).

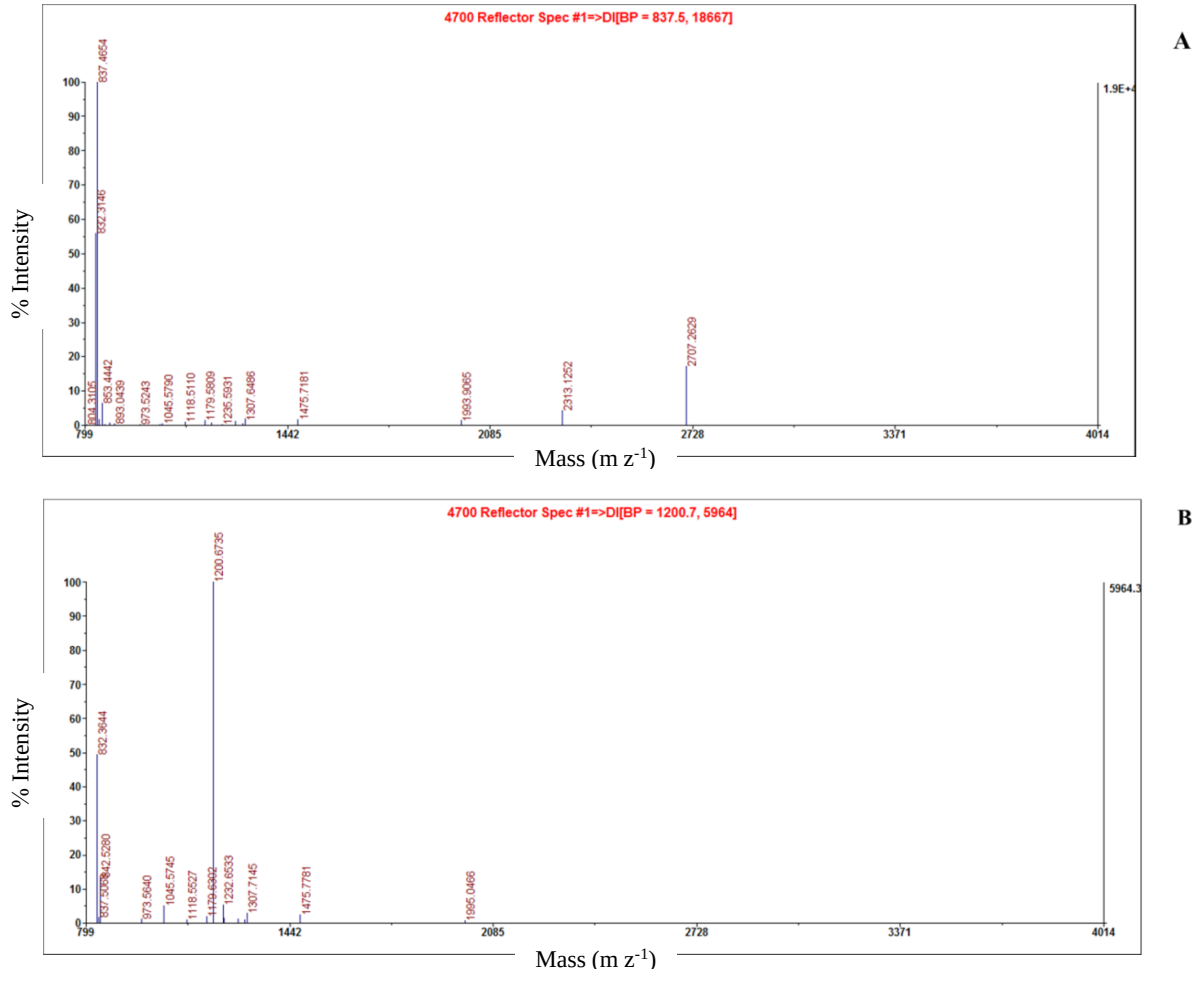


Figure 4. The MALDI-TOF mass spectra of a part of ~14 kDa band (A, α-lactalbumin) and ~18 kDa band (B, β-lactoglobulin) of the inoculated samples in SDS-PAGE gels

Table 6. Identified peptides in the concentrated fractions of 30 KDa from inoculated Doogh samples based on less than MALDI-TOF-MS method

Peptide sequence	Origin	Molecular weight in gel (kDa)
MMSFVSLLLV	α-Lactalbumin	14
AASDISLLDAQSAPLR	β-lactoglobulin	20

Regarding molecular masses, predicated proteins of β-lactoglobulin and α-lactalbumin included similar weights, which were detected in gel-electrophoreses. Predicted poly peptides, which were parts of β-lactoglobulin and α-lactalbumin, were similar to those of previous studies (Table 6) [42,43]. Abilities of yeast proteases to release peptides from food proteins have been reported by several studies [44,45]. Moreover, findings of other studies have verified proteolytic activities in *Kluyveromyces* spp. [46-48]. The β-lactoglobulin and α-lactalbumin, as the major members of whey proteins, were less affected that was due to their spherical structures; therefore, they were more resistance to proteolysis [42]. Hence, *K. marxianus* proteolytic activity led to degradation of stable proteins in Doogh samples of the current study.

4. Conclusion

Secondary contamination and consequently its metabolites cause adverse changes in food products; of which, some changes can be harmful. Regarding business sustainability management, contaminations decrease food marketability and cause great economic losses for the producers of popular dairy drinks in the country. The present study was one of the few studies that have extensively assessed diversity of yeast microbiota in spoiled Iranian Doogh and their effects on protein profiles of the drink using basic and advanced techniques. The current results showed that presence of *K. marxianus* with the highest proteolytic activity affected whey proteins in Doogh. Due to the production of *K. marxianus* proteases, short chain proteins were produced that might be directly related to the development of unpleasant taste in the product. Therefore, in order to purposeful and effective elimination of microbial contaminations, it is necessary to study the critical control points of Doogh production line as well as diversity of domestic species and/or their metabolite to adapt specific hygienic methods for each type of microbe. For bioactivity, development of novel protein hydrolysates in fermented dairy products may be a valuable achievement for food scientists to improve functional products for human health.

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6. Conflict of Interest

The present manuscript, as an original article, has not been submitted to nor is under review in other journals or publishing venues. Authors are not affiliated to organizations with direct or indirect financial interests in the subject discussed in the manuscript

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تنوع میکروبیوتای مخمری در دوغ فاسد ایرانی و اثرات *کلووروما یسس مارکسیانوس* بر پروفایل-های پروتئینی آن

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چکیده

سابقه و هدف: آلودگی میکروبی می‌تواند موجب تغییرات نامطلوب در فرآورده‌های غذایی شود. این تغییرات علاوه بر سلامت مصرف کننده می تواند بازار محصول را به چالش بکشد. دوغ به‌عنوان یکی از فرآورده شیری سنتی ممکن است توسط محدوده وسیعی از میکروب‌ها آلوده شود. آگاهی از انواع آلاینده‌ها می‌تواند یک استراتژی مناسبی برای حذف آلودگی‌ها باشد. با توجه به اهمیت ایمنی مواد غذایی و همچنین تقاضای صنعت، مطالعه حاضر تلاشی برای درک بهتر تنوع میکروبی مخمر در دوغ فاسد بود. همچنین تأثیر متابولیت‌های مخمر غالب بر پروفایل پروتئین‌ها و پپتیدهای دوغ مورد بررسی قرار گرفت. نتایج استراتژی‌های هدفمند صنعتی را برای تضمین پارامترهای کیفیت و افزایش ماندگاری دوغ نشان داد.

مواد و روش ها: بسته های متورم نمونه دوغ پاستوریزه از یک کارخانه لبنیات محلی در طی یک سال جمع آوری شد. شناسایی با استفاده از روش‌های ریخت‌شنلسی[۱]، بیوشیمیایی و مولکولی انجام شد. سپس پروفایل‌های پروتئینی و پپتیدی نمونه‌های دوغ تلقیح شده با مخمرهای جدا شده دارای فعالیت پروتئولیتیکی بالا با نمونه شاهد با استفاده از روش‌های SDS-PAGE و MALDI-TOF-MS مقایسه شد.

یافته‌ها و نتیجه‌گیری: در مجموع، ۱۳ جدایه[۲] متعلق به دو جنس *Kluyveromyces* و *Candida* جداسازی و شناسایی شدند. بر اساس توالی ژن 18S rRNA، هشت جدایه به‌عنوان *Kluyveromyces marxianus* ، چهار جدایه با *Kluyveromyces lactis* و یک جدایه به عنوان *Candida kefyr* شناسایی شدند. علاوه براین، آزمون phthaldialdehyde (OPA)- o نشان داد که *Kluyveromyces marxianus* بالاترین فعالیت پروتئولیتیکی را در مقایسه با سایر جدایه های شناسایی شده دارد. نتایج نشان داد که فعالیت *Kluyveromyces marxianus* پروفایل‌های پروتئینی را به ایزوفرم‌های β-لاکتوگلوبولین و α-لاکتالبومین و چندین پپتید با وزن مولکولی کمتر از ۱۰ کیلو دالتون پیوند می‌دهد.. تغییرات اندکی در خواص حسی می‌تواند مستقیماً بر بازاریابی محصول تأثیر بگذارد و بقای صنایع را به خطر بیندازد. با افزایش تدابیر لازم در نقاط بحرانی خطوط تولید، اتخاذ راهکارهای صحیح برای کاهش یا حذف آلاینده‌ها و در نتیجه ارتقای کیفیت محصولات امکان پذیر است.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

[۱] morphological
[۲] isolate