Prevalence of Pathogenic Microorganisms in Traditional Dairy Products of Mashhad, Iran

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ABSTRACT

Background: Milk and dairy products have an essential role in human health; however, they are easily contaminated and are likely to transfer pathogenic foodborne bacteria. Since traditional dairy products are very popular and are frequently used by the community, the aim of this study was to assess the contamination rate of raw milk and traditional dairy products by pathogenic bacteria and mold in Mashhad, Iran.

Methods: A total of 200 samples were collected from five districts of Mashhad, Iran in the summer of 2018. Samples were tested for Coliform, *E. coli*, *S. aureus*, *Salmonella* spp, *Listeria monocytogenes*, mold, and yeast according to the Iranian national standard methods.

Results: Results showed that 65% of the samples were positive for at least one pathogenic bacteria. The prevalence of Coliform, *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* spp., molds, and yeast in all dairy products were 62.5%, 32.5%, 59.5%, 27.5%, 26%, and 100% respectively.

Conclusion: The results indicated that considerable numbers of traditional dairy products were contaminated with pathogenic microorganisms in Mashhad, Iran which emphasized the need for stricter hygienic rules and general education about the safety of traditional foods.

1. Introduction

Foodborne diseases are one of the most common public health hazards in developing and developed countries [1].

Milk has high nutritional value containing calcium, essential amino acids, vitamin B2 (riboflavin), vitamin A, phosphorus, and proteins. Moreover, it is considered a rich medium for the growth of the pathogens that cause several Foodborne diseases [2, 3]. Milk contamination is the consequence of exogenous transfer (i.e. during or after the milking process) or endogenous transfer (i.e. by infections in the udder or direct transfer of blood) [4].

Bacillus cereus, *Salmonella* spp., *Campylobacter* spp., *Listeria Monocytogenes*, *Shiga toxin-producing Escherichia coli* (STEC), *Staphylococcus aureus*, *Mycobacterium bovis*, *Clostridium botulinum*, *Brucella* spp., *Helicobacter pylori*,

Leptospira spp., *Cryptosporidium parvum*, and *Toxoplasma gondii* are the main microorganisms associated with cow, sheep, and goat raw milk [4]. Traditional cheese can be the most contaminated dairy because of using unheated milk in the producing process or microbial survival during the ripening process [5]. Furthermore, cream is a by-product of milk and provides a suitable environment for the growth of microorganisms due to its high nutritional value and neutral pH [6].

Previous studies in Tehran, Zanjan, west Azerbaijan, Sistan and Baluchestan, and Markazi provinces have reported high loads of cheese microorganisms, particularly Coliforms, *Escherichia coli*, *Staphylococcus aureus*, molds, and yeasts which were below the Iranian National Standard [7-11]. A similar research conducted in Isfahan has shown that 11.11% of cream samples were contaminated with *Listeria* spp. [12].

Due to the lower cost and organoleptic properties, traditional dairy products are frequently used in Mashhad. Therefore, accurate data about the microbial quality of these products is necessary for future planning and control strategies. This study aimed to evaluate the microbial contamination rate of major traditional dairy products (cheese, milk, and, cream) of Mashhad, Iran.

2. Materials and Methods

2.1. Sample collection

Samples were randomly collected from retail markets in Mashhad, Iran by two-stage cluster sampling between July and October 2018. In the first stage, Mashhad was divided into 5 regions- considered as a district- based on the number of health centers. Each of the 5 regions was then divided into several headquarters which were managed under the health center supervision. Four headquarters were randomly selected in each region (Table 1). Then 40 samples were obtained from each district with a one-month interval (20 samples monthly including 4 milk, 4 cream and 12 cheeses). Four types of more common traditional cheeses were selected from each region including 3 lactic, 3 Onsori, 3 Kordi, and 3 Lighvan cheeses. Samples were aseptically transferred to the laboratory of Nutrition department of Mashhad University of Medical Sciences in sterile containers at 4 ℃.

2.2. Microbial culturing

Regarding solid foods like cheese and cream samples, 10 g were mixed with 90 mL of sterile 0.1% peptone water (w/v) by a mechanical blender (to obtain homogenous suspension). For liquid food sample, like raw milk, serially dilutions were prepared.

2.2.1. Total count

After initial preparation and serial diluting of the samples, total counts of the samples were carried out according to the method for the enumeration of microorganisms at 30 ℃ [13].

2.2.2. Coliform

Coliform enumeration was performed on Violet Red Bile Agar (VRBA) (Merck, Darmstadt, Germany) according to the Iranian national standard [14]. Based on this method, 1 mL of each dilution was added to sterile petri dish and 15mL of VRBA was pour plated and plates were incubated at 37 ° C for 24 h. Specific red-purple colonies were counted and to confirm colonies as coliform, lactose fermentation test was used.

2.2.3. Escherichia coli

After preparation for the suspension, 1 mL of the samples was streaked on VRBA and incubated at 37 ℃ for 24 h. Red-purple colonies were transferred to the Brilliant Green Bile Broth (Merck, Darmstadt, Germany) and incubated at 44℃ for 24-48 h. Gas positive tubes were selected for further confirmation by Eosin Methylene Blue agar (EMB, Merck, Darmstadt, Germany) and differentiation of E. coli was performed by Indole Methyl Red Voges-Proskauer Citrate Utilization test. A control positive sample (PTCC 1338) was provided from the Iranian research organizations for science and technology (IROST) [15].

2.2.4. E. Coli O_{157}: H_7

About 1 mL of sterile sample was added to 9 mL of lauryl sulfate tryptose medium (Merck, Darmstadt, Germany) - which contained novobiocin and then incubated at 37 ℃ for 24 h.

After the enrichment step, Sorbitol MacConkey agar medium (Merck, Darmstadt, Germany) containing Cefixime and potassium tellurite (CT-SMAC, Merck, Germany) was used for the selective and differential isolation of enterohemorrhagic *Escherichia coli* O_{157}: H_7 and then incubated at 37 ℃ for 24 h.

Colorless colonies on CT-SMAC were further confirmed on the EMB agar [16].

2.2.5. Staphylococcus aureus

25 mL of each sample was homogenized with 225 mL of Buffered Peptone Water (BPW) (Merck, Darmstadt, Germany) for about 10 min. Then 0.1 mL of the samples were spread cultured on the surface of Baird Parker agar (Merck, Darmstadt, Germany), supplemented with egg yolk–tellurite emulsion and incubated at 37℃ for 24 h.

Table 1: Samples collected from different regions of Mashhad city

	Region				
	1	2	3	Samen	5
subgroup	4 Headquarters	4 headquarters	4 headquarters	4 headquarters	4 headquarters
First month	4milk 4cream 3lactic 3Onsori 3Kordi 3 Lighvan	4milk 4cream 3lactic 3Onsori 3Kordi 3 Lighvan	4milk 4cream 3lactic 3Onsori 3Kordi 3 Lighvan	4milk 4cream 3lactic 3Onsori 3Kordi 3 Lighvan	4milk 4cream 3lactic 3Onsori 3Kordi 3 Lighvan
Second month	4milk 4cream 3lactic	4milk 4cream 3lactic	4milk 4cream 3lactic	4milk 4cream 3lactic	4milk 4cream 3lactic

Coagulase and DNase activity confirmation tests were used for the enumeration of *Staphylococcus aureus*. Grey to black colonies with clear halo developed around them were selected for this purpose [17].

2.2.6. Salmonella spp.

At first in order to prepare the suspension, 25 mL of each sample was dissolved in 225 g of BPW (Merck, Darmstadt, Germany) and incubated at 37 ℃ for 24 h. Following the enrichment and selective culturing of *Salmonella* spp., Tetrathionate, selenite cysteine broth, *Salmonella Shigella* Agar (SSA), and Xylose Lysine Deoxycholate (XLD) agar all provided from Merck, Germany were used, respectively, according to the method of Institute of Standards and Industrial Research of Iran. Finally, all suspected colonies were confirmed by using Triple Sugar Iron (TSI) agar (Merck, Darmstadt, Germany) [18].

2.2.7. Listeria monocytogenes

Listeria monocytogenes was detected by using Polymyxin Acriflavine Lithium chloride Ceftazidime Aesculin Mannitol (PALCAM) Listeria selective agar (Merck, Darmstadt, Germany) according to the Iranian National Standard protocol [19].

Briefly, after preparing the suspension in Listeria enrichment broth (Merck, Darmstadt, Germany) and incubation at 37 ℃ for 24 h, PALCAM agar was used for selective culturing at 37 °C for 48 h. Finally, black colored colonies with black halo were identified as presumptive *Listeria* spp.

For the confirmation, Tryptone Soya Agar (QueLab, Laboratories, and Canada) was used and plates were incubated for 24 h at 37 ° C. Single colonies were tested for gram-staining, catalase, glucose, and hemolysis tests.

2.2.8. Mold and yeast

Mold and yeast counting were done according to the Iranian National Standard [20] on Yeast extract glucose chloramphenicol agar (YGC) (QueLab Laboratories, Canada). After incubation at 25°C for 3-5 days, colonies were counted and yeast colonies were distinguished from molds by colony morphology.

2.3. Statistical analysis

All experiments were performed in triplicate and statistical analysis was performed using SPSS software (version 20).

P values less than 0.05 were considered statistically significant between regions.

The chi-square test was applied to compare the rate of microorganism contamination among various classes of dairy products.

3. Results and Discussion

A total of 200 samples including raw milk and traditional dairy products collected from different regions of Mashhad, Iran were examined in order to detect pathogenic bacteria. It was revealed that 130 samples (65%) were positive for at least one pathogenic bacteria. Figure 1 compares the ratio of contaminated samples in different regions. The ratio of positive results (microbial contamination) in different regions was almost the same. Although it was lower in the Samen region but the difference was not statistically significant ($P > 0.05$).

As shown in Table 2, 67.5% of milk, 62.5% of cream, and 64% of cheese samples were contaminated with pathogenic bacteria in all regions. Milk contamination was significantly lower in Samen region and in region "three" ($P < 0.05$). Cream contamination was also lower in Samen region, but more eminent in region "Two" ($P < 0.05$).

The Prevalence of pathogenic bacteria was significantly lower in region "Two" ($P < 0.05$). The prevalence of salmonella, *L. monocytogenes*, and *E. coli* in dairy products were 26%, 27.5%, and 32.5% respectively (figure 2). There were no statistically significant differences in the prevalence of *Salmonella* contamination amongst different regions ($P > 0.05$). The prevalence of *L. monocytogenes* contamination was 29.2%, 20%, and 30% in cheese, milk, and
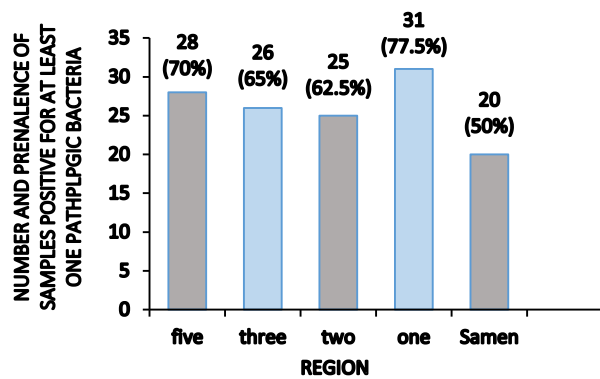
Figure 1: Comparing the contamination rate of dairy products (milk, cheese and cream) in different regions.

cream respectively. The prevalence of *L. monocytogenes* contamination was significantly higher in regions "one" and "three" (*P* < 0.05) and regions "two" and "five" had a greater number of E. coli contamination (*P* < 0.05). Results of *S. aureus, coliforms*, and total counts (above 5 Log10 cfu/ml) are presented in Table 3. Counts more than 5 Log10 cfu/ml of S. aureus and coliforms were detected in 59.5% and 62.5% of the samples respectively. In the present samples, all of the samples (milk+ cheese+ cream), were contaminated with mold and yeast more than the standard limits of Iran (10^3 cfu/ml) (Table 4). Samen region showed a significant difference with other regions (*P* < 0.05) regarding TNTC (too numerous to count) results.

Results of this study indicate that 65% of dairy product samples in Mashhad had inadequate hygienic condition. In the current study, 67.5% of milk samples had microbial contamination which was higher than the levels found in Zahedan (13.1%) and Azerbaijan (15%) [11, 21]. Another important finding was that 32.5% of all samples (milk+ cheese+ cream) were contaminated with *E. coli*. Similar studies found different prevalence in cheese samples from Tehran (54%), Markazi (24%) and even other countries like Italy (44%) and Turkey (18%) [7, 9, 22, 23]; that can be due to differences in raw milk contamination, processing, distribution, storage, salt concentration, and PH of the products [9].

Table 2: The prevalence of microbial contamination of dairy products in different regions of Mashhad

Region	Milk N (%)	Cream N (%)	Cheese N (%)
Five	6(75)	5(62.5)	17(71)
Three	3(37.5)*	6(75)	17(71)
Two	8(100)	7(87.5)*	10(45)*
One	8(100)	5(62.5)	17(71)
Samen	2(25)*	2(25)*	16(67)
Total	**27(67.5)**	**25(62.5)**	**77(64)**

*= indicates the statistically significant differences in prevalence of pathogens in all dairy products between different regions of Mashhad city (*P* < 0.05).

Another important finding was that 32.5% of all samples (milk+ cheese+ cream) were contaminated with E. coli. Similar studies found different prevalence in cheese samples from Tehran (54%), Markazi (24%) and even other countries like Italy (44%) and Turkey (18%) [7, 9, 22, 23]; that can be due to differences in raw milk contamination, processing, distribution, storage, salt concentration, and PH of the products [9].

In this study 26% of the samples were positive for *salmonella* spp*.* In a similar study by Rezaei *et al.* (2010) it was found that 8.75% of cheese samples were contaminated with *salmonella* spp. [9]. Pooled frequencies of *salmonella* spp. in Gonzales *et al.* (2017) systematic review were 1.4%-2.4% for sheep and goat raw milk [24]. However, Aygun *et al.* (2005) have reported that Salmonella was not detected in any of the cheese samples [22]. The main source of salmonella contamination of the cow milk tank is fecal shedding of the pathogen from asymptomatic animals [25]. The consumption of cheese from salmonella contaminated milk led to a large outbreak in France in 1993 [26]. Several studies have revealed different prevalence of Listeria species in raw milk and traditional dairy products. Rosengren *et al.* (2018) did not detect any *L. monocytogenes* in cheese samples [27]. In another study by Almeida *et al.* (2007), 11.4% of cheese samples were contaminated with *L. monocytogenes* [28]. The prevalence of *L. monocytogenes* in homemade white cheese in Turkey was 9.2% [29]. An earlier study in Mashhad found that 4% of raw milk samples were contaminated with *L. monocytogenes* [30]. In a systematic review published in 2017, frequencies of *L. monocytogenes* were in the range of 2.6-3.6% for sheep and goat raw milk in Australia, Brazil, China, Colombia, Costa Rica, Czech Republic, Egypt, Germany, Greece, Iran, Italy, Malaysia, Mexico, Norway, Poland, Portugal, Spain, Sweden, Switzerland, Turkey, UK, and the USA [24]. Prevalence of *L. Monocytogenes* in this investigation (27.5%) was higher compared to those of other studies in Iran (1.47%, 1.7%, 4.03%, and 5% in Isfahan, Yazd, Shahrekord, and Chaharmahal and Bakhtiari respectively.) [2, 12, 31, 32]. Dairy products, mainly cheese, are great sources for *L. monocytogenes* and has caused listeriosis outbreaks in many countries. *Listeria* spp. can grow over a wide temperature range (0–45 °C) and external stress, including salt concentrations up to 14%, water activity above 0.92, and extreme pH (4.4–9.4) [33-35]. *L. monocytogenes* can cause listeriosis, which is a serious infectious foodborne disease with a 20% mortality rate, correlated to food contamination level. Abortions, stillbirth, bacteremia, sepsis, meningitis, diarrhea, and fever are the main symptoms of listeriosis. These features make *L. monocytogenes* a serious foodborne organism [35, 36]. In the current study, 59.5% of the samples (milk, cream, and cheese) were contaminated with *S. aureus* more than 5 Log10 cfu/mL. This finding is in line with the previous studies in Mazandaran (62.2%) [38] and Mahabad (45%) [39], but higher than findings in Tabriz (20%) [40] and Brazil (15.1%) [41].
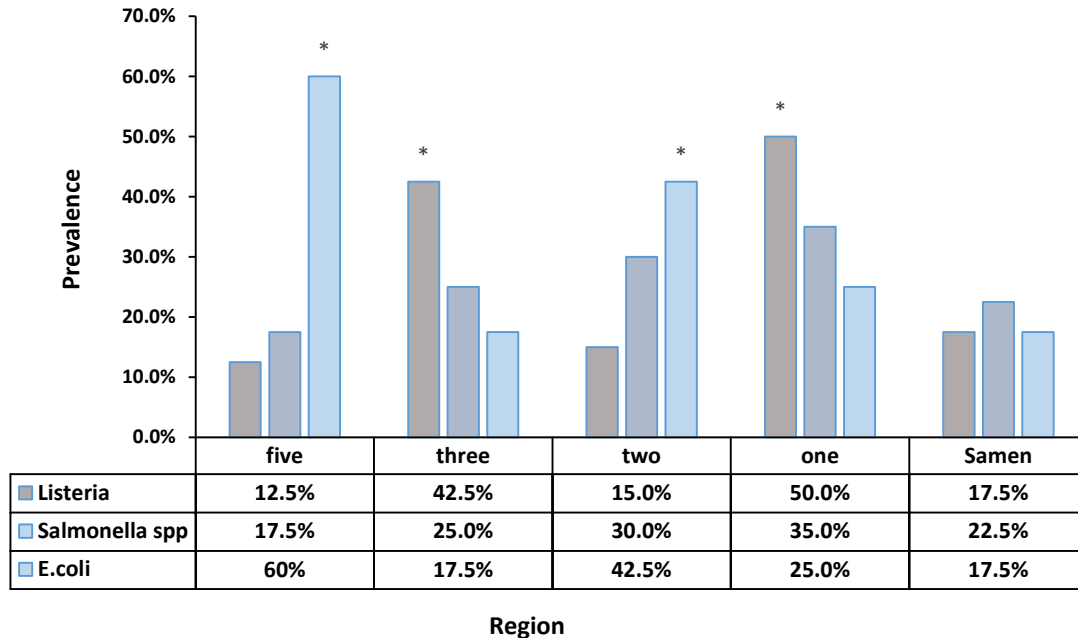
Region	five	three	two	one	Samen
■ Listeria	12.5%	42.5%	15.0%	50.0%	17.5%
■ Salmonella spp	17.5%	25.0%	30.0%	35.0%	22.5%
■ E.coli	60%	17.5%	42.5%	25.0%	17.5%

Region

Figure 2: Prevalence of *L. monocytogenes*, *Salmonella* spp. and *E. coli* in dairy products in different regions of Mashhad city
* indicates the statistically significant differences in the prevalence of pathogens in all dairy products (cheese, milk and cream) between different regions of Mashhad city ($P < 0.05$).

Staphylococcus aureus exists in the mammary ducts of cows with mastitis and has the ability to secrete in milk while milking; due to not using the pasteurization process in the traditional cheese production, bacteria survive in milk and can transfer to the cheese [42]. In this study, all of the samples (milk, cream, and cheese) had mold and yeast more than the standard level. This finding is consistent with the study by Rezaei *et al.* (2013) regarding cheese samples [9, 43].

Moreover, further analysis revealed coliforms in 62.5% of the samples (milk, cream, and cheese) which is in contrast with the previous studies that found higher contamination rate [44, 45]. However, Riadh *et al.* (2005) found that the prevalence of Coliforms was 10-fold greater in traditional cheese than industrial [42]. This study demonstrated a considerable contamination rate of dairy products in each region of Mashhad which will pave the way for better management in the future.

Table 3: Results of *S. aureus*, coliforms and total counts of dairy products in Mashhad city

	Mean(±SD) logs cfu/ml				Prevalence of samples (>5 logs cfu/ml)
	Milk	**Cream**	**Cheese**	**Total**	
Coliform	6.08 (±2.2)	4.39 (±2.7)	4.93 (±2.5)	4.83 (±2.6)	62.5
S. aureus	5.72 (±2.0)	5.9 (1±1.8)	4.35 (±2.6)	4.93 (±2.5)	59.2
Total counts	7.1 (±1.7)	7.14 (±1.0)	5.98 (±2.1)	6.43 (±1.9)	88.5

Table 4: Mold and yeast contamination in dairy products (cheese, milk and cream) of different regions of Mashhad city

Region	MOLD AND YEAST, N (%)
Five	40(100)
Three	40(100)
Two	40(100)
One	40(100)
Samen	40(100)

4. Conclusion

The purpose of the current study was to determine the prevalence of pathogenic microorganisms in traditional dairy products. The most striking result to emerge from the data is that pathogenic microorganisms in traditional dairy products in Mashhad are more than the standards levels and

these data highlight the public health risk associated in people who consume these products. In addition, the findings suggest that authorities need to consider cows' health as an important issue, apply better hygiene in farms and during transporting, educate the general population about the safety of unpasteurized dairy products. Moreover, the need to improve raw milk packaging in factories (according to the successful documents in other countries, including the United States and some European countries) under the supervision of relevant organizations along with the correct principles of GMP and HACCP is felt more than ever.

Authors' Contributions

Y.J.B., A.M.D., and M.R.T., carried out the experiment. H.Gh., wrote the manuscript with support from A.A., M.H., analyzed the data. A.A., supervised the project. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Conflicts of Interest

The authors report no actual or potential conflicts of interest.

Acknowledgements

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