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Risk Assessment and Antibiotic Resistance of Microbial Contaminants Isolated from Traditional and Industrial Ice creams in Shiraz City

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ABSTRACT

Background: Dairy products are considered as some staple food rich in nutrients. Among dairy products, ice cream is one of the most attractive and popular products. The popularity of ice cream is due to its great taste and unique texture. Considering the prevalence of traditional ice cream consumption in Iran and the risk of microbial contamination of these products, the microbial quality of industrial and traditional ice creams in Fars province was evaluated and compared. .

Materials & Methods: A total of 470 ice cream samples were tested to determine total viable count (TVC), total coliform count (TCC), and the presence of fungi. Biochemical properties and antibiotic sensitivity of isolated bacteria were investigated.

Findings: TVC in all industrial ice cream samples was lower than the limit specified by the national standard of Iran. Coliform levels in 37% (n=100) of traditional and 1.5% (n=3) of industrial ice cream samples were higher than the permissible limit. Furthermore, 74.8 and 40% of traditional samples contained *Escherichia coli* and fungi and mold, respectively. Antibiogram results showed that the highest antibiotic resistance of the isolates was related to ampicillin and trimethoprim/sulfamethoxazole.

Conclusion: Traditional ice creams are highly contaminated with microbial agents resistant to two or more drugs, which poses great risks for the consumers of these products, especially children. Therefore, it is necessary to implement appropriate hygiene practices to increase the safety of ice creams. Alerting people about the risks of foodborne diseases could significantly help prevent such diseases.

Keywords: Ice cream, Risk assessment, Foodborne diseases. *E. coli*, Antimicrobial resistance

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Introduction

Today, with increasing awareness about the role of nutrition in health, the consumption of dairy products has increased significantly. In many diets, dairy products are considered as a staple food rich in nutrients needed by the body [1]. Milk is a nutritious food rich in carbohydrates, protein, fat, phosphorus, calcium, and B vitamins [2]. Among dairy products, ice cream is one of the most attractive and popular products. The popularity of ice cream is due to its great taste and unique texture [3].

Ice cream originated from making a drink using snow. Discovering this phenomenon, Salt Peter created an ice-cooling mixture that could both cool and freeze drinks, finally leading to the creation of a cream-like product [4].

Ice cream is made from fresh milk, milk powder, cream, sugar, fat, stabilizers, emulsifiers, and additives such as colors, flavorings, nuts, fruits, jams, and eggs. Ice creams are produced in boxes or open packages and consumed mainly in the hot months of the year by a large number of people, especially children. Due to its high nutrient content, neutral pH, and long storage time, ice cream is susceptible to microbial contamination by a variety of organisms such as *Escherichia coli, Staphylococcus aureus, Salmonella enterica, Listeria monocytogenes*, coliforms, and bacilli [5-7].

In industrial production, pasteurization and freezing steps could destroy most disease-causing bacteria. However, the final product after pasteurization could also be contaminated by contaminated additives and improper handling [8].

But in traditional production, recontamination is very common due to the use of unpasteurized milk, non-compliance with the pasteurization temperature, and insufficient heat, or there may be a delay between cooking and freezing the milk. Also, non-compliance with personal and environmental health standards during production and distribution could mainly cause microbial contamination. Furthermore, since traditional ice creams are often sold in large quantities, improper storage conditions and non-compliance with cold chain requirements affect the microbiological quality of the product [9, 10].

From a risk assessment viewpoint, the health sensitivities of particular consumers, such as the elderly , young people, pregnant women, and immunocompromised individuals, deserve special attention because even low microbial loads may result in death [11]. In a study by Hassanzadazar et al. (2012), 78% of traditional ice cream samples contained a microbial load higher than 4.2×107 CFU/g. Moreover, 82.9% of the samples were contaminated with more than 10 CFU/g of Enterobacteriaceae. The results also revealed that 52.2 and 2.8% of the samples were contaminated with *E. coli* and coagulase positive S. aureus, respectively [12]. Moreover, Emami et al. (2013) reported that 77.5% of traditional ice cream samples had microbial loads higher than the standard limit. Also, 73.75 and 67.5% of the samples contained a large number of microorganisms and coliforms, respectively. Furthermore, 37.5 and 28.75% of the samples were contaminated with *E. coli* and *S. aureus,* respectively. However, Salmonella spp. were not found in any of the ice cream samples [13].

Previous studies conducted in Iran and other countries have also reported the microbial contamination of traditional ice creams [11-15].

Objectives: Despite global efforts to raise public awareness about foodborne diseases and their impact on public health, there are few studies on the prevalence of diseases caused by consuming improper food, especially in countries where there is no detailed epidemiological information on this issue.

Therefore, in this study, a comparison was made for the first time between the microbiological quality of traditional and industrial ice creams produced in Fars province. It is important to emphasize that conducting studies on food quality standards plays a crucial role in preventing the spread of foodborne illnesses. Alerting the public about the risks of diseases caused by consuming contaminated food and water as well as educating healthcare professionals on the importance of raising public awareness could significantly help prevent such diseases. Providing data to regulatory agencies to investigate suspected cases is essential and could assist policymakers to make informed decisions about the economic and social impacts of these diseases, which is crucial for developing effective control measures.

Materials and methods

Sample collection: This cross-sectional descriptive-analytical study was conducted on 470 ice cream samples (270 traditional and 200 industrial samples) collected from markets, sweet shops, and cafes producing and selling ice cream in Fars province from the beginning of 2020 to the end of 2023. The sampling method of this study was comprehensive. All the collected traditional and industrial ice cream samples were transported to the microbiology laboratory under sterile conditions and kept in compliance with cold chain requirements.

Evaluation of mycology testing: For mycological analysis, 10 g of each sample was first dissolved in Ringer's solution, then 0.5 cc of the solution was added to the YGC (yeast glucose chloramphenicol) culture medium and kept at 25 °C for 72 hours. Fungal pathogens were identified based on phenotypic characteristics such as background color and colony shape.

General measurement of total viable count (TVC) and total coliform count (TCC): TVC was measured based on the serial dilution method, streak culture technique, and colony counting, and TCC was measured by MPN (most probable number) technique based on the national standard of Iran (ISIRI 2406).

Isolation and biochemical identification of microorganisms: To isolate and identify *E. coli, S. aureus,* and *S. entrica* strains, streak culture method was performed on selective and differential media as follows: EMB (eosin-methylene blue agar), SS (Salmonella-Shigella agar), MacConkey agar, and MSA (mannitol salt agar). Growth and morphological characteristics were recorded based on biological techniques listed in Lieber's book. Biochemical tests such as catalase, fermentation of sugars, motility, TSI (triple sugar iron), KIA (Kligler's iron agar), indole, and urea and citrate consumption were used to accurately identify bacterial isolates.

DNA extraction and PCR (polymerase chain reaction): After preliminary phenotypic confirmation tests, test media containing *E. coli* strains were selected for DNA extraction. Boiling strategy was used for DNA extraction, and PCR reaction was done to identify the GADA gene under the conditions presented in Table 1. The sequences of primers used for PCR amplification are shown in Table 2 [16]. Finally, electrophoresis of PCR products was performed on a 1.5% agarose gel to differentiate specific fractions of 670 bp with a 100 bp measurement marker.

Antibiotic sensitivity testing of isolated bacteria: In this study, only *E. coli* was isolated; thus, antibiotic sensitivity testing was performed on this organism. The Kirby-Bauer disk diffusion method was used to measure the antibiotic sensitivity of the isolates. The antibiotics used in this study were selected based on the Clinical and Laboratory Standards Institute (CLSI) guidelines. Also, ten different antibiotic

Table 1) PCR conditions for amplification of GABA gene

Step		Temperature (˚C)	Time(min)	Cycle Repeat
Initial denaturation		96	5	1
Amplification	Denaturation	94	1	30
	Annealing	54	1	
	Extension	72	1	
Final Extension		72	7	1

Table 2) Sequence of primers used for amplification of GABA gene

Strains	Primers	Sequences	Size
E. coli	GADA/F	5′-ACCTGCGTTGCGTAAATA-3′	670 bp
	GADA/R	5′-GGGCGGGAGAAGTTGATG-3′	

discs, including ceftazidime (30 µg), ampicillin/sulbactam (10 µg), ampicillin (25 µg), ceftriaxone (30 µg), clarithromycin (15 µg), amikacin (30 µg), cefexime (5 µg), azithromycin (15 µg), ciprofloxacin (5 µg), and trimethoprim/sulfamethoxazole (5 µg), were purchased from Padtan Teb Company. In addition, *E. coli* ATTC 25522 was used as a positive control in this study.

Evaluating the risks of consuming ice creams contaminated with *E. coli:* In this study, Risk Ranger software was used to assess the risk of consuming ice creams contaminated with *E. coli*. The software calculates a score related to the risks caused by the presence of this bacterium in ice creams based on 11 questions that fall into the following three major groups: a) population sensitivity and severity of pathogenicity, b) probability of exposure, and c) amount of infectious dose consumed.

Statistical analysis: After completing the tests, the obtained data were analyzed using SPSS software Version 15 by employing t-test, Fisher's exact test, and descriptive statistics. The significance level was considered at $p< .05$.

Findings

Measuring total viable count and total coliform count in traditional and industrial ice cream samples: In this study, 270 traditional and 200 industrial ice cream samples of various types were tested. According to the national standard of Iran, the permissible limit of TVC is less than $5x10^4$ CFU/g. In this study, the amount of microbial load after 24 hours of incubation was $7.8x10^6$ CFU/g in traditional ice creams and $3.2x10^2$ CFU/g in industrial ice creams (Table 3).

The amount of TVC in all industrial ice creams was lower than the permissible limit specified by the national standard of Iran. But in traditional ice creams, 175 out of 270 samples (65%) had microbial loads higher than the normal level.

The permissible limit of coliforms is less than 10 CFU/g according to the national standard of Iran. In this study, 100 (37%) out of 270 traditional ice cream samples had higher coliform levels, while only three (1.5%) out of 200 industrial ice cream samples had higher coliform levels compared to the normal limit.

Prevalence of different bacterial isolates in traditional and industrial ice creams: Biochemical tests were performed on all collected ice cream samples to identify *E. coli, S. aureus,* and *S. enteritidis.* None of the tested organisms were detected in industrial ice cream samples, but in traditional ice creams, 202 (74.8%) samples were contaminated with *E. coli. S. aureus* and *S. enteritidis* were detected in none of the traditional and industrial ice cream samples (Table 3).

Table 3) Total count of' coliform, *Escherichia coli, Salmonella entrica*, and *Staphylococcus aureus* contaminants in traditional and industrial ice creams

	Traditional Ice-Cream (n=270)		Industrial Ice-Cream (n=200)		National Standard of Iran
	Average(CFU/g)	Percent	Average(CFU/g)	Percent	
Total viable count	$7.8x10^6$	175 (65%)	$3.2x10^2$	0	$<5x10^4$
Total coliform count	$2x10^2$	100 (37%)	8	3 (1.5%)	<10
Fungi and mold	$1.5x10^2$	110 (40%)	0	0	<100
Escherichia coli	-	202 (74.8%)	0	0	negative
Salmonella entrica	0	0	0	0	negative
Staphylococcus aureus	0	0	0	0	negative

Table 4) Antibiotic susceptibility of *E. coli* isolates (n = 202)

Antibiotics	Susceptible N (%)	Intermediate N (%)	Resistant N (%)
Ceftazidime	84(41.4)	34(17.2)	84(41.4)
Ampicillin/Sulbactam	97(48)	9(4.4)	96(47.6)
Ampicillin	14(6.9)	6(3)	182(90.1)
Ceftriaxone	143(70.8)	22(10.9)	37(18.3)
Clarithromycin	115(57)	2(1)	85(42)
Amikacin	101(50)	15(7.4)	86(42.6)
Cefexime	98(48.5)	32(15.8)	72(35.7)
Azithromycin	117(58)	24(11.9)	61(30.1
Ciprofloxacin	139(69)	19(9.4)	44(11.6)
Trimethoprim/ Sulfamethoxazole	30(14.8)	34(16.8)	138(58.5)

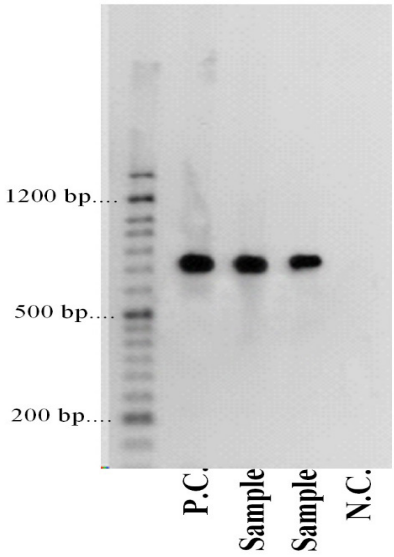

Figure 1) PCR detection specificity of *E. coli* (670 bp) in sample. P.C: positive control, N.C: negative control

Determining the presence of fungi in traditional and industrial ice creams: According to the results, fungi were detected in 12 and 85% of plain and flavored traditional ice cream samples, respectively. In contrast, no fungal growth was observed in ice creams industrially prepared from pasteurized milk (Table 3).

Detection of *E. coli* with PCR: According to PCR results, the presence of GABA was detected in 78% of traditional and 2% of industrial ice cream samples (Fig. 1).

Antibiotic sensitivity pattern of bacterial isolates from traditional ice creams: The antibiogram results indicated that the isolates showed the highest resistance to ampicillin and trimethoprim/sulfamethoxazole and

the highest sensitivity to ceftriaxone and ciprofloxacin, respectively. The results related to sensitivity to other antibiotics are presented in Table 4.

Risk ranking of *E. coli* with Risk Ranger: The final risk ranking for the presence of *E. coli* in ice cream was 62, meaning that more control measures are needed. Using this tool, it was determined that the probability of illness per consumer per day was 4.80×10^{-6}, the total number of predicted cases per year in the target population was 3.09×10^{4}, and the comparative risk within the group was 5.02×10^{-9}.

Discussion

One of the main concerns of people around the world is microbial contamination caused by antibiotic-resistant pathogens. Dairy products, especially ice cream, have the ability to create a favorable environment for the growth of pathogens.

Due to suitable nutrients, neutral pH, and long storage time, these products are easily contaminated by pathogenic bacteria. According to the national food standard of Iran, the permissible limits of TVC and TTC are less than $5x10^{4}$ and 10 CFU/g, respectively. But pathogenic species such as *E. coli, S. aureus,* and *Salmonella* should not be detected in ice cream. According to the information presented above, this research showed that 65% (n=175) of traditional ice cream samples had a TVC level higher than the national standard of Iran, while all industrial ice cream samples had a TVC level lower than the normal level. Counting *Enterobacteriaceae* and coliforms in food as an indicator of food hygiene is of great important.

The results showed that the amount of TTC in 37% (n=100) and 1.5% (n=3) of traditional and industrial ice cream samples was higher than the normal limit, respectively, indicating that the contamination level of traditional ice creams is much higher. The results of this study are in line with those of other studies conducted in Iran and other parts of the world, reporting high levels of bacterial contamination in traditional ice creams [17-22]. The cause of these high levels of contamination may be related to the use of unpasteurized milk, cream, fruits, or nuts in these ice creams. In addition, the supply of ice cream in centers that do not comply with the health principles and conditions of selling ice cream could also cause contamination of traditional ice creams. *S. aureus* is an important foodborne pathogen that could cause gastrointestinal diseases by producing enterotoxins. Fortunately, in the present study, this microorganism was not isolated from any of the traditional and industrial ice cream samples. This result is contrary to the results of most studies conducted in Iran. This may be due to the lower prevalence of this bacteria or better hygiene in this geographical area [8, 12, 17, 21].

Salmonella is the second leading cause of zoonotic diseases in humans. Foodborne salmonellosis is considered as a serious problem in the health systems of many countries. This bacterium is easily transmitted to food through water, soil, or food components contaminated with feces or wastewater.

The results of this study showed that this microorganism was not isolated from any of the 450 samples studied. Non-isolation of this bacterium has also been reported in other studies [12, 21, 23].

This may be due to the isolation of other bacteria with high loads and their inhibitory effect on this bacterium. Furthermore, the problems associated with their isolation and culture should not be ignored. Despite these problems, in some studies, the isolation rate of this bacterium has been reported to be between 3.7 and 46%, in contrast to this study [24, 25].

E. coli is one of the most important causes

of gastrointestinal diseases such as diarrhea. This bacterium is considered as a health indicator of food products, and the isolation of this organism indicates fecal contamination and poor health conditions. Ice creams produced by traditional and unhygienic methods could be an important source of *E. coli* bacteria. *E. coli* contamination in dairy products such as ice cream has been reported in many studies. In the studies conducted by Gundogan and Avci (2014) and Yaman et al. (2006), 56 and 20% of the samples were infected with *E. coli*, respectively [19, 26]. The overall results of these studies showed that the health status of traditional ice creams was poor and inappropriate.

The isolates showed the highest resistance to ampicillin and cotrimoxazole and the highest sensitivity to ceftriaxone and ciprofloxacin, respectively. In a study conducted by Moezi and colleagues (2015) in Shiraz on dairy products, the highest resistance was related to ampicillin [27]. In another study conducted by Ghadimi et al. (2016) in Hamedan city, the isolates showed the highest resistance to ampicillin and the highest sensitivity to ciprofloxacin [23], similar to the present study results.

High levels of ampicillin resistance have been reported in studies conducted in Turkey and Libya on traditional ice creams, which is consistent with this study results [26, 28]. In this study, the results also showed that the rate of fungal and mold contamination was very high in ice creams flavored with different ingredients. Unlike flavored ice creams, the contamination rate of plain ice creams was very low. These results show that most fungal and mold contaminations are related to flavoring additives added to ice creams, which may also be due to the storage of flavorings in unhealthy conditions.

The current research shows that there are high levels of contamination, especially by pathogenic bacteria, in traditional ice creams. On the other hand, the high resistance of these microorganisms as well as their simultaneous resistance to two or more drugs cause many risks for the consumers of these products, especially children. Therefore, it is necessary to take appropriate health measures to increase the safety of ice creams.

Using Risk Ranger, the risk ranking was estimated to be 62, which indicates that there is a moderate level of hazard for consumers, and more control and preventive measures are needed for this contamination level. As the second output of this method, it was determined that the total number of cases predicted to be infected with this food per year in the target population was 3.09×10^4. This index is the most understandable criterion among all obtained indices. As the last output, the comparative risk was determined to be 5.02×10^{-9}, which is actually a measure of relative risk. This criterion depends on the size of the consuming population and is the most useful criterion for measuring the risk of different pathogens in food in different populations. The study had limitations, including not isolating other pathogens, which resulted in not performing PCR testing on them. Antibiotic sensitivity testing was not conducted due to the non-isolation of these pathogens.

Conclusion

The present study shows that traditional ice cream could be a source of foodborne pathogens for consumers. The presence of *E. coli*, fungi, and molds was detected only in traditional ice cream samples. Given the complexity of the production process of ice cream, the whole production system is required to be guaranteed in terms of quality and safety. The use of high-quality raw materials, storage under proper hygienic conditions, and proper heat treatment during processing could prevent the growth and

proliferation of pathogenic microorganisms in ice cream. It is necessary to train people working in this field as well as to wash and keep clean dishes and machines.

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Ethical Permission: This study was approved by the Ethics Committee of Shiraz University of Medical Sciences (Ethical Code: IR.SUMS.REC.1402.181).

Authors' contributions: AR and MR conceived and designed the work, analyzed data, wrote and revised the article, and approved the final version for publication. PM, NK, MH, and SB collected and analyzed data. MJR was encouraged to investigate a specific aspect and supervised the findings of this work.

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