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ORIGINAL ARTICLE

Characterization of *Staphylococcus aureus* isolates from pastry samples by rep-PCR and phage typing

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ABSTRACT

Background and Objectives: *Staphylococcus aureus* is one of the most common causes of food poisoning. This study aimed to identify *S. aureus* isolated from pastries, the virulence factors, antimicrobial resistance patterns, biofilm formation, and then classification based on SCCmec types, phage types, and also Rep types.

Materials and Methods: In this study, 370 creamy and dried pastry samples have been randomly collected from different confectioneries in Hamadan city. The *S. aureus* isolates were identified by conventional microbiological methods and *nuc* gene amplification. The virulence factors and prophage genes were detected. After that, the biofilm production and antibiotic susceptibility assay of *S. aureus* isolates were examined. Finally, the isolates were classified by rep-PCR typing.

Results: Among 370 samples, 97 creamy (34.64%) and 3 dried (3.33%) pastry samples were contaminated with *S. aureus.* Antibiotic sensitivity results showed the highest resistance to penicillin (90%) but none of them were MRSA. According to biofilm formation assay, 14 strains (45%) were strongly adhesive. The dominant phage among isolates was SGF, especially SGFa subgroup. About half of the isolates carried SCCmec Types I and III. Analysis of the genetic linkage between isolates by rep-PCR showed ≥80% genetic similarity and also different rep-types of *S. aureus* isolates.

Conclusion: The presence of different prophage encoded virulence factors and antibiotic resistance enable *S. aureus* strains to produce a broad range of diseases. Thus, consumption of creamy pastries increases the risk of infection with *S. aureus* and it is a serious warning to the health system.

Keywords: *Staphylococcus aureus*; Pastry; Phage typing; Repetitive element sequence-based polymerase chain reaction

INTRODUCTION

Staphylococcus aureus as a main human pathogen causes a wide range of infections from mild clinical manifestations such as skin and soft tissue and food poisoning that may lead to severe life-threatening diseases like bacteremia, endocarditis, osteomyelitis, pleuropulmonary infections, and toxic shock syndrome. Food poisoning could be occurred by *S. aureus* toxins produced in contaminated food including sandwiches, meats, cold salads, tuna, chicken, macaroni, ham, and also bakery products such as cream-filled pastries, cream pies, and chocolate eclairs. Staph food poisoning is indicated by nausea,

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vomiting, and stomach cramps. Some patients also have diarrhea. Symptoms usually appear during 30 minutes to 8 hours after eating or drinking an item containing Staphylococcal toxins and last for 1 day (1-3).

There are several typing methods for *S. aureus* to reveal the major types of this bacterium for instance SCC-mec, Coagulase, Spa, MLST, capsule, *agr* operon-encoded quorum sensing system, pulse-field gel electrophoresis, VNTR loci, and also phage typing methods among methicillin resistance and sensitive *S. aureus* (MRSA & MSSA) or among hospital and community-acquired isolates. Among different molecular typing methods, phage typing is preference with two benefits. Not only, phage typing could classify the isolates, but also phage therapy is an alternative treatment method for *S. aureus* resistant strains (4, 5).

Although more sophisticated systems are available for differentiation between *S. aureus* isolates, such as ribotyping, random amplified polymorphic DNA-PCR fingerprinting, or pulsed-field gel electrophoresis of enzyme-digested DNA, but phage typing remains a simple, rapid, and cost-effective method (5, 6).

Phages as genetic material could incorporate into the genome of a bacterium; therefore, it has a major role to introduce the virulence genes to a bacterium through horizontal transduction then inducing pathogenesis (7). There are six functional modules of *S. aureus* phages that are involved in human diseases and organized into six categories: lysogeny, DNA replication, packaging, head, tail, and lysis which have siphoviridae genomes (8).

In recent years, molecular methods have been used instead of conventional methods for assessing the genetic relationships between different strains of bacteria in epidemiological studies. The rep-PCR is a practical technique that uses oligonucleotide primers based on short repetitive sequence elements distributed throughout the bacterial genome to create DNA fingerprints due to discriminate the different types of *S. aureus* isolates. The rep-PCR is a useful typing technique for a variety of bacteria. The rep-PCR technique fingerprints directly without the need for enzyme digestion (9, 10). There are few studies about the linkages between different rep types of phage patterns in *S. aureus* isolated strains compared with virulence factors, antibiotic resistance, SCCmec types, especially among foodstuffs. We designed this study to identify the Rep pattern types and compare these types with mentioned methods. So, this study aimed to rep-PCR typing of *S. aureus* isolated from pastry samples of Hamadan city, Iran.

MATERIALS AND METHODS

In this cross-sectional study, we randomly collected 370 samples of creamy 280 (75%) and dried 90 (25%) pastries from confectionaries located in Hamadan city, Iran in 2019. Then, 1 gram of each pastry sample was suspended in brain heart infusion broth (BHI) medium and then homogenized at 120-140 rpm for 5 min. 0.1 mL of suspension was cultured on mannitol salt agar and incubated at 37°C for 18 h. The isolated colonies were further sub-cultured on Baird-Parker agar and *S. aureus* isolates were confirmed by the conventional biochemical methods (catalase, coagulase, nitrate reduction, citrate, urease, and glucose fermentation). Also, *nuc* gene amplification was done using polymerase chain reaction. Finally, one hundred *S. aureus* isolates were confirmed.

The present study was approved by the Ethics Committee of Hamadan University of Medical Sciences, Hamadan, Iran (IR.UMSHA.REC.1397.193).

Biofilm formation assays. The biofilm formation was checked phenotypically by Congo red agar. In this process, we cultured the selected isolates on BHI agar containing 0.8 g/L Congo red and 36 g/L sucrose. After incubation, black, dark-red and red colonies were considered as strongly adherent, weakly adherent, and non-adherent isolate, respectively. The biofilm formation was also checked quantitatively by Microtiter plate (MtP) assay. In this procedure, we cultured the selected isolates in trypticase soy broth with 1% glucose. After incubation, we added the McFarland 0.5 turbidity of each isolate to the microplate and incubated for 20 h. After that, we stained the produced biofilm with Crystal Violet 1%. Then, Aston Alcohol released the absorbed stain that the optical density (OD) measured at 570 nm. Finally, the OD<0.35, 0.35-0.49, and >0.5 considered as non-adherent, weakly- adherent, and strongly-adherent isolates, respectively (11).

Antibiotic susceptibility assay. The antimicrobial susceptibility testing was performed based on CLSI guideline (2020) on disk diffusion agar for E (Erythromycin), CP (Ciprofloxacin), Cli (Clindamycin), GM

(Gentamicin), TE (Tetracycline), CF (Cefalotin), and P (Penicillin), Fox (Cefoxitin) antibiotic disks (HiMedia, India) (12).

Detection of virulence genes, and SCCmec types of *S. aureus*. The virulence *icaA, icaB,* and *icaD* genes that encode N-acetylglucosaminyl transferase, the enzyme involved in polysaccharide intercellular adhesion synthesis, and also SCCmec types (I and III) of *S. aureus* selected isolates were detected by PCR method (13).

Identification of *S. aureus* phages. The Prophage serogroups: SGL (648 bp), SGD (331 bp), SGA (744 bp), SGB (405 bp), and SGF (155 bp) and SGFa (548 bp) and SGFb (147 bp) as two prophage subtypes, were detected by Multiplex-PCR using specific primers as described previously (5, 14).

The rep-PCR typing. A total of 31 isolates were selected for rep-PCR typing based on differences in antibiotic resistance pattern, toxins, biofilm, and SCC profile genes using REP primer (15). The rep-PCR master mix contained: 1 µl of genomic DNA, at a concentration of 10 to 100 ng/µl, each PCR tubes containing 24 µl of a PCR mixture composed of 5 µl of PCR buffer (5×), 3.125 µl of deoxynucleoside triphosphates (10 mM each), and 15.47 µl of water, and also, 0.4 µl of rTaq. The PCR conditions were as follows: initial denaturation (95°C for 2 min) and amplification of DNA for 45 cycles consisting of denaturation (95°C for 30 sec), annealing (46°C for 1 min), extension (72°C for 2 min) temperature and time. After that final extension (72°C for 16 min) was done. Finally, ten microliters of the rep-PCR products were loaded and electrophoresed in a 1% agarose gel using 1× Tris-acetate-EDTA buffer mixed with 3.0 µl of SYBR Safe/ml with a voltage of 70 for 1 hour. DNA ladder 100 bp (SMOBIO, Taiwan) was used as a molecular-weight size marker.

The rep-PCR analysis. The REP band patterns were compared by Dice and unweight paired group (UPGMA) method and were clustered using inslico.ehu.es databases. After fingerprinting by rep-PCR, we compared the rep-PCR profiles with the antibiotic susceptibility, virulence factors, and biofilm formation of the isolates.

Statistical analysis. The results were analyzed by SPSS 16 and compared by ANOVA test.

RESULTS

Among 370 creamy and dried pastries, 100 *S. aureus* isolates were separated as 34.64% were from creamy and 3.33% from dried pastries. Antibiotic sensitivity results among the separated isolates showed the highest resistance place related to penicillin (90%) and the next place belonged to ampicillin. All of the isolates were susceptible to methicillin. The dominant phage among antibiotic resistance isolates was SGF (28%), and SGFa was the dominant subgroup. The other subgroups including SGA and SGD were not detected in any isolate.

The results of the antimicrobial susceptibility test showed all isolates were susceptible to ciprofloxacin, gentamicin, cephalothin, and erythromycin. Resistant to cefoxitin, penicillin, ampicillin, and tetracycline were detected in 34%, 75%, 20%, and 6% of isolates, respectively.

In the quantitative study of biofilm formation, 14 strains (45%) were strongly adhesive, while 7 (22.5%) were weakly adhesive and 10 (32.5%) strains showed lacking adhesive ability that is compatible with the qualitative study of biofilm formation. The dominant phage among biofilm-producing isolates was SGF, especially SGFa subgroup (Table 1).

Virulence genes detection showed that *icaB* was detected just among 5 isolates but 12 and 15 isolates had SCCmec type I and III, respectively. The virulence genes indicated a significant relationship with phages especially with SGF (SGFa subgroup) (Table 1).

The results rep-PCR patterns on agarose gel showed in Fig. 1. Analysis of the genetic linkage among isolates by rep-PCR showed ≥80% genetic similarity. Genetic similarity (closed genetic linkage) was established among the *S. aureus* isolates by detecting 8 different rep-PCR types with the similarity cut off ≥95%. Seven different rep-PCR profiles were identified, including five common types and 2 unique types (Fig. 2).

DISCUSSION

The results of this study showed that 34.64% of the creamy pastries and 3.33% of the dried pastries were contaminated with *S. aureus.* This outcome showed

Table 1. The antibiotic resistance patterns, biofilm formation, virulence genes and different phage and REP types of selected *S. aureus* isolates

	SGA	SGB	SGF	SGFa	SGFb	SGD	SGL	FOX	P	AM	Cli	TE	*icaA*	*icaB*	*icaD*	Sec I	Sec III	Biofilm	Rep-type
1	-	-	-	-	-	-	-	S	R	R	S	S					+	None-adherent	G
2	-	-	+	-	-	-	-	S	R	R	S	S	+		+			Strongly-adherent	E
3	-	+	+	-	-	-	+	S	S	S	S	S					+	None-adherent	E
4	-	-	+	-	-	-	-	R	R	R	S	S						Weakly-adherent	E
5	-	-	-	-	-	-	-	S	R	R	I	S				+		None-adherent	E
6	-	-	+	-	-	-	-	S	R	S	S	S	+	+	+	+	+	Strongly-adherent	D
7	-	-	+	-	-	-	-	R	R	R	S	R					+	None-adherent	E
8	-	-	-	-	-	-	-	S	R	R	S	S						None-adherent	E
9	-	-	+	-	+	-	-	S	S	S	S	S						None-adherent	E
10	-	-	+	-	-	-	-	S	R	S	S	S						Weakly-adherent	E
11	-	-	+	-	-	-	-	S	R	S	S	S						None-adherent	G
12	-	-	+	+	-	-	-	S	R	S	I	S	+		+	+		Strongly-adherent	E
13	-	-	+	+	-	-	-	S	R	S	S	S	+		+	+	+	Strongly-adherent	E
14	-	-	+	+	-	-	-	S	R	R	S	S	+		+	+	+	Strongly-adherent	E
15	-	+	+	-	-	-	-	S	S	S	S	S	+		+			Strongly-adherent	F
16	-	-	+	-	-	-	-	R	R	S	S	S	+		+	+		Weakly-adherent	A
17	-	-	+	+	-	-	-	S	R	S	S	R	+		+	+		Strongly-adherent	E
18	-	-	+	+	-	-	-	S	R	R	S	S				+	+	Weakly-adherent	C
19	-	-	+	-	-	-	-	R	R	S	S	S					+	None-adherent	A
20	-	-	-	+	-	-	-	S	R	S	S	S	+		+		+	Strongly-adherent	A
21	-	-	+	+	-	-	-	S	R	R	S	S	+	+	+		+	Strongly-adherent	A
22	-	-	+	+	-	-	-	R	R	R	I	S	+		+			Strongly-adherent	A
23	-	-	+	-	-	-	-	S	R	S	S	S	+	+		+	+	Strongly-adherent	E
24	-	-	-	-	-	-	-	R	R	S	S	S	+				+	Weakly-adherent	D
25	-	-	+	-	+	-	-	R	R	S	S	S	+	+	+	+	+	Strongly-adherent	D
26	-	+	+	-	+	-	-	R	R	S	I	S						None-adherent	D
27	-	+	+	-	-	-	-	R	R	S	S	S						Weakly-adherent	D
28	-	-	+	-	-	-	-	S	R	S	S	S				+		Strongly-adherent	D
29	-	-	+	-	+	-	-	R	R	S	S	S	+	+	+		+	Strongly-adherent	D
30	-	-	+	-	+	-	-	R	R	S	S	S						None-adherent	B
31	-	-	+	-	-	-	-	R	R	S	S	S				+	+	Weakly-adherent	B

*P: Penicillin, AM: Ampicillin, TE: Tetracycline, E: Erytromycin, Cli: Clindamycin, ice: intercellular adhesion

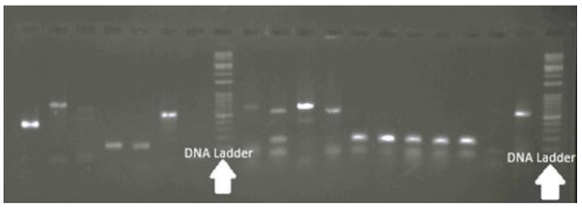

Fig. 1. The rep-PCR gel electrophoresis of *S. aureus* strains isolated from pastry samples

Lane 1-7, 9-19. The rep types of *S. aureus* isolates, Lane 8 and 20: DNA Ladder (100 bp)

the creamy-filled pastries could be a suitable environment for *S. aureus* growth than baked pastries. Thus, the consumption of creamy pastries increases the risk of infection with *S. aureus.* Also, we found high antibiotic resistance to penicillin and ampicillin but, fortunately, low resistance to erythromycin, ciprofloxacin, clindamycin, gentamicin, tetracycline, cefoxitin, and cefalotin. This indicates that antibiotic susceptible isolates are circulated within our community. The quantitative and qualitative study

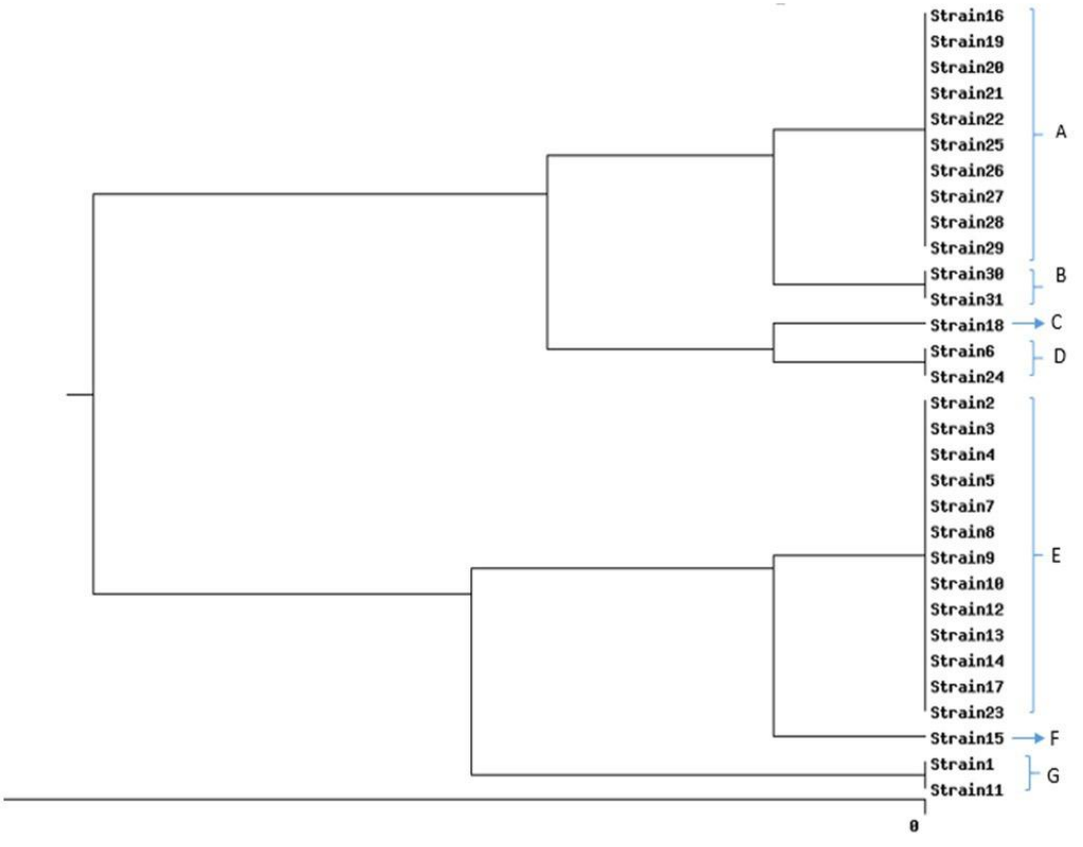

Fig. 2. Dendrogram of rep-PCR patterns of 31 *S. aureus* isolated from pastry samples in Hamadan

of biofilm production indicates half of the separated isolates have a strongly adherent ability due to biofilm formation. This data was compatible with *icaA* and *icaC* genes detection that they are necessary for biofilm formation. This information proved that biofilm prevention is the right way to eliminate *S. aureus* isolates from the pastries. The detection of SCC-mec types among half of the isolates showed the pathogenicity island could incorporate into the *S. aureus* genome as well as prophages.

According to our findings, SGF phage was the dominant phage, especially the SGFa subgroup, and also we used rep-PCR due to discriminating related and unrelated *S. aureus* isolates. Eight different rep-PCR types including four common types and four unique types were identified among *S. aureus* strains. This research showed the genetic diversity and determined diverse clones of *S. aureus* with different prognoses. The isolates defined in the common types had different phage patterns as well as virulence factors, antibiotic susceptibility, biofilm formation, and SCC-mec types. It is important in providing Hamadan services because these diverse isolates might have different abilities or might acquire them. There are some studies that report different results of the prevalence of *S. aures* and toxin strains isolated from creamy and dried pastry samples.

According to our results in a study by Fatahi et al. *S. aureus* was more isolated from creamy pastry samples. In their study, *S. aureus* was isolated from 34.64% and 3.33% creamy and dried pastry samples, respectively. According to our results, the highest resistance to penicillin (90%) was detected. Their funding of quantitative biofilm assay was agreed to our results, 42 strains (42%) were strongly adhesive, while 17 (17%) and 41 (41%) strains were weakly adhesive and lacked adhesive ability, respectively (16).

In some studies, different goals have been considered, as well as, Azizkhani et al. investigated the prevalence of MRSA isolates from 360 creamy pastry samples products from the local markets in Amol, North of Iran. Among 360 pastry samples 150 (41.6%) samples were contaminated by *S. aureus* with an average count of 4.94 log CFU/g in summer; 4.72 log CFU/g in autumn, 2.74 log CFU/g in winter, and 3.62 log CFU/g in spring. Eleven (3.05%)

samples have the *mecA* gene. No MRSA isolate was identified among winter specimens. 56% of isolated strains were sensitive to oxacillin, 7% of isolates were sensitive to penicillin, 23% to ampicillin, 82% to gentamicin, and 33% to tetracycline (17).

In a study by Sundararaja et al. from India, a total of 100 different food samples, including milk, cake, cheese, and chicken products were investigated for *S. aureus* and Staphylococcal Enterotoxin B (SEB) by PCR. Their findings showed a total of 34 isolates were *S. aureus*, 4 *S. aureus* isolates were detected in cake samples, and 14 (41%) isolates were found positive for SEB. Among, 4 isolates from cake sample 2 were positive for *sec*. Consistent with our study no resistance to ciprofloxacin and high resistance to penicillin was detected among *S. aureus* isolates (18).

Various typing methods and virulence genes detection have been done for epidemiological studies of *S. aureus.* There are several studies on the relationship between phage typing and molecular methods such as Pulsed-field gel electrophoresis (PFGE), rep-PCR, DLST, MLST, MLVA, and *spa* typing (19-22). Although, PFGE is a gold standard typing method for *S. aureus* and this method can be used for epidemiological studies and infection control but the results of the rep-PCR are reliable. The rep-PCR has high discriminatory power in analyzing but PFGE was able to distinguish a greater number of distinct patterns. Overall, because of non-typeability of some isolates, correspondence between PFGE and rep-PCR subgroups is necessary, especially for unrelated isolates. Therefore, the isolates that were non-typeable by rep-PCR could be recognized by PFGE or MLST (22).

Bacteriophages are mainly used in primary production, preservation, and sanitization in the food industry to ensure food safety (23). Phage typing is a phenotypic method that uses for detecting bacteria. Using this method to identify bacterial strains, especially in a food sample as well as in the confectionery industry can be very helpful and effective. In older studies, before the advent of molecular typing methods, this method was used to identify and type bacteria in food. New studies in this field are very limited. However, in clinical samples, we found studies that use phage typing for detection and characterization of *S. aureus* isolates. In a study by Dini et al. dissimilar results were obtained, the results of their study showed that out of 126 strains, SGB (88%) was the most prevalent prophage whereas in our study SGF is the most prevalent prophage, SGA and SGD were not detected in any isolate (24). In another study from Iran, prophage typing of clinical MRSA strains revealed that except for SGD prophage types, and similar to our results prophage SGF was the most common prophage among MRSA strains (5).

The rep-PCR is one of molecular typing that is used in molecular typing of bacteria as well as *S. aureus* strains. Reinoso et al. examined 45 *S. aureus* isolated from clinical specimens of humans, mastitis specimens in cattle, and food specimens by rep-PCR, which seven different genetic groups were identified with different virulence patterns and their results indicated the presence of various Rep types of *S. aureus* in different parts of Argentina (25). In a study by Dini and et al. the results of the rep-PCR analysis revealed 14 different patterns (seven common types and seven unique types) among the MRSA and MSSA isolates (24). There are few studies about rep-PCR typing among food subjects, especially pastries. Overall, the results obtained by rep-PCR in our study demonstrated diversity among selected *S. aureus* isolates. Isolates were recognized as belonging to five common types and two unique types.

Our findings showed diversity in prophage profiles, rep-types, and antibiotic-resistance patterns among *S. aureus* strains. The presence of SGF phage prognoses the virulence factors that it is possible to cause a wide range of diseases, some of which may be life-threatening diseases. It has been shown that typing a profile can be an acceptable way to predict the presence of these virulence factors. On the other hand, we found that differences in the geographical area can be a very important factor in influencing the model of the project. Based on our findings of the rep-PCR technique, the diversity in the strains collected from Hamadan pastries was proved and it was determined that we are facing serious challenges to control and manage strains.

There was some limitation of this study due to the costs of materials and lack of access to clinical samples and pastry samples simultaneously in order to compare these two types of samples. We also suggest other typing methods for further characterization of the isolates.

CONCLUSION

This research showed genetic diversity and the presence of different and diverse clones of *S. aureus*

in pastry samples in Hamadan city, Iran. The isolates with common rep types showed different Antibiotic-resistance patterns as well as different virulence genes. The presence of different phage-encoded virulence factors and antibiotic-resistant genes among *S. aureus* strains enables them to produce a broad range of diseases. Thus, diverse *S. aureus* strains can be considered as potential threat to the patient's health. Therefore, the consumption of creamy pastries increases the risk of infection with *S. aureus* and it is a serious warning to the health system. Pasteurization and storage of foodstuff in the refrigerator, continuous microbial control of pastries, and the screening of the confectionary staff can reduce the level of microbial contamination and the risk of staphylococcal poisoning.

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