JOURNAL OF NUTRITION FASTING AND HEALTH

# The Prevalence of *Staphylococcus aureus* and Its Enterotoxin Genes in Raw Milk and Dairy Products of Isfahan Province

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**ARTICLEINFO**

*Article type:*
Research Paper

*Article History:*
Received: 09 Dec 2023
Accepted: 03 Nov 2024
Published: 16 Nov 2024

*Keywords:*
Staphylococcus aureus
Enterotoxin
Raw milk
Dairy products
Food poisoning

**ABSTRACT**

Staphylococcus aureus is a primary causative agent of severe hospital-acquired infections, and its significant role in foodborne illness outbreaks has recently come to light. This study aimed to quantitatively assess the incidence and distribution of Staphylococcus aureus and the prevalence of enterotoxin-encoding genes among resistant isolates recovered from raw milk and dairy products. A total of 485 samples of raw milk and traditional dairy products (produced from cow's milk) were collected from Isfahan Province. The presence of *Staphylococcus aureus* was confirmed through microbial culturing techniques. The prevalence rates of Staphylococcus aureus in milk and dairy products were 17.20% and 20.42%, respectively, with cow's milk (25%) and cheese (44.44%) exhibiting the highest contamination rates. Among the Staphylococcus aureus strains, the most frequently detected enterotoxins were SEA (44.68%) and SEB (36.17%). The findings of this study underscore the role of milk and dairy products in transmitting highly pathogenic and resistant strains of *Staphylococcus aureus* to humans.

► Please cite this paper as:
Montazeri R, Rahimi E, Shakerian A. The Prevalence of Staphylococcus aureus and Its Enterotoxin Genes in Raw Milk and Dairy Products of Isfahan Province. J Nutr Fast Health. 2024; 12(4): 305-313. DOI: 10.22038/JNFH.2024.76601.1487.

## Introduction

Milk and dairy products are essential components of the diet in every household due to their rich nutritional content, including proteins, fats, lactose, and minerals vital for human health and well-being. Milk consumed daily by millions can be transformed into a wide range of dairy products, each offering diverse nutritional values. However, in many rural and nomadic areas, milking is often conducted using traditional hand methods without proper adherence to hygienic practices. This lack of hygiene can lead to contamination and spoilage of milk, posing significant health risks [1].

Throughout the production, distribution, and storage phases, dairy products are susceptible to contamination by microorganisms, mainly bacteria, often due to neglecting hygiene principles. Additionally, milk's diverse composition creates a conducive environment for microbial growth. Ensuring high-quality milk with an extended shelf life involves several key factors, including the health of the animals, proper milking procedures, and rapid chilling. Maintaining cleanliness in milking equipment, transport tanks, and cleaning water is also crucial in preventing spoilage [2].

Therefore, the milk consumed must exhibit high quality and be free from contamination to meet societal needs and prevent the transmission of various diseases. Foodborne illnesses from contaminated dairy products, including bacteria and molds, can lead to gastrointestinal infections and food poisoning. Bacteria have the capability and propensity to contaminate food items, particularly raw milk and dairy derivatives [3, 4]. Bacterial factors such as Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Clostridium perfringens, Bacillus cereus, Vibrio parahaemolyticus, Listeria monocytogenes, *Shigella* species, Yersinia enterocolitica, Campylobacter jejuni, and Pseudomonas aeruginosa are among the prominent causes of food poisoning associated with the consumption of contaminated food items of animal origin [5, 6].

Staphylococcus aureus, a cocci-shaped, gram-positive, facultatively anaerobic bacterium, is one of the most common factors in hospital infections and the primary cause of many food

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poisoning cases related to the consumption of animal-originated food. It is catalase-positive and is the most important species within the *Staphylococcus* genus [7]. In addition to causing skin infections, wound infections, meningitis, endocarditis, pneumonia, and toxic shock syndrome, Staphylococcus aureus is known to induce food poisoning with a short incubation period of 2 to 4 hours. Symptoms of this food poisoning include nausea, vomiting, abdominal cramps, and weakness, although diarrhea may also be reported in some cases [8].

This bacterium is one of the primary culprits behind widespread foodborne outbreaks, particularly food poisoning. Its significance stems from its high resistance to various environmental conditions, including salt concentration, water activity, and temperature. Additionally, the presence of heightened antibiotic resistance in Staphylococcus aureus strains, coupled with virulence factors and enterotoxins, further amplifies its importance [8, 9].

Improper milking techniques, mainly manual methods involving hand contact and the risk of mammary gland infections, contribute to easy milk contamination by *Staphylococci*. Consequently, there exists the potential for residual contamination in dairy derivatives produced from infected milk, as well as the transmission of toxin-producing bacteria from contaminated personnel in dairy processing facilities to these products [10].

In cases of Staphylococcus aureus-induced poisoning, the disease's short incubation period is primarily attributed to the ingestion of preformed toxins present in contaminated food substances. Specifically, the enterotoxins of Staphylococcus aureus (including enterotoxins A-E, G-R, and U) act on receptors located in the intestines, triggering a vomiting response in affected individuals [11].

These enterotoxins comprise small, single-chain peptides featuring a disulfide bond near the molecule's center. Due to their compact structure, these enterotoxins resist heat and intestinal proteases, becoming inactive only after prolonged boiling. Consequently, there is a possibility that consuming food devoid of live Staphylococcus aureus cells can still result in illness [11, 12].

Primarily, these stimulated receptors activate the vomiting center in the brain through both sympathetic and parasympathetic pathways. Although the exact mechanism by which enterotoxins induce diarrhea remains unclear, studies indicate that enterotoxins do not stimulate adenylate cyclase activity [13].

Identifying and detecting Staphylococcus aureus enterotoxins and their encoding genes in various food items can significantly enhance the understanding of new epidemiological aspects and risk factors associated with food poisoning caused by this bacterium. Research indicates that in cases of staphylococcal food poisoning, less than one milligram of pure toxin can induce disease symptoms [14]. Given the significance of Staphylococcus aureus as a foodborne pathogen, its high prevalence in food poisoning incidents within communities, the increased consumption of milk and dairy products, and the absence of microbiological, epidemiological, and sanitary studies on Staphylococcus aureus in milk and its derivatives in Iran, further investigation is essential. Therefore, this study aimed to evaluate the occurrence of resistant strains of Staphylococcus aureus and the presence of classical enterotoxins in various traditional milk and dairy products (derived from cow's milk) in the province of Isfahan.

## Materials & Methods

### *Materials*

The study utilized several materials, including various culture media such as Trypticase soy broth (TSB), Trypticase soy agar (TSA), Baird Parker Agar, Bordet-Gengou agar, Brain heart infusion agar (BHIA), DNAse agar, Mannitol salt agar (MSA), Alkaline peptone water (APW), and Blood agar, all sourced from Merck, Germany. Biological reagents included rabbit plasma from Modern Med, hydrogen peroxide from Merck, and histological slides from T&Q, China. Other reagents consisted of sterile distilled water, egg yolk tellurite, PCR buffer, $MgCl_2$, deoxyribonucleoside triphosphates (dNTPs), forward and reverse primers, a DNA extraction kit from Synagen, and agarose powder, all from various suppliers. The specific concentrations and procedures for using these materials are described in the related sections of the study.

### *Sample Size and Sampling Methods*

In this study, the population of interest included milk and dairy products produced during the summer of 2021 in Isfahan Province. A simple random sampling method was employed to

collect samples from various dairy sales outlets throughout the province. The samples comprised different types of raw milk, including 80 samples of cow's milk, 70 samples of sheep's milk, 50 samples of goat's milk, and 50 samples of camel's milk. Additionally, traditional dairy products made from cow's milk were analyzed, which included 50 samples each of clotted cream and butter, 50 samples of yogurt, 40 samples of curd, and 45 samples of cheese.

### *Sample Transfer and Preparation for Laboratory Analysis*

The visual and physical qualities of milk and dairy products, such as color and consistency, were essential criteria for selection. After collection, the samples were transported to the laboratory under sterile conditions within 2 to 4 hours. They were then stored in a refrigerator at a temperature of 4°C.

### *Isolating Staphylococcus aureus from Raw Milk and Dairy Products*

To isolate Staphylococcus aureus from raw milk and dairy product samples, the samples were first cultured in Tryptic Soy Broth (Merck, Germany) supplemented with a 10% salt solution. Incubation occurred at room temperature (37 °C) for 18 hours. The grown colonies in the Tryptic Soy Broth medium were transferred to Baird Parker Agar (Merck, Germany), enriched with egg yolk tellurite emulsion, and incubated at 37 °C for 24 hours. The black colonies with a surrounding precipitate were typical colonies indicative of Staphylococcus aureus. To confirm their identification, these colonies were then subjected to biochemical tests, including catalase, oxidase, O/F, urease, phosphatase, coagulase, DNAse, and mannitol fermentation.

### *Molecular Tracking of Genes Encoding Enterotoxins*

The Polymerase Chain Reaction (PCR) technique was employed to identify the genes responsible for encoding enterotoxins using primers listed in Table 1. The PCR reactions were conducted with a Gradient Thermal Cycler (Eppendorf, Germany) and involved a 25-microliter reaction mix containing 1 unit of Taq DNA Polymerase (Fermentas, Lithuania), 200 micromoles of dNTP (Fermentas, Lithuania), 5.2 microliters of 10x buffer solution (Fermentas, Lithuania), 1 micromole of manganese chloride (Fermentas, Lithuania), 10 picomoles of each primer, 3 microliters of DNA template, and 25 microliters of sterile distilled water. The amplification procedure for the genes encoding enterotoxins consisted of four temperature programs: for SEA-SED, denaturation for 2 minutes at 94 °C, annealing for 2 minutes at 55 °C, and extension for 1 minute at 72 °C (30 cycles); for SEE, denaturation for 2 minutes at 94 °C, annealing for 2 minutes at 57 °C, and extension for 1 minute at 72 °C (35 cycles); for SEG-SEI, denaturation for 30 seconds at 94 °C, annealing for 30 seconds at 55 °C, and extension for 60 seconds at 72 °C (30 cycles); and for SEJ, denaturation for 60 seconds at 94 °C, annealing for 60 seconds at 62 °C, and extension for 1 minute at 72 °C (30 cycles).

**Table 1.** List of primers used for the detection of staphylococcal enterotoxins (SEs) in *Staphylococcus aureus* isolates from milk and dairy products

| Enterotoxin | Primer sequence (5'-3') | Product size (bp) | Source |
|---|---|---|---|
| SEA | F: TTGGAAACGGTTAAAACGAA | R: GAACCTTCCCATCAAAAACA | [1] |
| SEB | F: TCGCATCAAACTGACAAACG | R: GCAGGTACTCTATAAGTGCC | [1] |
| SEC | F: GACATAAAAGCTAGGAATTT | R: AAATCGGATTAACATTATCC | [1] |
| SED | F: CTAGTTTGGTAATATCTCCT | R: TAATGCTATATCTTATAGGG | [1] |
| SEE | F: AGGTTTTTCACAGGTCATCC | R: CTTTTTTTTCTTCGGTCAATC | [2] |
| SEG | F: AAGTAGACATTTTTGGCGTTCC | R: AGAACCATCAAACTCGTATAGC | [3] |
| SEH | F: GTCTATATGGAGGTACAACACT | R: GACCTTTACTTATTTCGCTGTC | [3] |
| SEI | F: GGTGATATTGGTGTAGGTAAC | R: ATCCATATTCTTTGCCTTTACCAG | [3] |
| SEJ | F: CATCAGAACTGTTGTTCCGCTAG | R: CTGAATTTTACCATCAAAGGTAC | [4] |

### *Data Analysis*

The data from the experiments were compiled in Microsoft Office Excel and analyzed using SPSS software (Version 26). The statistical methods employed for data analysis included the chi-square and Fisher's exact tests. This study investigated the association between sample type, prevalence, and frequency of genetic factors. A P value of 0.05 or less was considered statistically significant.

## Result and Discussion

This study conducted additional tests to confirm the strains and assess the prevalence and

frequency of genes encoding enterotoxins in Staphylococcus aureus isolates collected from raw milk and dairy products in Isfahan Province. Figure 1 presents examples of Staphylococcus aureus strains isolated from the raw milk and dairy product samples. Staphylococcus aureus is identified by black colonies surrounded by a precipitate halo. This medium facilitates the proliferation of Staphylococcus aureus while specifically inhibiting the growth of most other bacteria. Almost all coagulase-positive staphylococci can reduce tellurite, forming black colonies, although other staphylococci may not always exhibit this characteristic. Therefore, this medium is a specific diagnostic tool for detecting and quantifying coagulase-positive staphylococci in food products.

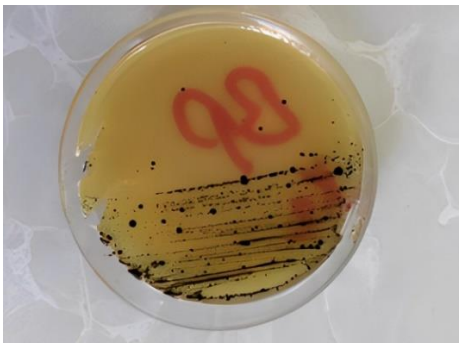

**Figure 1.** Staphylococcus aureus isolates from the samples examined on Baird Parker Agar (BPA).

Also, Figure 2 illustrates the ability of Staphylococcus aureus strains to produce beta-hemolysis (complete) on blood agar. This medium serves as a differential culture medium for detecting hemolysis, which is the destruction of red blood cells by cytolytic toxins produced by certain bacteria, including Staphylococcus aureus. As depicted in the figure, the bacteria lysed the red blood cells, resulting in the characteristic appearance of the medium.

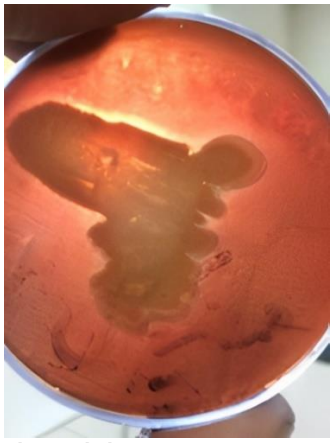

**Figure 2.** Beta hemolysis in blood agar medium by Staphylococcus aureus

The ability of Staphylococcus aureus strains isolated from milk and dairy products to grow on mannitol salt agar (MSA) and turn the medium yellow is illustrated in Figure 3. MSA agar is utilized as a selective and differential medium for the isolation and identification of Staphylococcus aureus from both clinical and non-clinical samples. This culture medium enhances the growth of a specific group of bacteria while inhibiting the growth of others. Mannitol salt agar is a selective medium for isolating pathogenic *Staphylococcus*, as non-staphylococcal bacterial species are inhibited from growing entirely or partially at a sodium chloride concentration of 7.5%. The bacterium can be identified by the acid produced during the fermentation of mannitol, a carbohydrate that is detectable using the phenol red indicator.

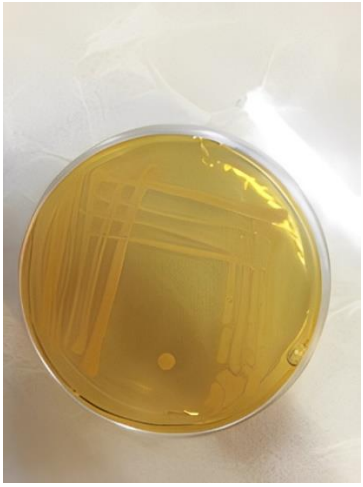

**Figure 3.** Staphylococcus aureus strains in mannitol salt agar (MSA) medium and changing the color of the medium to yellow

Coagulase-positive staphylococci (e.g., Staphylococcus aureus) create yellow colonies and a yellow medium, while coagulase-negative colonies produce red colonies with no phenol red indicator color shift. A 5% egg yolk emulsion is added to detect *Staphylococcus* lipase and mannitol fermentation. The salt in the medium demulsifies the egg yolk emulsion, revealing lipase production as a yellow, opaque zone surrounding the colonies.

**Figure 4.** Catalase reaction for Staphylococcus aureus strains isolated from milk samples and dairy products.

This research also differentiated staphylococci using *catalase*. This test distinguishes *streptococci* and *enterococci* from *staphylococci* (Figure 4). The enzyme *catalase* breaks down hydrogen peroxide into oxygen and water. When a small inoculum of the bacterial isolate is added to hydrogen peroxide, the presence of the enzyme produces oxygen bubbles quickly. In contrast, bubble generation is poor in the absence of *catalase*. The culture must be 24 hours old for accurate results.

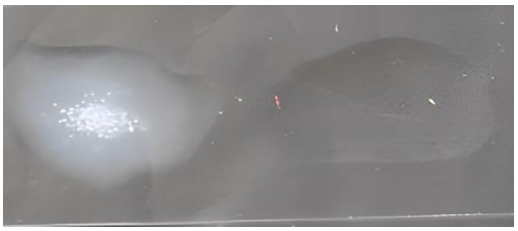

**Figure 5.** Coagulase reaction on slides for Staphylococcus aureus strains isolated from milk and dairy products.

Biochemical testing verified Staphylococcus aureus in samples. The biochemical coagulase test was performed to distinguish Staphylococcus aureus from other staphylococcal species (Figure 5). This test relies on microorganisms producing *coagulase*. The

coagulase test is crucial for separating *Staphylococcus* species into coagulase-positive and coagulase-negative groups. In certain species, the coagulase enzyme interacts with fibrinogen on the host cell to generate pathogenicity. Coagulase-positive organisms shield themselves from the immune system, which makes them a greater risk.

**Figure 6.** Positive reaction in DNAse agar medium for Staphylococcus aureus strains isolated from milk and dairy products. The appearance of a clear halo after the addition of normal HCl on the colonies indicates a positive reaction.

The ability to produce the enzyme *DNase* is another criterion for diagnosing Staphylococcus aureus bacteria (Figure 6). All strains of this bacterium produce the enzyme *DNase*. However, only 25% of strains of coagulase-negative staphylococci can produce this enzyme. The *DNase* enzyme is heat-resistant and is considered an extracellular enzyme. This enzyme breaks down DNA into smaller units called nucleotides, allowing for the identification of the type of bacteria.

**Table 2.** Prevalence of *Staphylococcus aureus* isolates in raw milk samples.

| Sample type | Number of samples collected | Prevalence of Staphylococcus aureus (%) |
|---|---|---|
| Cow milk | 80 | 20 (25) |
| Sheep milk | 70 | 15 (21.42) |
| Goat milk | 50 | 5 (10) |
| Camel milk | 50 | 3 (6) |
| Total | 250 | 43 (17.20) |

**Table 3.** Prevalence of Staphylococcus aureus isolates in dairy product samples.

| Product type | Number of samples collected | Prevalence of Staphylococcus aureus (%) |
|---|---|---|
| Clotted cream | 50 | 18 (36) |
| Butter | 50 | 10 (20) |
| Yogurt | 50 | - |
| Curd | 40 | - |
| Cheese | 45 | 20 (44/44) |
| Total | 235 | 48 (20.42) |

Tables 2 and 3 display the prevalence rates of Staphylococcus aureus strains in samples of raw milk and dairy products collected from Isfahan province. According to the results, the prevalence of Staphylococcus aureus in samples of milk and dairy products was 17.20% and 20.42%, respectively. A statistically significant difference in the prevalence of Staphylococcus aureus between milk samples and dairy products was observed ($P < 0.05$). Among the raw milk samples, cow's milk exhibited the highest contamination rate (25%), while camel's milk showed the lowest (6%). Among the dairy product samples, cheese had the highest contamination rate (44.44%), whereas butter had the lowest (20%). The techniques were ineffective in isolating Staphylococcus aureus from curd and yogurt samples. This study demonstrated that raw milk and dairy products can easily transmit virulent and resistant strains

of Staphylococcus aureus to human communities. Therefore, consuming raw or partially cooked food from contaminated raw milk can lead to widespread staphylococcal food poisoning. The contamination rates of raw milk samples from cow, sheep, goat, and camel with Staphylococcus aureus were 25%, 21.42%, 10%, and 6%, respectively. Additionally, 36%, 20%, and 44.44% contamination rates were observed in clotted cream, butter, and cheese samples. No confirmed cases of Staphylococcus aureus were detected in the yogurt and curd samples.

Among the potential reasons for the failure to isolate Staphylococcus aureus from curd samples is the continuous boiling during its production, which prevents the survival and growth of a broad spectrum of bacteria. Additionally, the acidic pH of curd impedes the growth of Staphylococcus aureus. In the case of yogurt samples, a robust bacterial flora hinders the growth and proliferation of foreign bacteria, including *Staphylococcus aureus*. This product also maintains an acidic pH level [15, 16].

However, cheese samples exhibited the highest prevalence of Staphylococcus aureus contamination at 44.44%. Notably, in some traditional cheese types, during cheese production and the formation of para-casein (curd), milk should not exceed temperatures higher than 42°C, as higher temperatures disrupt curd formation [17]. Therefore, it is likely that the milk used in cheese-making was contaminated and did not experience the appropriate temperature for elimination.

Traditional dairy products exhibited higher contamination levels with Staphylococcus aureus than raw milk. This may be attributed to respiratory droplets from coughing and sneezing and direct contact with the skin of dairy processing workers.

Several studies have investigated this issue. A study conducted in Nigeria reported the prevalence of Staphylococcus aureus in fresh milk samples and milk stored in tanks at 2.94% and 7.14%, respectively [18]. In 2022, Oliveira et al. examined the prevalence of Staphylococcus aureus in raw milk samples from animals in Portugal, finding a bacterial prevalence rate of 53%, which is significantly higher than the rates reported in the current study [19].

**Table 4.** Prevalence of Classic Enterotoxin Genes in Staphylococcus aureus Isolates from Raw Milk and Dairy Products in Isfahan Province, Iran.

| Samples | Prevalence of classic enterotoxin genes (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | ***SEA*** | ***SEB*** | ***SEC*** | ***SED*** | ***SEE*** | ***SEG*** | ***SEH*** | ***SEI*** | ***SEJ*** |
| Cow milk (10) | 50 | 30 | 20 | 20 | 20 | 10 | 10 | 20 | 10 |
| Sheep milk (8) | 50 | 37.5 | 37.5 | 25 | 25 | 12.5 | 12.5 | 12.5 | 12.5 |
| Goat milk (2) | 50 | 50 | 50 | 50 | - | 50 | - | - | - |
| Camel milk (1) | 100 | 100 | 100 | - | - | - | - | - | - |
| Clotted cream (10) | 40 | 40 | 20 | 20 | 10 | 10 | 20 | 10 | 10 |
| Butter (4) | 50 | 50 | 50 | 25 | 25 | - | - | 25 | 25 |
| Cheese (12) | 33.33 | 33.33 | 16.66 | 16.66 | 16.66 | 8.33 | 16.66 | 8.33 | 16.66 |
| Total (47) | 44.68 | 36.17 | 27.65 | 21.27 | 17.02 | 10.63 | 12.76 | 12.76 | 12.76 |

Table 4 illustrates the frequency of genes encoding enterotoxins in resistant Staphylococcus aureus strains isolated from raw milk and dairy product samples. According to the results presented in this table, the genes encoding enterotoxins SEA (44.68%) and SEB (36.17%) exhibited the highest frequencies in Staphylococcus aureus strains isolated from these samples. The lowest prevalence of genes encoding enterotoxins in Staphylococcus aureus strains was associated with SEG (10.63%), SEH (12.76%), SEI (12.76%), and SEJ (12.76%). Overall, the prevalence of genes encoding classical enterotoxins in Staphylococcus aureus strains isolated from dairy products was higher than in raw milk. A statistically significant difference ($P < 0.05$) in the frequency of enterotoxin-encoding genes among different types of samples and their associated Staphylococcus aureus strains was observed.

Resistant Staphylococcus aureus strains that produce enterotoxins are among the most virulent and pathogenic. The genes that encode enterotoxins contribute to their synthesis. In this investigation, Staphylococcus aureus strains exhibited high levels of SED, a poultry-specific enterotoxin, which may indicate proximity between poultry and livestock or a potential infection. Additionally, SEA and SEB, recognized as the most dangerous enterotoxins, were found

to be the most prevalent in the isolated Staphylococcus aureus strains, suggesting that these strains are particularly virulent. Although emerging enterotoxins (SEJ-SEG) are less common in food poisoning, they remain significant due to their high resistance to heat and proteolytic enzymes such as pepsin and trypsin [20].

Vicosa et al. conducted a study in Brazil in 2013 to investigate the prevalence of enterotoxins in raw milk. Their results indicated that out of 92 Staphylococcus aureus strains isolated from this food group, at least 91 had one gene encoding staphylococcal enterotoxin. Their findings revealed that genes SEN, SEM, SEI, SEG, and SEU exhibited a notably high prevalence among the traced enterotoxins [21]. In other studies, the significance of enterotoxins in *Staphylococcus* has also been emphasized. For instance, Chiang et al. conducted a survey in Taiwan (2008) focusing on strains of Staphylococcus aureus isolated from foodborne outbreaks. Their research findings revealed that out of a total of 147 examined Staphylococcus aureus strains, 135 strains (91.8%) contained at least one or multiple genes encoding enterotoxins such as SEA, SEB, SEC, SED, and SEE [22].

Rosec and Gigaud (2021) studied classical and developing enterotoxins in 159 food samples from diverse French areas. Of the 332 Staphylococcus aureus strains investigated, 57% had at least one enterotoxin gene. In Staphylococcus aureus strains, enterotoxins SEA, SEB, SED, SEC, and SEE were the most common, which supports the present examination [23].

In line with our study, Beygi and Kargar (2020) reported findings from an investigation on dairy product samples collected from Fars province. Of the 300 examined samples, 123 isolates (40%) were identified as Staphylococcus aureus. The highest contamination frequency was observed in cheese (66.31%), while the lowest was found in yogurt (33.8%). About 75.83% of the contaminated samples carried enterotoxin genes, with SEA exhibiting the highest prevalence (71.54%). The prevalence of enterotoxins B, G, E, D, and C was 50%, 33.28%, 67.16%, 17.14%, and 50.3%, respectively [24].

Consistent with findings from other studies, the present research demonstrates a logical correlation between the prevalence of Staphylococcus aureus in various dairy products and the abundance of its enterotoxin genes, aligning with similar trends observed in related studies.

## Conclusion

The present study revealed a significant prevalence of Staphylococcus aureus strains in raw milk and dairy products, particularly cheese. Moreover, there was a high incidence of enterotoxin-coding genes among isolates from raw milk and dairy products. These findings collectively indicate a substantial risk to food safety associated with raw milk and dairy products produced in small, sometimes traditional, workshops, especially within the Isfahan province. The presence of enterotoxins signifies the high virulence of resistant Staphylococcus aureus isolates. Implementing stringent control measures within milk production workshops and ensuring thorough pasteurization of milk before processing dairy products and even before consumption can prevent outbreaks of food poisoning caused by Staphylococcus aureus strains.

## Declarations

### *Conflits of Interest*

The authors declare no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### *Acknowledgments*

The authors would like to thank the Food Hygiene Research Centre staff in Iran for their significant support.

### *Funding*

No funding was received.

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