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ORIGINAL ARTICLE

Evaluation of Physicochemical and Microbiological Properties of Milk Collected from Traditional Sale Centers in Shiraz, Southern Iran

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ABSTRACT

Background: Milk and dairy products have important roles in the human diet. Therefore, quality control and monitoring of the hygiene standards of dairy products during production, transportation, storage, and distribution are of particular importance. Due to the poor sanitary conditions during storage, improper heat treatment, and post-contamination, traditional dairy products can be a good media for the growth of a wide range of microorganisms

Methods: This study aimed to evaluate traditional dairy products in Shiraz including 25 samples of raw cow's milk (cold and warm seasons), for significant risk factors. Physical, chemical, and microbial tests were performed according to the national standard of Iran.

Results: The trend of changes in fat, solids-non-fat, and freezing point of raw milk samples in cold seasons was more than in hot seasons. In addition, instability against alcohol and somatic cells was higher in warm-season samples. The milk density was unrelated to seasons. The microbial quality of the samples was not within the national standard of Iran, which could be due to improper storage conditions and lack of cold chain control from livestock to supply centers. PCR analysis confirmed the absence of two pathogenic bacteria *Escherichia coli* O157:H7 and *Listeria monocytogenes* in raw milk samples.

Conclusion: According to these findings, the quality of traditional dairy products distributed in Shiraz was not good. Therefore, having a specialized technical manager for traditional dairy units is essential. Furthermore, more serious and more supervision are needed from livestock to distribution centers by relevant organizations and centers.

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Introduction

Milk is considered as a complete food due to its variety for minerals and vitamins and plays an important role in the growth and maintenance of human health. Milk and dairy products are the main sources of calcium and their consumption is recommended to maintain the health and strength of bones, teeth, and skin (1, 2). Dairy products

like milk contain beneficial proteins, fat, vitamins and the required electrolytes that should be in a daily household basket. According to WHO per capita, consumption of 200 litters of milk and its derivatives per year is one of the effective factors on raising IQ level, life span and learning ability (1). People's lack of knowledge about the nutritional value of milk and its derivatives and the actual consumption pattern are important determining factors in the consumption of milk and other dairy products (3).

In unsanitary conditions, milk and dairy products as well as their improper storage can cause premature spoilage by microorganisms. Milk contaminated to bacteria not only reduces the product quality, but also poses a serious threat to consumer health. One of the most important types of these bacteria is *Escherichia coli*, which is considered as an indicator of fecal contamination in food hygiene laws and related standards (4, 5). *Listeria monocytogenes* is a widely distributed food pathogen that can have adverse health effects such as intrauterine infection, meningitis, and sepsis. Major sources of listeriosis are dairy products, unpasteurized milk, and soft or semi-soft cheeses (6). Somatic cell count is an important criterion to assess the quality and health of raw milk. Raw milk contains some somatic cells that increase dramatically in the presence of bacterial infection, tissue damage, or other factors that cause inflammation. Important microorganisms such as Staphylococci, Streptococci, coliforms, and even fungi can cause mastitis (7, 8). Aflatoxin M_1 (AFM_1) in milk and dairy products is another serious problem. It is heat and processing stable and storage conditions are ineffective in reducing the concentration of AFM_1 in milk and milk products (9, 10). Therefore, to prevent or minimize the health risks of AFM_1, immediate steps must be taken, including strategies to reduce it and develop diagnostic methods.

Antibiotics are used in livestock to control and treat infectious diseases in domestic animals and livestock, as well as to stimulate livestock growth. Due to the harmful effects that veterinary drug residues have on humans, the presence of antibiotics in milk and dairy products is not tolerable; because it may cause allergic reactions and digestive problems in the consumer (11, 12). Traditional dairy products have received a lot of attention from consumers in recent years, so the review of these products is very important for both government agencies and consumers. Therefore, the analysis of these products in order to identify risk factors is very important in increasing consumer awareness. The aim of this study was to find the most important health risk factors of traditional dairy products from 25 samples of raw cow's milk (cold and warm seasons) distributed in Shiraz, southern Iran by evaluating their physical, chemical, and microbial properties. The results were compared with the allowable limit reported by the Iranian National Standard.

Materials and Methods

Samples of raw milk were randomly purchased from retail markets in Shiraz, southern Iran. The samples included 50 raw milk samples [25 samples of the cold seasons (November-February) and 25 samples of the warm seasons (April-July)] (13). Freezing point is the most stable property of milk, which is directly related to the number of particles in milk. The samples were collected at 4-6°C and then transported to the laboratory inside a cold box. Chemical properties of raw milk including chemical composition [fat, protein, solid non-fat (SNF), and lactose] and freezing point were assessed by a rapid milk analyzer (Milkoscan FT2, Foss, Hillerød, Denmark). As the pH is a good indicator of initial milk quality, traditional measurement of titratable acidity that shows bacterial growth in milk is less precise*; m*ilk is slightly acidic or close to neutral pH *and f*resh cow milk typically has a pH between 6.5 and 6.7, which changes over time (14)*;* therefore, the present study assessed the pH in samples. The pH and acidity of the milk samples were measured using a pH meter (Mi 180 Bench Meter, Szeged, Hungary) and titration method, respectively, according to the method described in the INSO No. 2852 (15, 16). The acidity measure makes it possible to control the quality of raw milk. One Dornic degree (1°D) is equal to 0.1 g of lactic acid per liter. The acidity of raw milk as mentioned before must be in the range of 14-16°D (15). Acidity was calculated as percentage of lactic acid. Milk density quickly indicates deviations from the normal milk composition such as water addition. The density of raw milk is dependent on its composition and temperature, usually in the range of 1.026-1.034 g/cm^3 at 20°C (15) that was determined in this study. As described before that the lactose content of milk should be in the range of 4.6-4.8%; this variable was measured in this study (15).

The moisture content of the samples was measured based on the INS No. 1753. For determination of antibiotic residues in the milk samples, antibiotic test kits provided by Unisensor (KIT035, Seraing, Belgium) were used in this study. The principle of detection in this rapid test was based on immunochromatographic method. The presence of antibiotic residue in milk is a major problem that can affect both human health and the production of some dairy products such as yogurt. The presence

of tetracycline and beta-lactam antibiotics was monitored in the milk samples as antimicrobial detection kits are very accurate, sensitive and ease to use for rapid detection of low levels of antibiotics. Raw milk adulteration is an important concern especially in developing countries due to the lack of monitoring and policies. In this study, the presence of formalin, chromate, and potassium bchromate in the raw milk samples was investigated using the method described by Hossain *et al.* (17). In this study, immunochromatographic rapid test strips (rapid test KIT041, Unisensor, Seraing, Belgium) were used for specific detection and determination of AFM_1. This quantitative test strips utilized the high affinity of monoclonal antibody against AFM_1, which could easily identify its contamination in the milk samples without any instrument. The detection limit of the kit met both European and USA MRLs (maximum residue level), when employed properly. In this method, test strips were inserted into the milk samples and then placed into a heating block. After 10 minutes, the results were recorded by a portable strip reader (Read sensor L018010, Unisensor, Seraing, Belgium). As the total viable count is the most common test to estimate the total number of aerobic bacteria in the product, successive dilutions of raw milk were prepared from raw milk samples. Then 1 mL of the sample was added to 9 mL of physiological saline and finally the culture medium plate count agar was added by the pour plate method. The plates were incubated at 30°C for 48 h (18). Also, somatic cells count in the raw milk samples were performed using a Lactoscan SCC device (Lactoscan SCC, Milkotronic Ltd, Nova Zagora, Bulgaria). One of the major problems that threaten milk and other dairy products is mycotoxin contamination. Therefore, it is important to measure AFM_1 levels in milk and dairy products to protect consumers from its potential hazards (19) that were assayed in the present study. Raw milk is also graded based on the number of somatic cells. Generally, somatic cells are an important indicator in milk, and somatic cell count is used as an indicator of udder health and milk quality that was investigated in this study.

Milk is extremely susceptible to spoilage by microorganisms due to the presence of various nutrients which provides an excellent medium for bacterial growth. Regarding the appropriate conditions of milk, it can act as a carrier of disease-causing microorganisms transferred from cows to humans. Raw milk is graded in many countries (including Iran) based on its microbial population. The polymerase chain reaction (PCR) was performed to identify *L. monocytogen* and *E. coli* O157:H7 bacteria in the raw milk samples. A DNA extraction kit (Dena Zist, Tehran, Iran) was used to extract DNA from *L. monocytogenes* and *E. coli* O157:H7 in the raw milk samples. Oligonucleotide primers for the PCR assay were synthesized against the sequence of *hly* gene to detect *L. monocytogenes* and sequence of *stx* gene to detect *E. coli* O157:H7. A pair of primers including forward: 5′-AGC ACA ACA AAC TGA AGC AAA GGA-3′ and reverse: 5′-ATT GTG ATT CAC TGT AAG CCA TTT CGT CAT-3′ were used to amplify a 596 bp fragment of the *hly* gene in *L. monocytogenes*, and a pair of forward: 5′-GCC GGG TTC GTT AAT ACG GCA-3′ and reverse: 5′-GAA CGT TCC AGC GCT GCG ACA-3′ were utilized to amplify a 391 bp fragment of the *stx* gene in *E. coli* O157:H7. The conditions of the PCR method for *E. coli* and *L. monocytogenes* were listed Table 1. The PCR products were separated by electrophoresis on a 1.5% agarose gel. In the present study, the DNA extracted from *L. monocytogenes* PTCC 1297 and *E. coli* O157:H7 ATCC 43895 was used as the positive control (6, 20). All tests were performed in three replications. Data analysis was performed using Microsoft Office Excel, version 2016 and results were expressed as mean±standard error. Data were compared with the Iranian National Standardization Organization (INSO).

Results

The physicochemical properties of raw milk samples during the hot and cold seasons in comparison with the standard values were represented in Table 2. According to INSO number 164, the minimum fat and protein contents of raw milk samples was 3.2% and 3%, respectively. In the present study, 32% of the cold-season raw milk samples and 28% of the warm-season raw milk samples were out of the standard range in terms of fat content. In addition,

Table 1: Thermal cycles used in PCR process for Escherichia coli O157:H7 and Listeria monocytogenes.

Phase	Temperature (ºC)	Time (s)		Number of cycles
		L. monocytogenes	*E. coli*	
Hot start	94	120	300	1
Denaturation	94	45	60	35
Annealing	58	45	30	
Extension	72	60	90	
Final extension	72	300	600	1

in terms of protein content, 28% of the cold season samples and 40% of the warm season samples were out of the standard range. According to Table 2, the average fat and protein contents in the cold season samples were higher than those of the warm season samples. Climatic conditions and geographical locations could impact on the amount of these variables. In terms of SNF content, in comparison with the standard value (minimum 8%), 12% of cold and hot season samples were out of the range. Lactose, the major carbohydrate in milk, was found in most mammals, but other carbohydrates were also found in small amounts in milk. The lactose content of milk was 64% among cold season samples that was out of the standard range.

In this study, 60% of warm-season samples and 52% of cold-season samples showed acidity outside the standard range. It was demonstrated that 8% of the cold season samples and 40% of the warm season samples were not within the standard range. Cold season samples showed a higher average value.

As shown in Table 2, there was no change in the density of raw milk samples between warm and cold seasons. Therefore, 16% of cold and warm season samples were outside the range set by the INSO. The freezing point of raw milk was in the range from -0.565°C to -0.525°C. As shown in Table 2, 32% of cold and 24% of warm season raw milk samples were out of range. In this study, the stability of raw milk samples to ethanol (68%) was 72% and 40% in cold and warm season samples, respectively. Examination of antibiotic residues in raw milk in cold seasons showed the presence of tetracycline and beta-lactam antibiotic residue in 4% of the samples. However, the results confirmed the absence of antibiotics in the warm season samples.

In this study, 28% and 12% of cold and warm season samples, respectively, contained formalin as an antimicrobial agent. Potassium chromate and potassium dichromate were not detected in any of the samples. AFM_1 concentration in cold and warm season samples was 4% and 16%, respectively (more than the permitted level of 0.05 ng/mL). As shown in Table 3, microbial grading of raw milk showed that 68% of cold season samples and 48% of warm season samples with a total count of higher than 106 CFU/mL were classified as unacceptable. The high microbial population of samples can be due to the

Table 2: Physicochemical properties of raw milk samples in comparison with INSO* levels.

Property	INSO	Warm seasons			Cold seasons		
		Mean	Range	Out of range (%)	Mean	Range	Out of range (%)
Fat (%)	Min 3.2	3.472	2.85-3.77	28	3.585	1.24-11.70	32
Protein (%)	Min 3	3.080	2.85-3.64	40	3.054	2.70-3.30	28
Lactose (%)	4.6-4.8	-	-	-	4.62	4.05-5.11	64
Solids-non-fat (SNF, %)	Min 8	8.288	7.57-9.19	12	8.346	7.34-9.27	12
Freezing point (°C)	-0.525 to -0.565	-0.539	-0.571 to -0.501	24	-0.535	-0.577 to -0.453	32
Density (g/cm^3)	1.029-1.033	1.029	1.027-1.032	16	1.029	1.022-1.034	16
pH	6.6-6.8	6.532	5.86-6.76	40	6.71	6.48-6.87	8
Acidity	14-16	14.709	11.97-21.06	60	14.842	12.78-19.17	52

*INSO: Iranian National Standardization Organization

Table 3: Grading the quality of raw milk samples based on somatic cell number and microbial population (total viable count).

Degree	Quality of raw milk	Standard	Conformity	
			Warm seasons (%)	Cold seasons (%)
Excellent	Microbial population	Max 3×10^4 CFU/mL	0	4
	Somatic cells	Max 10^5 cells/mL	8	8
Degree 1	Microbial population	Max 3×10^4-10^5 CFU/mL	8	4
	Somatic cells	Max 3×10^5 cells/mL	4	16
Degree 2	Microbial population	Max 10^5-5×10^5 CFU/mL	16	4
	Somatic cells	Max 4×10^5 cells/mL	32	12
Degree 3	Microbial population	Max 5×10^5-10^6 CFU/mL	28	20
	Somatic cells	Max 5×10^5 cells/mL	8	12
Unacceptable	Microbial population	Max 10^6 CFU/mL	48	68
	Somatic cells	Max of 5×10^5 cells/mL	48	52

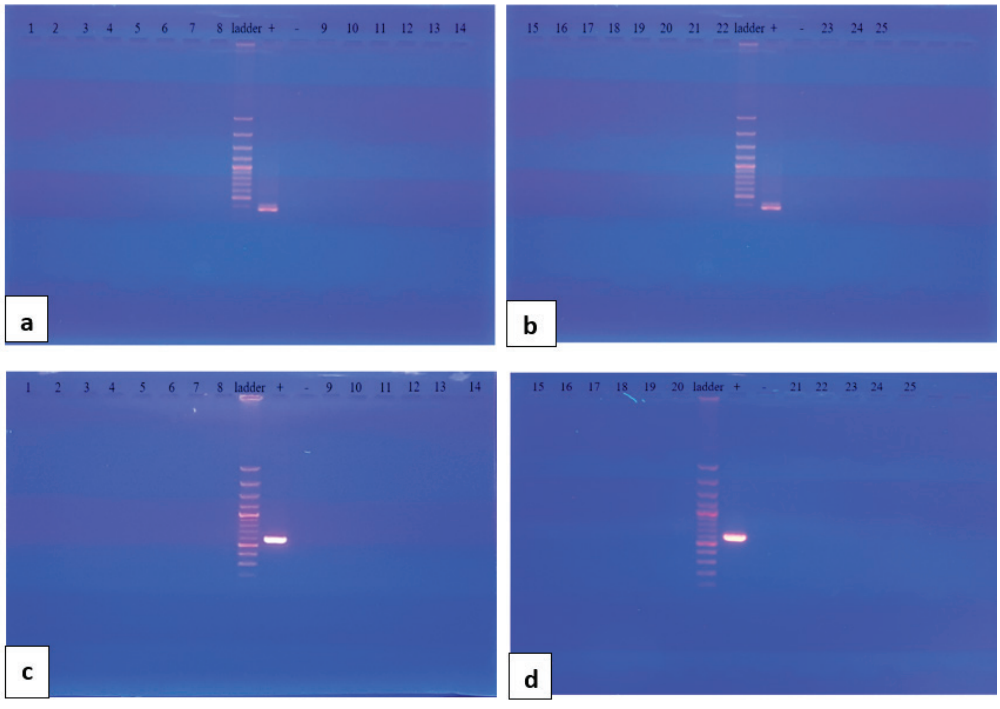

Figure 1: a and b: Electrophoresis patterns of the PCR product resulting from the amplification of the *stx* gene in *Escherichia coli* O157:H7. Lanes 1 to 8, 12 to 25, and 29 to 31 samples of raw milk, lanes 9 and 26 markers; lanes 10 and 27 positive controls, lanes 11 and 28 negative control. c and d: electrophoresis patterns of the PCR product resulting from the amplification of the *hly* gene in *Listeria monocytogenes*. Lanes 1 to 8, 12 to 23, and 27 to 31 samples of raw milk, lanes 9 and 24 markers, Lanes 10 and 25 positive controls, lines 11 and 26 negative control.

storage of raw milk samples at ambient temperature, especially in the cold seasons, for more than one day, combining several types of milk with different qualities and/or poor hygiene during milking, storage, and transportation. According to Table 3, the number of somatic cells in cold-season samples was higher than in warm-season samples. Indeed, 13% of cold season samples and 12% of warm season samples contained more than 5×10^5 cells/mL. Out of 50 raw milk samples screened for the presence of *E. coli* O157:H7 and *L. monocytogenes*; as shown in Figure 1a and 1b, *E. coli* O157:H7 was not detected by PCR in any raw milk samples. Also, as presented in Figure 1c and 1d, *L. monocytogenes* was not observed in any raw milk samples after PCR analysis.

Discussion

In physical and chemical analysis of raw milk samples, the results were in agreement with findings by Chen *et al.*, who reported that raw milk produced in the autumn had a significantly higher fat content than in other seasons, while protein and casein contents showed less variability. However, significant higher protein content was observed in spring when compared to the summer and autumn periods, which reflects the particular feeding regime used for the herd (21). According to Chen *et al.*'s findings, the total solids of milk in the fall were higher than the summer time, but no significant seasonal changes were observed during the spring and winter (21). The reason for the lack of change in lactose level during warm season can be due to the breakdown of fat stored in the cow body, through which the level of blood glucose and subsequently milk remains constant (22). According to Chen *et al.*'s results, lactose content did not change significantly throughout the year since lactose is one of the ingredients of raw milk with the least change.

Acidity above 16°D indicates microbial activity that breaks down lactose and converts it to lactic acid. In addition to microbial activity, vigorous stirring during transport and freezing in the storage tank can be the reasons for increased acidity. Acidity less than 14°D can be related to adulteration in raw milk (e.g. addition of soda, phosphate, and bicarbonate compounds), mastitis, and an increase in somatic cells. Milk bacteria convert the sugar lactose into lactic acid and makes the milk goes sour, while it becomes more acidic and the pH decreases too. The first milk secreted by a cow contains colostrum, which leads to a lower pH. If the cow has diseases such as mastitis, the pH of the milk can be higher or more basic (14), while the pH of a normal raw milk should be around 6.6-6.8. Chen *et al.* reported that the pH value shows a similar seasonal trend to the protein content, which is significantly higher in spring when compared to summer and autumn seasons. However, buffering capacity does not

display any significant seasonal trend (21).

Chen *et al.* demonstrated that there was no significant difference in raw milk density with seasonal variations throughout the year (21). The literature indicates that the freezing point of milk varies within relatively narrow limits. Some of the variations were indicated to be related to seasonal effects, feed, water intake, time of day (i.e., morning milk versus evening milk), breed of cow, and method of handling the samples (23). As milk becomes more diluted, the freezing point will rise closer to zero (23). Therefore, freezing point measurement is an appropriate indicator that can be used to control the quality of the milk production chain, especially to detect a possible adulteration. An alcohol test is used on fresh milk to indicate whether it will coagulate in thermal processing or not. This test is especially important for manufacturing Ultra-high temperature (UHT) milk, evaporated milk, and milk powder. This parameter is significantly related to the pH, protein, casein, and fat (21).

Improper administration of antibiotics by farmers and veterinarians without observing the withdrawal time for treated animals can result in antibiotic residues in milk and milk products, which contributes to the development of microbial drug resistance and the spread of resistant bacteria, including those with serious health consequences (24). Milk adulteration includes the addition of toxic substances such as formaldehyde, hydrogen peroxide, hypochlorite, dichromate, salicylic acid, melamine, and urea. These compounds are usually added to spoiled and sour milk to regulate the pH and prevent milk coagulation. To assure food safety and avoid health risks to consumers, novel analytical procedures have been proposed for the detection of these adulterants (25). In a previous study, 30% of raw milk samples contained hydrogen peroxide, 44% contained formaldehyde, and 55% contained urea (26).

Mycotoxins are natural pollutants and secondary toxic metabolites of molds that are produced mainly by specific species such as Fusarium, Penicillium, and Aspergillus under certain conditions of temperature and humidity. Among these toxins, aflatoxins (AF) are primarily important. AFB_1 is the most toxic type of aflatoxin family. When livestock are fed with contaminated food, AFB_1 is converted to AFM_1 during the hydroxylation in the liver and subsequently is secreted in the milk of lactating livestock. The major effect of aflatoxins is the creation of various cancers, especially liver cancer (27). Although AFM_1 has a potency approximately one magnitude lower than that of AFB_1, the presence of AFM_1 in milk is a major concern due to its high stability during heat processing as well as fermentation (28). In another study, the AFM_1 concentrations exceeded the maximum permitted levels in 2.9% of the samples, and the highest detected concentration was 408.1 ng/kg (28). It was shown that 2.2% of the raw milk samples contained AFM_1 more than the permitted level of 50 ppt (29).

The prevalence of foodborne pathogens including *Campylobacter jejuni*, Shiga toxin-producing Escherichia coli (STEC), *L. monocytogenes*, and *Salmonella spp.* in milk varies considerably (30). The prevalence of foodborne pathogens in milk is influenced by numerous factors such as farm size, number of animals in the farm, hygiene, farm management practices, variation in sampling and types of samples evaluated, differences in detection methodologies, geographical location, and seasons (30). The total number of bacteria, number of coliforms, and number of somatic cells were evaluated in raw milk collected from different regions. Results showed significant differences between various locations. Milk handling and adulteration practices were the most likely causes for the observed differences in the microbial quality of raw milk. Breed, parity number, feeding system, farming experience, and distance from dairy technology dissemination centers had a significant influence on the fat and protein contents of milk samples (31).

In another study, the microbial quality of raw milk was evaluated and was shown that in 48 samples, the number of total bacteria was very high. Among them, two milk samples contained the pathogenic bacteria of *L. monocytogenes* and *Staphylococcus aureus*, which pose potential health risks to the consumer. The findings denoted to poor hygiene during milking and in delivery to the consumer (32). The quality of raw milk was reported not to be satisfactory when 71.4% of raw milk samples were prepared from retail centers revealing a total number of bacteria of more than 10^5 CFU/mL (33).

The number of somatic cells is affected by several factors such as mastitis, stress, diet, and seasonal changes. The number of somatic cells indicates the health status of the mammary gland (32). Raw milk samples with high levels of somatic cells and bacteria are associated with increased enzyme activity that can result in product defects. The use of raw milk with somatic cell counts higher than 10^5 cells/mL has been shown to reduce cheese yields, while somatic cell count (SCC) higher than 4×10^5 cells/mL have been associated with textural and flavor defects in cheese and other dairy products (34). Moreover, the increase in the number of somatic cells is associated with the changes in bovine milk components such

as lactose, fat, and casein contents. According to a previous the research, the amount of raw milk somatic cells produced in winter was significantly higher than in other seasons, which probably reflects the difference in herd management (21).

The *E. coli* O157:H7 is a major cause of food-borne illnesses in humans. Symptoms include diarrhea, intestinal bleeding, and severe inflammation. Molecular tests such as PCR are the best way to identify bacteria because they are less expensive and more sensitive than other methods (35). *S. aureus* and *E. coli* were isolated from milk and milk products in a previous study (36). The results of this study showed that 10.3% of the samples were infected with *S. aureus* and 8.1% were infected with *E. coli*. These organisms are significant in terms of public health because they have been associated with the onset of food poisoning in humans. In agreement with are findings, *E. coli* contamination was not observed in raw milk and ice samples evaluated by PCR method (36). *L. monocytogenes* is abundant in the environment, water, and a wide range of food processing media and can appear in all raw food products (37); while its contamination was not seen in raw milk and ice samples assessed by PCR technique.

Conclusion

When the obtained data were compared with the Iran National Standards Organization, it is necessary to have more accurate and principled supervision over the production of traditional products and to provide the necessary training for farmers and producers for better quality products.

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Authors' Contribution

M.E: Software, Methodology, Data curation, Writing – original draft. M/M: Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. R.NS: Software, Methodology, Data curation, Writing – original draft. SM.HH: Validation, Methodology. M.G: Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Conflict of Interest

None declared.

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