Volume 10, Issue 4, 2020, 5923 - 5931 ISSN 2069-5837

Biointerface Research in Applied Chemistry

www.BiointerfaceResearch.com

https://doi.org/10.33263/BRIAC104.923931

Original Research Article Open Access Journal

Received: 01.04.2020 / Revised: 20.04.2020 / Accepted: 21.04.2020 / Published on-line: 23.04.2020

Isolation and identification of contaminant microorganisms in industrial and non-industrial Iranian fermented milk (Doogh)

Sadegh Khoshgozaran-Abras [1], Niloofar Fallah [2], Sara Sohrabvandi [2,*], Amir M. Mortazavian [2,*], Hedayat Hosseini [2,*], Reza Mohammadi [3,*], Fatemeh Zendeboodi [4], Roya Bahrami [3], Mohammad Mahdi Gholian [5]

[1]Department of Food Science and Technology, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran

[2]Department of Food Technology, Faculty of Nutrition Sciences and Food Technology/National Nutrition and Food Technology Research Institute, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[3]Department of Food Science and Technology, School of Nutrition Sciences and Food Technology, Kermanshah University of Medical Sciences, Kermanshah, Iran

[4]Student research committee, Department of Food Science and Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition and Food Technology, Shahid Beheshti University of Medical Sciences, , Iran

[5]Grape Processing and Preservation Department, Research Institute for Grapes and Raisin (RIGR), Malayer University, Malayer, Iran

*corresponding authors e-mail addresses: ***sohrabv@sbmu.ac.ir*** | Scopus ID 12793070400

mortazvn@sbmu.ac.ir | Scopus ID 12792054500

hedayat@sbmu.ac.ir | Scopus ID 55445059500

r.mohammadi@sbmu.ac.ir | Scopus ID 36716645500

ABSTRACT

Doogh is a traditional fermented milk based drink, that is a very popular product in Iran, its microbial safety is important. The purpose in this study, 16S based rDNA PCR, cloning and sequencing was to investigated the exploration microbial contaminants in commercial pasteurized Doogh specimens. To isolate and identify the contaminants in 24 different Doogh samples (17 different industrial and 6 different non-industrial samples) Polymerase chain reaction (PCR) was applied. The experimental results revealed that lactic acid bacteria (*Lactobacillus brevis, L. fermentum, L. paracasei* and *L. gallinarum*) gram-positive spore-forming bacteria (*Bacillus anthracis, B. licheniformis.* and *B. subtilis*) and acetic acid bacteria (*Acetobacter indonesiensis* and A. tropicalis) were identified in Doogh samples. Likewise, result obtained from sequencing of D1/D2 26S rDNA determined the presence of different yeasts including *Saccaromyces sp.* (*S. unisporous, S. pastorianus*) *Kazachstaia sp.* (*K. servazzi, K. unispora*) *Pichia fermentans, Galctomyces candidum*, and *Cryptococcus magnus.* The results demonstrated that some types of bacteria and yeasts and molds were identified as the main contaminants in Iranian Doogh.

Keywords: *Doogh; Polymerase chain reaction; Contaminant microorganism; Bacillus; Acetobacter; yeasts.*

1. INTRODUCTION

Doogh is a “composite milk product”, which could be easily obtained as a result of mixing yoghurt with potable water along with salt (sodium chloride) or direct fermentation of milk (mixed with potable water and salt). *Streptococcus thermophilus* and *Lactobacillus delbrueckii ssp. Bulgaricusare* are used as the typical starter microorganisms in the direct fermentation method of doogh production. Doogh could be marketed as plain, flavored, carbonated or non-carbonated [1]. Doogh could be bottled and marketed with or without heat treatment (90˚C, 10-15 min) [2].

Microbial contamination, especially in dairy based products, is a challenging issue. Therefore, the methods, culture based or culture independent, for characterization of contaminants have been developed. Wherein, various techniques employing DNA assays have been recognized as the most precise and time saving for the detection of specific microbes in food products [3-5].

Polymerase chain reaction-based assays (PCR) is a unique possible method for the precise determining of different microorganisms as it is possible to amplify different genomic with PCR method. One of the widely amplified and sequenced genomic is 16S rRNA (16S rDNA), as a specific tool for assessing microorganisms. Since Doogh is a traditional and the most consumed soft drink in Iran, its microbial safety is important. Therefore, in the recent study the PCR method, cloning and sequencing were applied to identify microbial contaminants in Iranian industrial and non- industrial produced Doogh samples [6-8].

2. MATERIALS AND METHODS

2.1. Samples.

51 Industrial Doogh samples from 17 different brand names (all available brand names in Iran market), and 6 non-industrial Doogh samples which were all freshly produced within a week, were collected randomly and transferred to the analytical laboratory in sterile condition and cold chain. All samples were stored at 4°C prior to analysis.

2.2. Conventional identification.

Sterile NaCl solution (0.85%) was used to prepare decimal dilutions of milk samples. Standard plate count method was used for each serially prepared aliquot 1 to 10 samples on Brom Cresol Purple (BCP agar, Nissui Pharmacy, Tokyo, Japan). For enumerating the total Lactic acid bacteria, the inoculated plates were incubated under anaerobic conditions for 48 h at 30˚C. To

inhibit the growth of fungi (yeast and mold) on plates Cycloheximide (0.01% V/V) was added.

1 mL of milk sample was poured into 9 mL sterile skim milk contained 0.1% yeast extract (Difco Laboratories, MD, USA), and incubated at 30˚C. After coagulation of the milk sample, one loopful of the sample was streaked on MRS agar and incubated at 30˚C for the duration of 2–3 days. After incubation time a single colony was selected from the plates based on morphological differences of colonies, such as color and shape determined by light microscopy. Gram-positive, catalase-negative bacterial isolates were selected and were transferred in to skim milk containing sodium glutamate (0.1%), and kept at 40˚C or 80˚C for future experiments.

Light Microscope (BH-2, Olympus Corporation, Tokyo, Japan) equipped by digital camera was used to differentiate the properties of the isolated organism, such as Gram stain reactions and cell morphology. CO_2 production and growth at 15˚C and 45˚C in MRS broth was applied to determine rod-shaped microorganisms. However, identification of cocci has been done by determining the growth ability of organisms at temperatures under 10, 40 and 50˚C. Moreover, D- and L-lactate dehydrogenases were used to investigate the presence of the lactic acid isomer. The carbohydrate fermentation ability and salt and acid tolerance of each isolate were tested for identifying the species of isolates based on the method described by Sneath et al, [9].

2.3. 16S RDNA Identification.

2.3.1. DNA isolation.

5 mL of inoculated MRS broth centrifugated at 5000 g. The pelleted cell re-suspended in buffer (500 µL, pH=8.0, 1 mM EDTA and 10 mM Tris–HCl,), and then lysed by using 50 µL of SDS and 10 µL proteinase K. The mixture incubated at 50˚C for 1 h. 100 µL CTAB⁄NaCl solution and 10 µL of NaCl (5M) were added which incubated at 65ºC for 10 min. Protein removal has been done by extraction with chloroform –phenol–isoamyl alcohol (24:25:1). The aliquot phase was separated by twice extraction with isoamyl alcohol – chloroform (1:24). DNA was precipitated by adding 0.1 volume of sodium acetate (3 M) to the aliquot phase and by addition of one volume of ice-cold dimethyl carbinol. The DNA was pelleted by centrifugation at 10000 g for 15 min, and then the collected DNA was washed with analytical ethanol and solubilized in 20 mL of sterile ultrapure water [10].

The amplification of 16S rDNA and sequencing the 16S rDNA have been done by PCR using the universal primer 27 F and 1495 R. The primer sequences were: 27 F, 5 ¢ -AGAGTTTGATCCTGGCTCAG-3, and 1495 R, 5 ¢ -CTACGGCTACCTTGTTACGA-3 ¢ .

2.3.2. Extracted DNA amplification using PCR.

The 16S rRNA sequences were amplified by the PCR method using general primers of 27 F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1495 R (5'-CTACGGCTACCTTGTTACGA-3') (CinnaGen, Iran). The sequences with approximately 1500 bp in primers were associated with the number of nucleotides, 27 nucleotides between 1415 and 1515. Thus, the PCR mixture (25 µl) was mixed with Tag polymerase enzyme (0.3 µL), 2.5 µL of 10× PCR buffer (without Mg^{2+}), 2 µL of $MgCl_2$ (25 mM), 2µL of dNTP (2.5 mM), 0.3 µL of forwarding primer (50 pM), 0.3 µL of reveres primer (50 pM), 1 µL of DNA template (100 ng/µL) and 16.6 µL of ddH_2O (Sigma Aldrich, USA). The reaction program was 94°C for 5 minutes, 94°C for 1 minute, 58°C for 1 minute, 72°C for 2 minutes, 31 cycles and then 72°C for 10 minutes, 4°C for 30 minutes. The amplified sequences were electrophoresed on 1% agarose gel (Merck, Germany), and were observed using GelRed staining (CinnaGen, Iran). The sequencing was carried out in collaboration with the Iranian Biological Resource Center.

2.3.3. Sequencing.

PCR purification kit was used to purifying the PCR products based on the kit instructions (Qiagen, Netherlands). First, 500 µL of PB buffer was mixed with 100 µL of PCR samples and agitate well. For DNA binding, the specimen was entered into the QIAquick column and then centrifuged for 30-60 s. The solution suspended on the outer surface and the column was retransferred to the same tube. Next, 0.75 mL of PE buffer was added to the column to rinse and re-centrifuged (30-60 s). The supernatant was removed and again the QIAquick column was returned to the same tube and centrifuged for 1 minute at the highest speed. The mentioned column was then transferred to clean centrifuge tube (1.5 mL). Afterward, 50 µL of buffer (10 mM of Tris-Cl, pH=8.5) was transferred to the QIAquick membrane and centrifuged for one minute to dilute the DNA (Kiagen, Netherlands) [11].

The DNA strands were sequenced directly by Macrogen using the automatic MegaBACE™ 1000 Sequencing System. The chromaspro 1.7.5 software was used to set the sequences with the sequences of test isolates and homologous sequences found on the EzBioCloud site. The sequences were searched in EzBioCloud site [12].

2.4. Identification of yeast isolates using PCR.

2.4.1. DNA extraction.

The nucleic acid of cultured yeast cells was separated in Yeast Malt Broth medium (3 g of malt and yeast extract, 5 g of peptone and 10 g of glucose per liter of distilled water). The yeast cells suspended in 20 µl of distilled water and lysed using a pipette tip, which was then boiled for 10 minutes at 96°C. The yeast species were cultured on peptone, yeast extract, and D-glucose in 16ml sealed test tubes at 30°C for 48 h along with shaking. The cells were isolated from the cultured medium using centrifugation at 20000 g and lysed by adding 500 µL of DNA lysis buffer together with glass beads. The solution was stirred vigorously and was frozen for 5 minutes. Ammonium acetate (275 µL, 7 M, pH=7) was added to the solution, and then the solution was incubated at 65°C for 5 minutes and then became cold on ice for 5 minutes. Next, chloroform was added and the resulting mixture centrifuged at 20000 g (2 minutes, 4°C). The immersed materials poured to a new tube and the DNA was deposited by adding isopropanol and centrifuged at 20000 g for 5 minutes at 4°C. The sediment separated with ethanol was washed and dissolved in 100 µl of TE (10 mM of Tris-HCl and 1 mM of EDTA, pH=8) [13].

2.4.2. Extracted DNA amplification using PCR.

The PCR for the 26SrDNA D1/D2 region was performed by application of fungal primers of NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3') (CinnaGen, Iran). A 50µL solution containing DNA, $MgCl_2$, dNTP, each primer, and Taq polymerase was prepared in order to perform the PCR reaction. The amplification was performed through GenAmp PCR (Model 2400)

within 35 cycles in accordance with the following procedure: annealing temperature of 52°C, expansion temperature of 72°C for 2 minutes and denaturation temperature of 74°C for 1 minute [14]. PCR products were electrophoresed on 1.5% agarose in TAE 1X buffer (0.04 M of Tris-acetate, 0.001 M of EDTA, pH= 8) and were observed using GelRed staining (Sigma Aldrich, USA) [15].

2.4.3. Sequencing.

The sequencing of DNA strands was performed base on the Moreira et al, [11] as described, previously. The sequences were searched in CBS site [16].

2.5. Statistical analysis methods.

The statistical design used in this research was a completely randomized design. One-way analysis of variance was applied to compare significant differences. Duncan's test compared the pH of yoghurt samples at 5% significant level. The statistical software was SPSS 16.0.0 [17].

3. RESULTS

3.1. pH values.

pH value of Doogh is critical for its shelf life and based on Iran national standard, it should not be exceeding 4.5 [2]. As given in table 1, the pH values of samples, ranging from 3.475 to 4.040, were all meeting the Iran standard limit. There was no significant statistical difference between pH values of samples ($p > 0.05$). The samples number 2 and 5 had the highest pH value (Table 1).

3.2. Microorganisms identification.

3.2.1. Lactobacillales or lactic acid bacteria.

The initial biochemical identification was conducted on samples. The biochemical distinguishing factors were including the ability to generate CO_2 as a function of glucose fermentation, growth capability at temperatures of 10 and 45°C, ability to survive and grow at saline environment (salt content 4 and 6.5%), and capability of survival and growth at conditions with pH values 4.4 and 9.6. The obtained results are illustrated in Table 2. The comparison of results with reference Table, which is given by Kenneth Todar [18], showed that the isolated colonies from industrial Iranian Doogh samples were from Lactobacilli or lactic acid bacteria.

To conduct the complementary identification, the DNA of suspicious colonies from pure cultures was isolated, amplified and sequenced. Then, the sequences were uploaded on EzBioCloud site to differentiate lactic acid bacteria at species level [19]. The output of EzBioCloud site showed that the isolated species of lactic acid bacteria were including: *L. fermentum, L. paracasei, L. brevis* and *L. gallinarum* (Table 3). As it is shown in Table 3, *L. fermentum* (access no., AJ575812) was identified in samples 2, 3, 4, 7, 9, 10 and 13 with 100% similarity, which was followed by its identification in samples of 1, 8, 11 and 15 with 99.73% similarity, and in samples of 6, 12, 14, 16 and 17 with 99.72% similarity. *L. paracasei* (access no., ACGY01000162) was differentiated in samples of 6, 8, 9, 10, 11, 15 and 17 with 100% similarity and samples of 1, 7, 12, 13 and 14 with 99.93% similarity. *L. brevis* (access no., CP000416) was distinguished in samples of 4, 5, 9 and 14 with 100% similarity and *L. gallinarum* (access no., AJ417737) was only found in samples of 2 and 3 with similarity 99.52% (Table 3).

Lactic acid bacteria are widely used in dairy. As shown in present study, lactic acid bacteria, based on biochemical characterization (Table 2) and sequencing based on 16S rRNA (Table 3), was dominant microbial flora in Doogh samples. Ogier and his colleagues employed TTGE method, showed that lactic acid bacteria were dominant microbial flora in different types of dairy products [20]. El-Baradei and colleagues [21], examined the bacterial biodiversity of traditional fermented milk called Zabady by the method of PCR-DGGE, and discovered lactic acid bacteria at the highest population. García-Cayuela and colleagues [22], also, reported the same and found lactic acid bacteria prudent, while detecting and enumerating viable lactic acid bacteria and bifidobacteria in fermented milk by using propidium monoazide and real-time PCR, Simultaneously.

In the present study, the isolated and identified species of lactic acid bacteria were catagorized as rod shapped non-starter ones [23]. The persuasive explaination to such observation is the capability of lactobacilli to survive and proliferation at environments with low pH in comparison to other species of lactic acid bacteria [24]. Isono *et al* [25] reported the identification of *L. brevis* in Masai fermented milk in Northern Tanzania. Mathara *et al*. [26] found *L. fermentum* and *L. paracasei* in kule naoto, the Maasai traditional fermented milk in Kenya, which is characterized by pH value < 4.5. Chen *et al*. [27], isolated and identified *fermentum* at a high ratio (31%) in traditional fermented milk of Tibet in China. The pH values of Doogh samples all were in the range of 3.475 to 4.040 (Table 1).

Non-starter lactobacilli are resistant to the environment with high NaCl content, as well. Since, it was reported that *L. gallinarum* was resistant to salt content up to 4% [28]. As can be observed in Table 2, these bacteria are capable to survive and multiply at saline cultures with salt content as much as 6.5% (Table 2). Where the maximum salt content of Doogh has been set at 1% [28]. Ultimately, it can be concluded that the defensive criteria of NaCl content (0.2-1%) and pH value (< 4.5) by defined Iran standard for Doogh [2] are not enough to halt or delay the growth of non-starter lactobacilli.

In general, non-starter lactic acid bacteria are not heat resistance against heat treatments such as pasteurization. Therefore, these bacteria should have been introduced to Doogh as post contamination through production environment, processing equipments, water, air and raw milk or inefficient heat treatment of the finished product, failing to destroy or inactivate them [11]. As mentioned, one of the main ways for post contamination of dairy products by non-starter lactic acid bacteria is processing equipments. In fact, these bacteria create biofilms on the surface of equipments, which are resistant to heat treatment, bactericidal, and bacteriostatic agents, so passed on finished dairy products [12].

3.2.2. Gram positive spore forming bacilli.

The biochemical analysis on preliminary identification of gram positive spore forming bacilli was based on criteria such as nitrate and simmons citrate reduction, starch haemolysis, gelatin

melting, Voges-Proskauer, manitol consumption, beta-haemolysis and antibiotic disc. Gram positive spore forming bacilli were identified in 7 samples of industrial dooghs (Table 4). Comparing the obtained results with the identification table presented by Mac Faddin [13] showed that the isolated bacillus in samples of 1, 5 and 14 was *Bacillus circulans*, since the results of simmons citrate and Voges-Proskauer were negative, and the results to other tests were all positive (Table 4). In the samples of 2 and 4, *B. firmus* was the identified bacillus bacteria (Table 4). As given in Table 4, the results for simmons citrate reduction, starch haemolysis, manitol consumption, and antibiotic disc were negative, while other tests were positive. *B. sphaericus* was also identified in the samples of 9 and 12 (Table 4) since the gelatin melting and beta-haemolysis results were positive.

The biochemical identification was followed by purification, isolation, amplification and sequencing of DNA of suspicious colonies. The introduction of isolated sequences to EzBioCloud site illustrated the different results and the obtained results of the biochemical analysis were not confirmed (Table 5). The identified bacilli were *B. subtilis* (99.91% similarity with access no., EU138467), *B. anthracis* (100% similarity with access no., AB190217) and *B. licheniformis* (99.86% similarity with access no., AB190217).

The identified gram positive spore forming bacilli are sporothermotolerant [29]. It means that these bacteria are able to generate heat tolerant endospores, which can tolerate the heat treatments such as pasteurization (72 ˚C, 15 seconds), sterilization (115-120 ˚C, 15-20 minutes) and ultra-heat temperature treatment (135-142 ˚C, 4-5 seconds) [30]. In addition to their ability to generate thermo-tolerant spores, these bacteria are resistant to high salt media and acidic pH as well. *B. licheniformis* is able to stand saline media with salt content as high as 7% [28] and the species of *B. licheniformis* and *B. subtilis* are capable to grow up at acidic media such as fermented dairy products [15].

The present study showed that 41% of samples of Iranian industrial Dooghs were contaminated by gram positive spore forming bacilli. The presence of these bacteria in fermented dairy products was reported in other studies. Hosseini et al [14] observed that 28% of Iranian kefir samples were contaminated by B. cereus and *B. subtilis*. Banykó and Vyletělová [15] isolated *B. licheniformis* in yogurt.

Gram positive spore forming bacilli can contaminate fermented dairy products in different ways. The possible ways are including heat treated or pasteurized milk [14, 16, 17], water [18], air [14] and equipments, whose internal surface is contaminated to biofilms containing spore of gram positive bacilli [12].

However, there is no report on the ability of isolated bacilli to spoil fermented dairy products, but they are able to rotten other dairy types by producing heat resistant proteolytic and lipolytic enzymes, specially during cold seasons [31, 32]. For instance, *B. licheniformis* is considered as a cause of the sour rot spoilage in condensed milk [13] and *B. subtilis* created sweet coagulative spoilage in milk and cream [33].

It is vital to minimize or even eliminate the presence of these bacilli in Iranian industrial Dooghs, since these bacteria are potential pathogens. There is a suspicious connection between *B. licheniformis* and *B. subtilis*, and some clinical syndromes such as intestinal disorders [34]. *B. anthracis* is capable to develope human respiratory and intestinal upsets [17]. *B. licheniformis* and *B. subtilis* can, also, create food poisoning by producing heat resistant toxins [35].

3.2.3. Gram negative bacilli.

The initial determination of gram negative bacilli was conducted by employing nutrient agar culture and gram staining. The observation showed that the samples of 2, 3 and 4 were containing these bacteria. To confirm the observation and to identify the species, the suspicious colonies were sequenced, after being isolated, and the sequences were uploaded on EzBioCloud site. The isolated gram negative bacilli were all from Acetobacter bacteria family and the species were including *A. tropicalis* (99.8% similarity, access no., AB032354) in sample of number 2 and *A. indonesiens* (99.7% similarity, access no., AB032356) in samples of 3 and 4 (Table 6).

It seems unlikely to identify Acetobacter bacteria in fermented dairy products since they are mostly involved in oxidative processes of different materials such as ethanol, acetic acid, acetate and lactate to water and carbon dioxide, and oxidizing glucose to dihydroxyacetone in fruits such as grape, banana, mango and in products such as vinegar and wine [36, 37]. Though, these bacteria were identified in 17.6% of samples. The identification of other species of Acetobacter bacteria was reported by other authors in different fermented dairy products as well. For instance, *A. lovaniensis* in Brazilian kefir (32 isolates) [38], *A. aceti* in Tibetan fermented milk (*39*), *A. orientalis* in Caucasusian fermented milk [40], *A. pomorum* in Sudanese fermented sour milk (Robe) [41].

Acetobacter bacteria are not micro flora of fermented dairy products and the optimum temperature for them is 20-37˚C and will be dead or inactive at temperatures below 20˚C and above 40˚C, but they are able to survive and grow at pH range 3-9. Therefore, the samples of Doogh in the present study were post contaminated with air, packaging materials, and equipments [36].

3.2.4. Yeasts and molds.

In order to identify and isolate yeasts, the culture media YGC was used. Then, the purified, isolated, sequenced DNAs were uploaded on CBS gene bank. The identified yeast species in industrial Doogh were including *Cryptococcus magnus* (100% similarity, access no., CBS4685) in samples of 15 and 17, *Pichia fermentans* (99.8% similarity, access no., CBS4611) in samples of 3, 5, 14 and 16 and *Saccharomyces unisporus* (100% similarity, access no., CBS398) in samples of 1, 2, 5, 8 and 15 (Table 7). In non-industrial samples the identified yeasts and molds species were including *Kazachstaia servazzi* (99% similarity, access no., KY103666) in samples 1 and 2, *Galactomyces candidum* (100% similarity, access no., KM1151135) in sample 2, *Kazachstania unispora* (100% similarity, access no., KY103684) in sample 3, *Penicillium granulatum* (100% similarity, accesss no., LN482444), *Saccharomyces cerevisiae* (97% similarity, access no., KF442632) and *Aspergillus awamori* (100% similarity, access no., MH345886) in sample 4, *Aspergillus flavus* (100% similarity, access no., MH345959) and *Kluyveromyces marxianus* (100% similarity, access no., KF851351) in sample 5, and *Mucor circinelloides* (96% similarity, access no., JN205940) and *Saccharomyces pastorianus* (100% similarity, access no., KY105227) in sample 6 (Table 7 and 8).

Table 1. pH of industrial Doogh samples.

Samle code	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
pH	3.645 ±0.012^{c}	3.826± 0.011^{b}	3.645± 0.012^{c}	3.645± 0.012^{c}	3.655± 0.009^{c}	3.650± 0.011^{c}	3.554± 0.013^{d}	3.551± 0.012^{d}	3.5550± 0.014^{d}	3.548± 0.008^{d}	3.547± 0.010^{d}	3.544± 0.009^{d}	3.531± 0.012^{ed}	3.491± 0.007^{e}	3.483± 0.014^{fe}	3.481± 0.008^{fe}	**3.475± 0.013^{f}**

Table 2. Biochemical identification of lactic acid bacteria in industrial Doogh samples.

Sample code	Shape	Coloring gram	CO_2 production of glocuse	Growth (C°)		Growth in salty sollution		Growth pH		Closest detected microoraganisms
				10	45	٪4	٪6.5	4.4	9.6	
2, 3, 4, 7, 9, 10, 13 and 16	filamentary	+	-	-	+	+	-	+	+	**Lactobacillus**
1, 7, 12, 13 and 14	filamentary	+	-	+	-	+	+	-	+	**Lactobacillus**
1, 8, 11 and 15	filamentary	+	-	-	+	+	-	-	+	**Lactobacillus**
6, 12, 14 and 17	filamentary	+	-	+	+	+	-	+	+	**Lactobacillus**
4, 5, 6, 8, 9, 10, 11, 15 and 17	filamentary	+	-	+	+	+	+	+	+	**Lactobacillus**
2 and 3	filamentary	+	-	+	-	+	+	+	+	**Lactobacillus**
4, 5, 9 and 17	filamentary	+	-	-	+	+	-	+	+	**Lactobacillus**

Table 3. Identification of isolated acidic bacteria from industrial Doogh samples based on 16S rRNA gene sequence

Sample code	Sequence size	Similarity Percentage	Species identified	Access number
2, 3, 4, 7, 9, 10, 13 and 16	nucleotide 939	٪100	CECT 562 *L. fermentum*	**AJ575812**
1, 7, 12, 13 and 14	nucleotide 1472	٪99.93	ATCC 25302 *L. paracasei*	**ACGY01000162**
1, 8, 11 and 15	nucleotide 1494	٪99.73	CECT 562 *L. fermentum*	**AJ575812**
6, 12, 14 and 17	nucleotide 1423	٪99.72	CECT 562 *L. fermentum*	**AJ575812**
4, 5, 6, 8, 9, 10, 11, 15 and 17	nucleotide 1446	٪100	ATCC 25302 *L. paracasei*	**ACGY01000162**
2 and 3	nucleotide 1442	٪100	ATCC 367 *L. brevis*	**CP000416**
4, 5, 9 and 17	nucleotide 1466	٪99.52	ATCC 39199 *L. gallinarum*	**AJ417737**

Table 4. Biochemical identification of Gram-positive Bacillus spider from industrial Doogh samples.

Sample code	Coloring gram	Nitrate revitalization	Cimon citrate	Starch hyrolize	Gelatin melt	Voges-Proskauer	Manitol Consumption	Beta Hemolysis	Antibiotic test	Closest detected microorganisms
1, 5 and 14	+	+	-	+	+	-	+	+	+	**Bacillus Circulars**
2 and 4	+	+	-	-	+	+	-	+	-	**Bacillus Esfricus**
9 and 12	+	-	-	-	+	-	-	+	-	**Bassillus Fables**

Milk and milk based products are suitable media for yeasts and mold growth. Chen *et al* [27], isolated *Pichia fermentans* from raw milk and Álvarez-Martín *et al* [42], isolated this yeast from blue cheese, using RFLP method. Callon *et al* in two seperated studies isolated *Saccharomyces unisporus* and *Cryptococcus magnus* in cheese [43], and in goat milk [44], respectfully. Identification and isolation of *Pichia fermentans* and *Saccharomyces unisporus* has been reported in variety of fermented dairy products as well such as kefir and Koumiss [10, 27, 45].

Table 5. Identification of gram-positive bacilli of sporadic isolates from samples based on 16S rRNA gene sequence.

Sample code	Sequence size	Similarity Percentage	Species identified	Access number
1, 5 and 14	1433 nucleotide	99.91٪	BGSC 3A28 *B. subtilis*	**EU138467**
2 and 4	1497 nucleotide	100٪	ATCC 14578 *B. anthracis*	**AB190217**
9 and 12	1430 nucleotide	99.86٪	*Bacillus licheniformis* ATCC 14580	**AE017333**

Table 6. Identification of gram-negative bacilli isolated from samples based on the sequence of 16S rRNA gene.

Sample code	Sequence size	Similarity Percentage	Species identified	Access number
2	Nucleotide 1469	99.8٪	NRIC 0312 *Acetobacter tropicalis*	**AB032354**
3 and 4	Nucleotide 1368	99.7٪	NRIC 0313 *Acetobacter indonesiensis*	**AB032356**

Table 7. Identification of isolated yeasts and moulds based on the D1/D2 26S rDNA gene in industrial and non-industial Doogh samples

Sample origin	Code	Sequence size	Similarity Percentage	Species identified	Access number
Industrial	2, 5, 14 and 16	Nucleotide 519	99%	*Pichia fermentans*	**CBS4611**
	15 and 17	Nucleotide 622	100%	*Cryptococcus magnus*	**CBS4685**
	1, 2, 5, 8 and 15	Nucleotide 585	100%	*Saccaromyces unisporous*	**CBS398**
Non-industrial	1	Nucleotide 685	99%	*Kazachstaia servazzi*	**KY103666**
	2	Nucleotide 315	100%	*Galactomyces candidum*	**KM1151135**
		Nucleotide 682	99%	*Kazachstaia servazzi*	**KY103666**
	3	Nucleotide 683	100%	*Kazachstania unispora*	**KY103684**
	4	Nucleotide 543	100%	*Penicillium granulatum*	**LN482444**
		Nucleotide 61	97%	*Saccharomyces cerevisiae*	**KF442632**
		Nucleotide 542	100%	*Aspergillus awamori*	**MH345886**
	5	Nucleotide 532	100%	*Aspergillus flavus*	**MH345959**
		Nucleotide 54	100%	*Kluyveromyces marxianus*	**KF851351**
	6	Nucleotide 532	96%	*Mucor circinelloides*	**JN205940**
		Nucleotide 750	100%	*Saccharomyces pastorianus*	**KY105227**

Table 8. Number and type of microorganisms isolated from each sample

Sample origin	code	*Lactobacillus*	Other microorganisms			
			Bacillus	*Acetobacter*	Yeast	**Mold**
Industrial	1	2	1	-	1	-
	2	1	1	1	2	-
	3	2	-	1	-	-
	4	3	2	2	-	-
	5	2	1	-	2	-
	6	2	-	-	-	-
	7	2	-	-	-	-
	8	2	-	-	1	-
	9	3	1	-	-	-
	10	2	-	-	-	-
	11	2	-	-	-	-
	12	2	1	-	-	-
	13	2	-	-	-	-
	14	2	1	-	1	-
	15	2	-	-	2	-
	16	1	-	-	1	-
	17	3	-	-	1	-
Non- industrial	1	-	-	-	1	-
	2	-	-	-	2	-
	3	-	-	-	1	-
	4	-	-	-	1	**2**
	5	-	-	-	1	**1**
	6	-	-	-	1	**1**

Yeasts are sensitive to heat treatment (*46*) and their growth and activity is limited by starter lactic acid bacteria presence and fermentation temperature (40-45˚C) (*47*). Therefore, these yeasts have entered Iranian industrial and non-industrial Doogh through post contamination. The susceptible ways of entry are including biofilms (*48*), environment (*47*) and packaging cap (*49*).

Since yeasts are potential spoiling and pathogen microorganisms and are able to survive and grow in media with a high content of salt, low water activity, acidic pH, and low temperatures [50], their presence in Iranian industrial and non-industrial Doogh should be limited. Yeasts are able to generate gas, which would result in blowing and deforming of packages, to create yeasty taste and other unpleasant off-tastes, fermentation odor, discoloration and texture damage in fermented dairy products [49]. However, yeasts are not able to spoil dairy products in absence of lactose [51], but if fermented dairy products are infected by yeasts, yeasts would create proper media for growth of other spoiling bacteria through consuming lactic acid, producing vitamins and amino acids, and increasing the pH of media [47]. Fleet [52] demonstrated the potential of yeasts to create gasterotestinal disorders and allergies in humans, which makes yeasts potential hazart for human health.

Mold growth in food products is a quality deficiency that may cause economic losses for food manufacturers. Molds can grow in fermented dairy products such as yogurt due to some intrinsic (pH, water activity, ingredients of product, and type of initial contamination) and extrinsic (packaging material and condition and temperature of storage) factors. *Penicillium*, *Mocur*, and *Aspergillus* are the most frequent mold that contaminate yogurt and yogurt based products [52]. Non-industrial Doogh is obtained from yogurt dilution with tap water in Iran, so initial yogurt contamination can be a reason for Doogh Contamination. Molds can affect on organoleptic properties of Doogh and develop some undesired flavors in Doogh. Molds contamination of non-industrial Doogh may be due to the unhygienic storage condition or other post contaminant such as packaging materials and unclean atmosphere.

4. CONCLUSIONS

In the present study, microbial contaminants of Iranian industrial Doogh were identified by employing biochemical and molecular methods. The identified and isolated microorganisms were including lactic acid bacteria (*Lactobacillus brevis*, *L. fermentum*, *L. paracasei* and *L. gallinarum*); gram-positive spore-forming bacteria (*Bacillus licheniformis*, *B. anthracis* and *B. subtilis*); acetic acid bacteria (*Acetobacter tropicalis* and *A. indonesiensis*) and yeasts. However, these microorganisms are not sever spoiling and pathogens, but their presence should be limited since they have the potential to spoil dairy products and to be a pathogen. These microorganisms possibly have entered Doogh by post contamination, which are biofilms, environment, packaging and raw materials. The identified bacteria and yeast species in the present study are able to grow up in Doogh, since they are resistant to heat treatment, high salt content, acidic pH, low temperature and water activity. Therefore, it is vital to block their entrance through good hygiene practice (GHP), Hazard analysis and critical control points (HACCP), appropriate heat treatment and storage conditions (refrigerator temperature, 4±1¬°C).

5. REFERENCES

1 Codex Alimentarius Commission. Joint FAO/WHO food standards programme FAO/WHO coordinating committee for the Near East. Eighth Session. **2015**.

2 ISIRI. Doogh – Specifications ans test method. *Institute of Standards and Industrial Research of Iran* **1996**, 2453.

3 Intanoo, M.; Kongkeitkajorn, M.B.; Pattarajinda, V.; Bernard, J.K.; Callaway, T.R.; Suriyasathaporn, W.; Phasuk, Y. Isolation and screening of aflatoxin-detoxifying yeast and bacteria from ruminal fluids to reduce aflatoxin B1 contamination in dairy cattle feed. *Journal of applied microbiology*. **2018**,125, 6, 1603-1613, https://doi.org/10.1111/jam.14060.

4 Szymczak, P.; Vogensen, F.;, Janzen, T. Novel isolates of Streptococcus thermophilus bacteriophages from group 5093 identified with an improved multiplex PCR typing method. *International dairy journal*, **2019**, 1, 91, 18-24, https://doi.org/10.1016/j.idairyj.2018.12.001

5 Ohshima, C.; Hajime, T. Detection Method of Food-borne Pathogens in Seafood. In *Seafood Safety and Quality*, CRC Press, **2018**, 72-89, https://doi.org/10.3923/ajft.2011.87.102.

6 Sambo, F.; Finotello, F.; Lavezzo, E.; Baruzzo, G.; Masi, G.; Peta, E.; Falda, M.; Toppo, S.; Barzon, L.; Di Camillo, B. Optimizing PCR primers targeting the bacterial 16S ribosomal RNA gene. *BMC bioinformatics*, **2018**, 19(1):343, https://doi.org/10.1186/s12859-018-2360-6.

7 Simam, J.; Rono, M.; Ngoi, J.; Nyonda, M.; Mok, S.; Marsh, K.; Bozdech, Z.; Mackinnon, M. Gene copy number variation in natural populations of Plasmodium falciparum in Eastern Africa. *BMC genomics*, **2018**, 19(1):372, https://doi.org/10.1186/s12864-018-4689-7.

8 El-Senosi, YA.; Atwa, HE.; Prince, AM.; Afify, AF. Development of Simple Multiplex Real-Time PCR Assays for Some Probiotics Detection. *Benha Veterinary Medical Journal*. **2017**, 33(2):52-7.

9 Sneath, P.H.; Mair, N.S.; Sharpe, M.E.; Holt, J.G. Bergey's manual of systematic bacteriology. *Williams & Wilkins*, **1986**, 2.

10 Wang, S.Y.; Chen, H.C.; Liu, J.R.; Lin, Y.C.; Chen, M.J. Identification of yeasts and evaluation of their distribution in Taiwanese kefir and viili starters. *Journal of dairy science* **2008**, *91*, 3798-3805, https://doi.org/10.3168/jds.2007-0468.

11 Moreira, S.R.; Schwan, R.F.; Carvalho, E.P.D.;Wheals, A.E. Isolation and identification of yeasts and filamentous fungi from yoghurts in Brazil. *Brazilian Journal of Microbiology* **2001**, *32*, 117-122, https://doi.org/10.1590/S1517-83822001000200009.

12 Dat, N.M.; Hamanaka, D.; Tanaka, F.; Uchino, T. Control of milk pH reduces biofilm formation of Bacillus licheniformis and Lactobacillus paracasei on stainless steel. *Food Control* **2012**, *23*, 215-220, https://doi.org/10.1016/j.foodcont.2011.07.013.

13 Mac Faddin, J.F. *Biochemical tests for identification of medical bacteria.* Williams & Wilkins Co, 1976.

14 Hosseini, H.; Hippe, B.; Denner, E.; Kollegger, E.; Haslberger, A. Isolation, identification and monitoring of contaminant bacteria in Iranian Kefir type drink by 16S rDNA sequencing. *Food Control* **2012**, *25*, 784-788, https://doi.org/10.1016/j.foodcont.2011.12.017.

15 Banykó, J.; Vyletělová, M. Determining the source of Bacillus cereus and Bacillus licheniformis isolated from raw milk, pasteurized milk and yoghurt. *Letters in applied microbiology* **2009**, *48*, 318-323, https://doi.org/10.1111/j.1472-765X.2008.02526.x.

16 Li, F.; Hunt, K.; Van Hoorde, K.; Butler, F.; Jordan, K.; Tobin, JT. Occurrence and identification of spore-forming bacteria in skim-milk powders. *International dairy journal*, **2019,** 97:176-84, https://doi.org/10.1016/j.idairyj.2019.05.004.

17 Pereira, AP.; Sant'Ana, AS. Diversity and fate of spore forming bacteria in cocoa powder, milk powder, starch and sugar during processing: A review. *Trends in food science & technology*, **2018** , 76:101-18, https://doi.org/10.1016/j.tifs.2018.04.005

18 Ghadimi, D.; Herrmann, J.; de Vrese, M.; Heller, KJ. Commensal probiotic bacteria affect host cellular lipid metabolism through various cellular metabolic pathways: Role of mTOR, FOXO1, and autophagy machinery system. *Zeitschrift für Gastroenterologie*, **2018**, 56(05), 5. https://doi.org/ 10.1055/s-0038-1648567.

19 Ibarburu, I.; Puertas, A.I.; Berregi, I.; Rodríguez-Carvajal, M.A.; Prieto, A.; Dueñas, M.T. Production and partial characterization of exopolysaccharides produced by two Lactobacillus suebicus strains isolated from cider. *International journal of food microbiology* **2015**, *214*, 54-62, https://doi.org/10.1016/j.ijfoodmicro.2015.07.012.

20 Ogier, J.C.; Son, O.; Gruss, A.; Tailliez, P.; Delacroix-Buchet, A. Identification of the bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis. *Appl. Environ. Microbiol.* **2002**, *68*, 3691-3701, https://doi.org/10.1128/AEM.68.8.3691-3701.2002.

21 El-Baradei, G.; Delacroix-Buchet, A.; Ogier, J. Bacterial biodiversity of traditional Zabady fermented milk. *International journal of food microbiology* **2008**, *121*, 295-301, https://doi.org/10.1016/j.ijfoodmicro.2007.11.014.

22 García-Cayuela, T.; Tabasco, R.; Peláez, C.; Requena, T. Simultaneous detection and enumeration of viable lactic acid bacteria and bifidobacteria in fermented milk by using propidium monoazide and real-time PCR. *International dairy journal* **2009**, *19*, 405-409, https://doi.org/10.1016/j.idairyj.2009.02.001.

23 Chou, Y.E.; Edwards, C.; Luedecke, L.; Bates, M.; Clark, S. Nonstarter lactic acid bacteria and aging temperature affect calcium lactate crystallization in Cheddar cheese. *Journal of dairy science* **2003**, *86*, 2516-2524, https://doi.org/10.3168/jds.S0022-0302(03)73846-6.

24 Caridi, A. Identification and first characterization of lactic acid bacteria isolated from the artisanal ovine cheese Pecorino del Poro. *International Journal of Dairy Technology* **2003**, *56*, 105-110, https://doi.org/10.1046/j.1471-0307.2003.00081.x.

25 Isono, Y.; Shingu, I.; Shimizu, S. Identification and characteristics of lactic acid bacteria isolated from Masai fermented milk in Northern Tanzania. *Bioscience, Biotechnology, and Biochemistry* **1994**, *58*, 660-664, https://doi.org/10.1271/bbb.58.660.

26 Mathara, J.M.; Schillinger, U.; Kutima, P.M.; Mbugua, S. K.; Holzapfel, W.H. Isolation, identification and characterisation of the dominant microorganisms of kule naoto: the Maasai traditional fermented milk in Kenya. *International journal of food microbiology* **2004**, *94*, 269-278, https://doi.org/10.1016/j.ijfoodmicro.2004.01.008.

27 Chen, L.S.; Ma, Y.; Maubois, J.L.; Chen, L.J.; Liu, Q.H.; Guo, J.P. Identifcation of yeasts from raw milk and selection for some specific antioxidant properties. *International Journal of Dairy Technology* **2010**, *63*, 47-54, https://doi.org/10.1111/j.1471-0307.2009.00548.x.

28 Bergey, D.H.; Boone, D.R.; Castenholz, R.W.; Garrity, G.M. *Bergey's manual of systematic bacteriology. Archaea and the deeply branching and phototrophic bacteria*. Springer, Volume 1, 2001.

29 Heyndrickx, M. The importance of endospore-forming bacteria originating from soil for contamination of industrial food processing. *Applied and Environmental Soil Science* **2011**, *2011*, https://doi.org/10.1155/2011/561975.

30 Phillips, J.; Griffiths, M. Factors contributing to the seasonal variation of Bacillus spp. in pasteurized dairy products. *Journal of applied bacteriology* **1986**, *61*, 275-285, https://doi.org/10.1111/j.1365-2672.1986.tb04288.x.

31 Burgess, S.A.; Lindsay, D.; Flint, S.H. Thermophilic bacilli and their importance in dairy processing. *International journal of food microbiology* **2010**, *144*, 215-225, https://doi.org/10.1016/j.ijfoodmicro.2010.09.027.

32 Sutherland, A.; Murdoch, R. Seasonal occurrence of psychrotrophic Bacillus species in raw milk, and studies on the interactions with mesophilic Bacillus sp. *International journal of food microbiology* **1994**, *21*, 279-292, https://doi.org/10.1016/0168-1605(94)90058-2.

33 Van Heddeghem, A.; Vlaemynck, G. Sources of contamination of milk with Bacillus cereus on the farm and in the factory. *Bulletin-International Dairy Federation* **1992**, 19-19.

34 Salkinoja-Salonen, M.S.; Vuorio, R.; Andersson, M.; Kämpfer, P.; Andersson, M.; Honkanen-Buzalski, T.; Scoging, A. Toxigenic strains of Bacillus licheniformis related to food poisoning. *Appl. Environ. Microbiol.* **1999**, *65*, 4637-4645.

35 Tatzel, R.; Ludwig, W.; Schleifer, K.H.; Wallnöfer, P.R. Identification of Bacillus strains isolated from milk and cream with classical and nucleic acid hybridization methods. *Journal of Dairy Research* **1994**, *61*, 529-535, https://doi.org/10.1017/S0022029900028454.

36 Bartowsky, E.J.; Henschke, P.A. Acetic acid bacteria spoilage of bottled red wine—a review. *International journal of food microbiology* **2008**, *125*, 60-70, https://doi.org/10.1016/j.ijfoodmicro.2007.10.016.

37 Drysdale, G.; Fleet, G. The growth and survival of acetic acid bacteria in wines at different concentrations of oxygen. *American Journal of Enology and Viticulture* **1989**, *40*, 99-105.

38 Magalhães, K.T.; Pereira, G.V.D.M.; Campos, C.R.; Dragone, G.; Schwan, R.F. Brazilian kefir: structure, microbial communities and chemical composition. *Brazilian Journal of Microbiology* **2011**, *42*, 693-702, https://doi.org/10.1590/S1517-83822011000200034.

39 Yang, X.; Fan, M.; Shi, J.; Dang, B. Isolation and identification of preponderant flora in tibetan fermented linggu milk. *China Brewing* **2007**, *6*, 10-6.

40 Ishida, T.; Yokota, A.; Umezawa, Y.; Toda, T.; Yamada, K. Identification and characterization of lactococcal and Acetobacter strains isolated from traditional Caucasusian fermented milk. *Journal of nutritional science and vitaminology* **2005**, *51*, 187-193, https://doi.org/10.3177/jnsv.51.187.

41 Sulieman, A.; Dirar, H.;Hyndriykx, M. Isolation and characterization of microorganisms associated with the Sudanese fermented sour milk (Robe). *Gezira Journal of Agricultural Science (Sudan)* **2004**.

42 Álvarez-Martín, P.; Flórez, A.B.; López-Díaz, T.M.; Mayo, B. Phenotypic and molecular identification of yeast species associated with Spanish blue-veined Cabrales cheese. *International dairy journal* **2007**, *17*, 961-967, https://doi.org/10.1016/j.idairyj.2006.11.005.

43 Callon, C.; Duthoit, F.; Delbès, C.; Ferrand, M.; Le Frileux, Y.; De Crémoux, R.; Montel, M.C. Stability of microbial communities in goat milk during a lactation year: molecular approaches. *Systematic and applied microbiology* **2007**, *30*, 547-560, https://doi.org/10.1016/j.syapm.2007.05.004.

44 Callon, C.; Delbès, C.; Duthoit, F.; Montel, M.C. Application of SSCP–PCR fingerprinting to profile the yeast community in raw milk Salers cheeses. *Systematic and Applied Microbiology* **2006**, *29*, 172-180, https://doi.org/10.1016/j.syapm.2005.07.008.

45 Todar, K. Online textbook of bacteriology. *University of Wisconsin-Madison Department of Bacteriology* **2008**.
46 Jodral, M.; Liñan, E.; Acosta, I.; Gallego, C.; Rojas, F.; Bentabol, A. Mycoflora and toxigenic Aspergillus flavus in Spanish milks. *International Journal of Food Microbiology* **1993**, *18*, 171-174, https://doi.org/10.1016/0168-1605(93)90222-3.
47 Fox, PF.; Guinee, TP.; Cogan, TM.; McSweeney, PL. Microbiology of cheese ripening. *In Fundamentals of cheese science*, Springer, Boston, MA, **2017**, https://doi.org/10.1007/978-1-4899-7681-9_11
48 Davis, J. microbiology of yoghourt. In: *Lactic acid bacteria in beverages and food: proceedings of a symposium* 1975.
49 Perricone, M.; Gallo, M.; Corbo, MR.; Sinigaglia, M.; Bevilacqua, A. Yeasts. *In The Microbiological Quality of Food*, Woodhead Publishing, **2017**, 121-131, https://doi.org/10.1016/B978-0-08-100502-6.00008-X
51 Davis, J.; Wilbey, R. Microbiology of cream and dairy desserts. *Dairy microbiology* **1990**, *2*, 41-108.
52 Fleet, G. Spoilage yeasts. *Critical reviews in biotechnology* **1992**, *12*, 1-44, https://doi.org/10.3109/07388559209069186.

6. ACKNOWLEDGEMENTS

Support for this study provided by Shahid Beheshti University of Medical Sciences is gratefully acknowledged.