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Safety Evaluation of Doogh, a Traditional Iranian Dairy Product, in Khuzestan, Iran: A Cross-Sectional Study on Microbiological, Chemical and Toxicological Aspects

Reza Zadeh-Dabbagh [a] | **Abdolazim Behfar** [b] | **Marjan Javadi** [c] | **Naji Saiahi** [d] | **Maryam Alborzi** [d] | **Maryam Seyedtabib** [e] | **Seyyed Mohammad Ali Noori** [a*]

a. Department of Nutrition School of Allied Medicine Jundishapur University of Medical Sciences, Ahvaz, Iran.
b. Food and drug Laboratory Research Center (FDLRC), Iran Food and Drug Administration (IFDA), Ministry of Health and Medical Education, Ahvaz, Iran.
c. Department of Food and Drug Control, Faculty of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.
d. Food and Drug Administration, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.
e. Department of Biostatistics and Epidemiology, School of Public Health, Hamadan University of Medical Sciences, Hamadan, Iran.

***Corresponding author:** Department of Nutrition School of Allied Medicine Jundishapur University of Medical Sciences, Ahvaz, Iran. Postal code: 15794-61357.
E-mail address: sma.noori@gmail.com

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ABSTRACT

Background: A lot of people consume traditional foods throughout the world, however there are concerns for their safety. The purpose of the current study is to evaluate the safety of traditional doogh (a traditional fermented dairy beverage) with respect to microbiological, chemical, and toxicological aspects.
Methods: 89 samples of traditional doogh from Khuzestan province, Iran, were analysed according to microbiological (*Staphylococcus aureus*, *Echerichia coli*, coliforms, mold and yeast), chemical (pH, total solid, fat, and salt content), and toxicological (aflatoxin M1, potassium sorbate, sodium benzoate, and natamycin) aspects.
Results: Results showed that 89.88% of samples would be rejected according to the guidelines specified by the Institute of Standards and Industrial Research of Iran (ISIRI). 78 sample had higher mold and yeast than standard. The salt content of five out of 89 samples were higher than the established Iranian standard.
Conclusion: The most challenging problem was mold and yeast contamination, indicating poor hygienic manufacturing conditions. However, the unfavorable conditions for microbial growth such as low pH may prevent the toxic metabolites formation, and therefore, consuming it presents no major concerns to public health. Nonetheless, the authors suggest that efforts should be made to improve hygienic conditions in traditional doogh manufacturing environments.

1. Introduction

Many traditional fermented dairy products are consumed worldwide for their beneficial impact on individual health [1]. Doogh is a traditional dairy product known as 'drinking yogurt' or 'doogh'. The Food and Agriculture Organization (FAO) defines it as "a savory yogurt-based beverage popular in Iran, Afghanistan, Armenia, Iraq, and Syria [2].

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Similar dairy beverages are common in other countries, for example, ayran (Turkey) [3], koumiss (China) [4], and kefir (Argentina) [5]. Different aspects of fermented dairy beverages have been studied by researchers. However, fewer studies have investigated the safety of these products, which requires additional consideration to lessen public health concerns.

Many types of dooghs are produced and consumed in Iran such as carbonated doogh, dooghs with different flavours (e.g. mint, *Ziziphora clinopodioides*, *Zataria multiflora*), and dooghs with special dried and ground vegetables. Traditional dooghs produced in Khuzestan province are well-known for their sour taste and desirable flavour, and often particular vegetables are being added to them such as celeriac (*Apium graveolens var. rapaceum*) and *Mentha pulegium*. There is a high consumers' demand for them due to the pleasant organoleptic properties of Khuzestan dooghs.

Two different types of doogh products can be found in the Iranian market: industrial and traditional. Industrial dooghs are supervised by the Food and Drug Administration of Iran (IFDA) and the Institute of Standards and Industrial Research of Iran (ISIRI), while almost no monitoring exists for traditional doogh manufacturing. Some consumers demand traditional dooghs due to the assumption that less adulteration occurs in this type of product, and as such, they provide better health benefits.

The production processes of traditional doogh are outlined in Figure1. Briefly, following milk delivery, milk fat, and total solids are standardized to 1% and 0.85, respectively. After heating (90 °C for five mins) and cooling (42 °C) processes, a starter culture (*S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*) is added and kept at 42 °C (120 Dornic acidity). Subsequently, pasteurized water at a ratio of 1:1 and salt (up to 1%) are added. Following on, homogenization and pasteurization (75 °C for 15 S) are carried out and finally, flavoring and dried vegetables are added [6].

Dairy products are prone to microbial contamination such as *Staphylococcus aureus*, *Escherichia coli*, coliforms, Yersinia, and etc. which are threatening human health [7]. In addition to microbial contamination, chemical and toxicological contamination of dairy products are also important for consumers' safety [8, 9].

Although traditional doogh is extensively consumed in Iran [10], no thorough study has been carried out on its safety in terms of microbiological, chemical, and toxicological aspects. Although several studies have been evaluated the safety of dairy products [8, 11, 12], the safety of dooghs has been neglected. Therefore, the aim of this study was to evaluate the microbiological (including *S. aureus, E. coli*, coliforms, and yeast and mold), chemical (including pH, total solid, fat, and salt content), and toxicological (aflatoxin, potassium sorbate, sodium benzoate, and natamycin) quality of traditional dooghs in Khuzestan province.

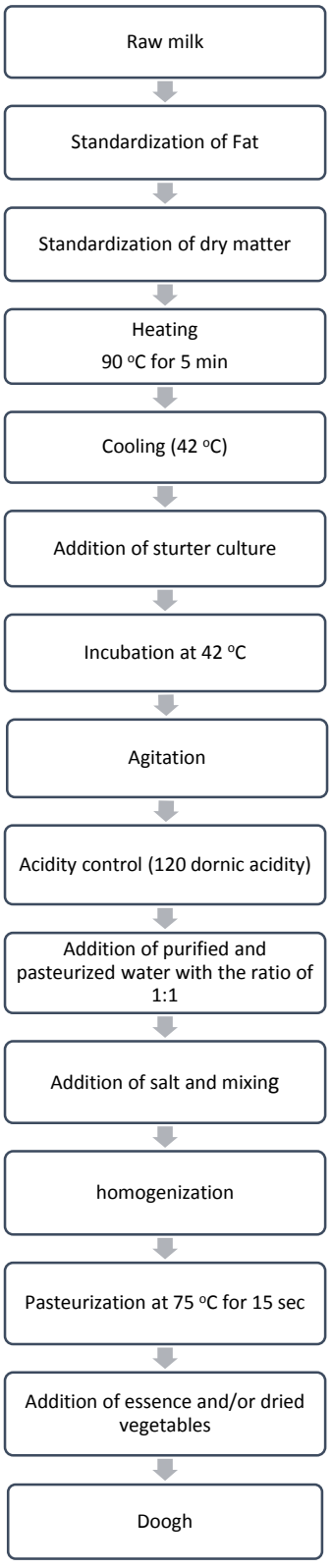

Figure 1: Manufacturing method for traditional doogh

2. Materials and Methods

2.1. Sample collection

A total of 89 traditional doogh samples were obtained randomly from producers without IFDA approval in Khuzestan province, southwest of Iran, from June 2017 to March 2019. All the samples contained dried vegetables (*Apium graveolens var. rapaceum* and *Mentha pulegium*). They were transferred to the FDA laboratory of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, under aseptic condition adjacent to cold boxes and held in a refrigerator (4 °C) until analysis.

2.2. Microbiological analysis

A 20 mL sample, weighed aseptically, was mixed with a 180 mL ringer solution (Oxoid, Hampshire, UK) and shaken to obtain a homogenous mixture. Decimal dilutions were provided by ringer solution and all microbiological tests were carried out in duplicate.

For enumeration of *Staphylococcus aureus*, a Giolitti-Cantoni broth (Merck, Darmstadt, Germany) was used as a selective enrichment media and incubated at 37 °C. After 24 and 48 h, a loopful was cultivated in Baird-Parker agar (Merck, Darmstadt, Germany) and placed in an incubator at 37 °C for 48 h [13, 14]. Shiny black colonies on Baird-Parker agar were considered to be *S. aureus* positive. Subsequently, colonies susceptible to coagulase positive were transferred to a sterile glass tube of brain-heart infusion broth (Merck, Darmstadt, Germany) at 37 °C for 24 h; then, 0.1 mL from this tube was added to 0.3 mL rabbit plasma fibrinogen, in a sterile tube. Formation of a clot in this tube was considered to reflect coagulase positive *S. aureus* [14].

The most probable number (MPN) technique was used to enumerate coliforms (3×3 tubes). Briefly, in the first step, a glass tube of lauryl sulphate tryptose broth (Merck, Darmstadt, Germany) was used and incubated at 35 °C for 24 h. Observing turbidity and gas formation was considered as positive. If the tube was negative, it remained at 35 °C for an additional 24 h to ensure progress. Positive samples, whether after 24 or 48 h, were transferred to a lactose bile brilliant green broth (Merck, Darmstadt, Germany), and incubated at 35 °C for 24 to 48 h, and turbidity and/or gas formation were considered as reflecting coliform positive doogh samples [14].

Yeast extract glucose chloramphenicol agar (Merck, Darmstadt, Germany) was used to enumerate mold and yeast. Media pH was 6.6 and incubated at 25 °C for 5 days. Blank plates were used to ensure the sterility of entire analysis [14].

E. coli was enumerated using the MPN method. Samples were added to lauryl sulphate tryptose broth (Merck, Darmstadt, Germany) and incubated at 35 °C for 24 to 48 h. Tubes were examined for gas formation, and negative tubes were re-incubated for an additional 24 h. A loopful of positive tubes was transferred to an E. coli broth (Merck, Darmstadt, Germany), incubated at 44.5 °C for 24 h, and examined for gas formation. Negative tubes were re-incubated for an additional 24 h and examined again thereafter. A loopful of positive EC broth was cultivated on Eosin Methylene Blue (EMB) agar (Merck, Darmstadt, Germany) and placed in an incubator for 24 h at 35 °C. Shiny metallic colonies were collected and analyzed for IMViC test [14, 15].

2.3. Chemical analysis

2.3.1. pH

The pH measuring was performed by a digital pH meter (Sartorius, Brentwood, NJ, USA). Calibration was carried out using standard buffer solution with pH 4 and 7.

2.3.2. Total solid and fat

Doogh samples were shaken at 20 °C and 2 g was transferred to a dish. The dish was placed in a boiling water bath and oven at 95 ± 2 °C and 102 ± 2 °C, respectively, to obtain a constant weight [16]. Fat content (%) was measured using the Gerber method, as stipulated by the ISIRI [17].

2.3.3. Salt content

Salt content was established according to the method of Altahir *et al.* (2014) with slight modification [18]. Briefly, 5 mL doogh sample was added to a flask with 100 mL warm water (50 °C) and stirred for 1 min. Then, 2 mL of 5% potassium chromate (Merck, Darmstadt, Germany) was added to the flask and titrated with a 0.0855 M solution of silver nitrate (14.53 g/L) (Merck, Darmstadt, Germany). Observing a red color due to the precipitation of silver chromate was considered the end point of the experiment. A blank test was performed with all the reagents and without a doogh sample. The percentage of salt content was calculated using the following formula:

$$W\ (\%) = \frac{(Vs - V0)}{10} \times 100$$

W (%): salt content
Vs: mLs of silver nitrate consumed for titration of sample
V0: mLs of silver nitrate consumed for titration of blank

2.4. Aflatoxin M1

Doogh bottles were shaken well to ensure homogeneity; 2 mL sample was transferred to a glass tube, and 12 mL dichloromethane (Merck, Darmstadt, Germany) was added to it. Ultrasonic (Haver and Boecker, Oelede, Germany) was used for 20 min. The sample was filtered using a Whatman

paper filter 42, and 4 mL of filtrate was dried in a conical tube with a mild steady stream of nitrogen at 50 °C. A 1 mL dilution buffer was used to dissolve the remaining from the previous stage. Subsequently, 1 mL heptane (Merck, Darmstadt, Germany) was added and vortexed for roughly 15 min. After centrifugation at 2000 rpm for 5 min, 100 µl from lower phase was transferred to an ELISA well. An aflatoxin M_1 sensitive ELISA kit (EuroProxima, BV, Arnhem, and Netherland) was used to determine aflatoxin M_1 [9, 19, 20].

2.5. Potassium sorbate, sodium benzoate, and natamycin

A 5 mL doogh sample was transferred to a volumetric flask and diluted with deionised water to 50 mL. The mixture was transferred to a 50 mL falcon tube, centrifuged at 9000 rpm for 10 mins, and a 0.45 µm cellulose acetate syringe was used to filter it. Finally, 20 µL was injected into a high performance liquid chromatography (HPLC) [21]. Determination of potassium sorbate, sodium benzoate, and natamycin was performed using the HPLC device (Waters, Milford, MA, USA), equipped with a UV detector. A C18 reverse phase column (250 × 46 mm, 5 µ m) was used. The mobile phase comprised an ammonium acetate buffer (Merck, Darmstadt, Germany) and acetonitrile (Merck, Darmstadt, Germany) at a ratio of 73:27, and the flow rate for the mobile phase was 1mL/min. A 254 nm wavelength was used to detect peaks. Analytical characteristics of method and spiked data are presented in Tables 1 and 2. Chromatogram is shown in Figure 2.

2.6. Statistical analysis

IBM SPSS Statistics version 16 was used to analyse the data, with descriptive statistics representing frequencies, percentages, means, and standard deviations (mean ± SD). The Kolmogorov-Smirnov (K-S) one-sample Z-test was used to check the normality of data distribution. The student t-test (one-sample t-test) was used to compare the means of the variable with a reference level. A two-tailed significance level of $P < 0.05$ was used for all tests.

3. Results and Discussion

The main results of the study have been provided in Figure 3 indicating high contamination of doogh samples.

3.1. Microbiological analysis

S. aureus and *E. coli* were not isolated from doogh samples. This was similar to the study of Hatamkia *et al.* (2012) [22]. In a study conducted by Çardak and Yilmaz (2011) [23] ayran samples were analysed to investigate the microbial contamination. They stated that 35% and 10% of samples were contaminated with *E. coli* and *S. aureus*. Cetinkaya and Elal (2012) [24] conducted a study to investigate the microbial contamination of 50 samples of kefir. They reported 2.4×10^2 cfu/ml contamination of *S. aureus*. Doogh and fermented dairy products have some characteristics such as low pH, high salt, and/or low water content, which can affect pathogenic bacteria and decrease their population.

To the best of our knowledge, only Hatmikia *et al.* (2012) investigated the microbiological qualities of doogh in Iran. They reported no *S. aureus* or *E. coli* being isolated from 200 samples, which is in complete accordance with the present study [7]. We suggest that low pH, high salt content, and beneficial bacteria in doogh prevented the growth of pathogens. This condition was observed for the evaluation of coliforms, where all doogh samples were < 3 MPN/mL, except for one sample with >3 MPN/mL coliforms.

Molds and yeasts presented the most substantial problem in the current study. The population distributions of mold and yeast are presented in Table 3. According to the ISIRI (No 2453), the maximum acceptable mold and yeast count in doogh is 100 CFU/mL [14]. The results showed that only 11 samples (12.4%) were conform to this criterion and the remaining 78 doogh samples were therefore unacceptable in this regard. Hatamikia *et al.* (2012) reported that 90% of traditional dooghs were unacceptable due to high contamination via mold and yeast, which is in accordance with the current study [7]. They also stated that only 7.3% of industrial dooghs were unacceptable according to microbiological analysis.

A selection of studies has evaluated microbiological contamination in fermented dairy products. Ayran is a fermented dairy drinking product, produced either homemade (traditionally) or industrially in Turkey, with different amounts of mold and yeast. According to Altay *et al.* (2013), traditional ayran was contaminated with mold and yeast in the range of 3.23 to 7.23 log cfu/mL [25]. High contamination of traditional ayran with mold and yeast supports the data obtained in the current study.

Table 1: Analytical characteristics of the method for potassium sorbate, sodium benzoate, and natamycin

Analyte	Calibration equation	R^2	Linear range (mg/kg)	LOD (mg/kg)	LOQ (mg/kg)	RSDr % (n=3)	Measurement uncertainty (mg/kg)	Accreditation
Sodium benzoate	y = 6064.2x + 569.07	0.9997	0.5-100	0.0623	0.2077	0.7027	2.023	Yes
Potassium sorbate	y= 191993x +223.18	0.9996	0.02-4	0.0029	0.0099	1.6314	0.085	Yes
Natamycin	y= 41223x + 210.17	0.9995	0.05-10	0.0104	0.0348	1.3315	0.491	Yes

Table 2: Spiked data for potassium sorbate, sodium benzoate, and natamycin

	Non-spiked	Spiked			
	(0 mg/kg)	(25 mg/kg)	(100 mg/kg)	(25 mg/kg)	(100 mg/kg)
Sodium benzoate		Found		Recovery	
Sample 1	ND	24.043	100.257	96.172	100.257
Sample 2	ND	25.462	103.461	101.848	103.461
Sample 3	ND	24.939	102.480	99.756	102.480
	(0 mg/kg)	(0.8 mg/kg)	(4 mg/kg)	(0.8 mg/kg)	(4 mg/kg)
Potassium sorbate		Found		Recovery	
Sample 1	ND	0.782	4.329	97.75	108.225
Sample 2	ND	0.797	4.002	99.625	100.05
Sample 3	ND	0.808	3.941	101	98.525
	(0 mg/kg)	(2 mg/kg)	(10 mg/kg)	(2 mg/kg)	(10 mg/kg)
Natamycin		Found		Recovery	
Sample 1	ND	1.946	10.235	97.3	102.35
Sample 2	ND	1.901	9.923	95.05	99.23
Sample 3	ND	1.979	10.089	98.95	100.89

However, another study carried out by Baruzzi *et al.* (2016) showed 1.8 log cfu/mL mould and yeast in ayran, which is lower than for doogh results in the current study [26].

The high contamination of mold and yeast in doogh samples in the present study may have been due to the entry of these microorganisms into the production line through contamination of equipment, containers, or storage tanks [27]. We suggest that among all possible sources of contamination, dried and ground vegetables added to doogh is the most susceptible factor for effecting high mold and yeast contamination. In addition, our inspection from doogh manufacturing plants revealed that these dried vegetables are prepared from low-quality vegetables that have been stored in undesirable conditions for a long time. The poor condition of cold chain supply processes in the storage and distribution of traditional doogh may also be a factor. Although mold and yeast in foods can be a major risk for consumers, the undesirable biological environmental factors of doogh including high salt content and low pH–did not allow for the formation of toxic metabolites [28].

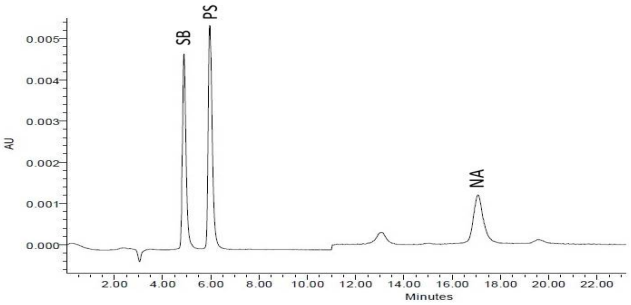

Figure 2: Chromatogram for standard solutions (SB 8 mg/kg, PS 0.32 mg/kg, and NA 0.8 mg/kg) SB: sodium benzoate PS: potassium sorbate, and NA: natamycin.

Therefore, it seems that the high levels of mold and yeast in doogh is not a concern for consumer safety.

3.2. Chemical analysis

The chemical analysis experiments for doogh samples are shown in Table 4. The lower pH of doogh compared to milk is beneficial for preserving and better shelf-life of this dairy product. ISIRI (National Standard No. 2453) sets a pH of 4.5 as the limit for doogh [14]. In this study, the pH in doogh samples was significantly lower than the ISIRI limitation ($P < 0.05$). According to ISIRI (No 2453), the total solids of doogh must be at least 3.2 g/100 mL [14]. With regard to this measure, seven of 89 samples were below the limit, which can be considered as adulteration. Nevertheless, doogh samples had significantly a higher level of total solid than 3.2 g/100 mL ($P < 0.05$). The salt content of five out of 89 samples were higher than the established Iranian standard (1 g/100 mL), but overall samples were significantly lower than the limitation set by this standard ($P < 0.05$). Although the higher salt content of doogh supports a longer shelf-life, it may be a risk for consumers predisposed to cardiovascular disease. Additionally, 83 samples (93.3%) met Iranian standards, and six samples contained more fat compared to standard limitations.

Since doogh production includes a dilution process using water, it is obvious that fat, pH, and the total solids in doogh will be lower than in milk. Kurut is a naturally fermented yak milk that is produced and consumed in China. Zhang *et al.* (2008) reported 14.3 g/100mL, 5.37 g/100mL, and 3.72 for total solids, fat and pH in kurut, respectively, which was higher than in doogh [29].

Chemical analysis of kefir, another fermented drinking dairy product, was carried out to select studies [30-32].

The total solids in these papers was higher than for doogh, and in the case of fat, Magalhaes *et al.* (2011) reported 2.34% fat for kefir which was higher than for doogh [32].

Table 3: Population distribution and mean of molds and yeasts in 89 doogh samples

Population groups (cfu/ml)	Frequency	Percent	Cumulative per cent	Mean (cfu/ml)	Standard deviation	Minimum	Maximum
<100	11	12.4	12.4	33.64	21.10	20	80
100-500	2	2.2	14.6	320	113.13	240	400
500-1000	7	7.9	22.5	654.29	109.97	600	900
1000-2000	2	2.2	24.7	1250	353.5	1000	1500
>2000	67	75.3	100	5705.97	964.503	2000	6000
Total	**89**	**100**	-	**4386.40**	**2470.1**	**20.0**	**6000.0**

However, other studies reported lower measures for fat [30]. Sarkar (2007) reported 0.2% which were much lower than in doogh. Fontan *et al.* (2006) reported that the pH of kefir decreased during the first 24 h of fermentation and reached 4.24 which was higher than the pH for doogh [33].

3.3. Aflatoxin M1, potassium sorbate, sodium benzoate, and natamycin

Aflatoxin M_1 was undetected in all the samples in the present study. Mohajeri *et al.* (2013) evaluated 45 white cheese samples and Lighvan cheese samples from Rafsanjan, Iran, to detect aflatoxin M_1, and reported that contamination was found in 64.4% of white cheeses (with a mean of 135 ng/kg), and in 27% of Lighvan cheeses (with a mean of 90.8 ng/kg) [34]. In one of the rare studies that evaluated aflatoxin M1 in doogh samples, industrial and traditional dooghs were compared. The data obtained by the mentioned study show that aflatoxin M_1 was detected in 22.5% of industrial dooghs, while only 13.8% of traditional dooghs were contaminated [35]. Low amounts of aflatoxin M_1 which was found in traditional dooghs in Fallah *et al.* (2009) supports the results of our study. Additionally, the mean concentration of aflatoxin M1 in their study was 0.007 ± 0.002 ng/kg and 0.003 ± 0.001 ng/kg for industrial and traditional dooghs, respectively. Furthermore, 4.2% of industrial dooghs exceeded ISIRI limitations, while all traditional dooghs were within the standard range. A study conducted by Shahbazi *et al.* (2017) evaluated aflatoxin M_1 in 360 cheese samples. The results show that 194 (53.8%) of total samples contained aflatoxin M_1 (mean 139.4 ± 2.4), and 38 of 360 cheese samples (10.5%) had higher amounts of aflatoxin M_1 than indicated by Iranian standards [9].

Potassium sorbate, sodium benzoate, and natamycin were negative in all the samples. This denotes the high number of mold and yeast in the samples of the current study.

According to ISIRI, potassium sorbate and sodium benzoate is forbidden as preservatives. Zamani Mazdeh *et al.* (2014) studied the preservation used in Iranian dooghs and found that sodium benzoate was present in all of 130 doogh samples, while only 13% of them contained potassium sorbate [36]. This disagreement with the results of the current study may be related to sampling. In Zamani Mazdeh *et al.* (2014), samples were obtained from supermarkets in Tehran, Iran, and had been produced by large dairy plants. In the current study, samples were collected from small and local producers. It seems that these producers may not have the knowledge for applying these types of preservations when making doogh. Another possibility is improvements in the recent years in the monitoring of preservative applications in food production which may have led to their removal in Iranian doogh samples. The concentration of these preservatives varied significantly based on the amounts that manufacturers used.

Table 4: Chemical analysis of doogh samples

	Mean	Standard deviation	Minimum	Maximum
pH	3.38	0.20	2.99	4.3
Total solid (g/100ml)	4.49	1.01	2.25	8.57
Salt content (g/100ml)	0.74	0.14	0.43	1.33
Fat (%)	0.97	1.01	0.1	9

4. Conclusion

The overall results show that 89.88% of all the samples would be rejected by the ISIRI No 2453; furthermore, mold and yeast contamination was the primary challenge for traditional dooghs. The undesirable hygienic conditions of these manufacturers were revealed and efforts should be made to address this problem. Future studies are highly suggested on the different stages of the manufacturing process to determine contamination sources for mold and yeast. Further, personnel should be provided with essential training and education.

Authors' Contributions

R.Z., A.B., and S.M.N., conceived and developed the idea for the article. R.Z, M.J., M.A., N.S., and S.M.N., designed the study. M.S., S.M.N., and A.B., contributed to data collection and data analysis. S.M.N., obtained funding, executed the research, and approved the final manuscript. R.Z., provided resources. All the authors discussed, revised, and commented on the final manuscript.

Conflicts of Interest

The Authors declare that there is no conflict of interest.

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