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DETECTION OF ENTEROTOXIN GENES OF *Staphylococcus aureus* ISOLATES FROM FOOD CONTACT SURFACES IN THE DAIRY INDUSTRY OF JALISCO, MEXICO

DETECCIÓN DE GENES DE ENTEROTOXINAS DE *Staphylococcus aureus* AISLADOS DE SUPERFICIES EN CONTACTO CON LOS ALIMENTOS DENTRO DE LA INDUSTRIA LÁCTEA DE JALISCO, MÉXICO

María Guadalupe Avila-Novoa, Maricarmen Iñiguez-Moreno, Jean Pierre González-Gómez, Eduardo Zacarías-Castillo, Pedro Javier Guerrero-Medina, J. Jesús Padilla-Frausto, Claudia Luz Navarro-Villarruel, Melesio Gutiérrez-Lomelí*
Laboratorio de Alimentos, División de Desarrollo Biotecnológico, Centro Universitario de la Ciénega, Universidad de Guadalajara, Av. Universidad 1115, Col. Linda Vista, 47820, Ocotlán, Jalisco, México

ABSTRACT

Staphylococcus aureus is widely distributed in nature, and two of the main sources of *S. aureus* contamination in the food industry are food handlers and Food Contact Surfaces (FCS). This pathogen is responsible for outbreaks of foodborne illnesses associated with the consumption of milk and dairy products. The aim of this study was to determine the prevalence of toxin-encoding genes in *S. aureus* isolates from FCS in the Jalisco dairy industry. The presence of enterotoxin genes was investigated by PCR. In this study, we identify 84 *S. aureus* isolates. Overall, 35.7% of the isolates tested positive for at least one of the nine studied enterotoxin genes, and 15.4% harbored 2-4 enterotoxin genes. The most predominant genes were *sej* and *sed*. This study demonstrates that FCS are a source of contamination for this food rotation and that there is a diversity of enterotoxin genes in *S. aureus* isolates. Therefore, vigilant food safety practices need to be implemented regarding FCS to prevent foodborne infections and intoxications due to *S. aureus* contamination.

Keywords: *Staphylococcus aureus*; enterotoxin genes; food contact surface-stainless steel; food contact surface-polypropylene.

RESUMEN

Staphylococcus aureus se encuentra ampliamente distribuido en la naturaleza, sin embargo, en la industria de alimentos dos de las principales fuentes de contaminación son el manipulador y las superficies en contacto con los alimentos (SCA). Este patógeno ha sido responsable de la generación de brotes de enfermedades transmitidas por alimentos donde los vehículos implicados fueron la leche y derivados lácteos. El objetivo del presente trabajo fue determinar la prevalencia de genes de enterotoxinas en *S. aureus* aislados de SCA dentro de la industria láctea de Jalisco. La detección de genes de enterotoxinas se realizó mediante PCR. Se identificaron 84 aislamientos de *S. aureus*. En el 35.7% de los aislados se detectó al menos un gen de los nueve estudiados y en el 15.4% de dos a cuatros genes. Los dos principales genes detectados fueron *sej* y *sed.* El estudio demuestra que las superficies de contacto de los alimentos son una fuente de contaminación para este giro alimentario y que existe una diversidad de genes de enterotoxinas en los aislamientos de *S. aureus*. Por lo tanto, se deben de implementar prerequisitos sanitarios en las SCA para prevenir intoxicaciones alimentarias debido a la contaminación por *S. aureus*.

Palabras claves: *Staphylococcus aureus*, genes de enterotoxinas, superficies en contacto con los alimentos de acero inoxidable, superficies en contacto con los alimentos de polipropileno.

INTRODUCTION

Staphylococcus aureus is a foodborne pathogen that is considered one of the world's leading causes of disease outbreaks related to food consumption (Marino *et al.*, 2011). Staphylococcal Enterotoxins (SE) are a series of intracellular proteins that are primarily produced by *S. aureus* strains. The major characteristics of SE are that they are single-chain proteins with a low molecular weight (27-31 kDa) and are rich in lysine, aspartic acid, glutamic acid, and tyrosine residues (Le Loir *et al.*, 2003). SE are heat-stable and resist the activity of proteolytic enzymes (pepsin, trypsin, and chymotrypsin), maintaining their activity in the digestive tract after ingestion (Le Loir *et al.*, 2003). Twenty-three serologically distinct staphylococcal enterotoxins and Staphylococcal Enterotoxin-like (SEl) proteins have been identified to date (Argudín *et al.*, 2010; Sato'o *et al.*, 2014; Rong *et al.*, 2017). It has been reported that 95% of Staphylococcal Food Poisoning (SFP) outbreaks are associated with classical biotypes, while new biotypes are responsible for the remaining 5% (Tang *et al.*, 2011).

To clarify the roles played by these newly identified SE in food poisoning, the development of reliable methods of SE detection is essential. Recently, Polymerase Chain Reaction (PCR) assays have been developed to detect the presence of many SE genes and were reported to be efficient and reliable (Fisher *et al.*, 2009).

Very recently, multiple studies have investigated the prevalence of SE genes within *S. aureus* strains isolated from milk, fresh cheeses, food handlers hands, Food Contact Surfaces (FCS)-stainless steel and workers gloved hands (Marino

*Autor para correspondencia: Melesio Gutiérrez-Lomelí
Correo electrónico: mele.gtz@gmail.com
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et al., 2011; Torres-Vitela *et al.*, 2012; Djekic *et al.*, 2016). In Mexico, Torres-Vitela *et al.* (2012) determined the incidence of pathogens (*Salmonella*, *Escherichia coli* O157:H7 and *Listeria monocytogenes*) in panela and adobera fresh-cheese samples, but staphylococcal enterotoxins were not detected in any of 200 samples of fresh cheese collected from wholesale distribution centers in the Guadalajara metropolitan area, Jalisco State, Mexico. These studies demonstrate the health risk of consuming fresh cheeses in Mexico as well as the inadequate conditions throughout the production and sales. In addition, errors in the cheese-production chain, inadequate heat treatment, and poor personal hygiene, as well as incorrect conservation temperature and time are among the risk factors for staphylococcal intoxication (Gucukoglu *et al.*, 2012). However, dust and FCS can be vehicles for the transfer of *S. aureus* in food (Marques *et al.*, 2007; Argudín *et al.*, 2010; Sospedra *et al.*, 2012). The aim of this study was to determine the prevalence of enterotoxin genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg, seh*, *sei*, and *sej*) in *S. aureus* isolates from FCS of the Jalisco dairy industry.

MATERIALS AND METHODS

Sample collection. A total of 293 samples were collected from FCS (183 FCS-stainless steel and 110 FCS-polypropylene). Six dairy industries in the Mexican state of Jalisco were tested. In each of the selected FCS (containers, pasteurizers, curd cutters, workshops, molds, and mixers), a 200 cm^2 area was sampled. Samples were taken aseptically from FCS by rubbering the surfaces for 20 s with sterile swabs moistened with either Letheen broth base (BD Diagnostic Systems) or saline solution supplemented with sodium thiosulfate. Disinfectant dependent neutralization agents (dodecyldimethylammonium chloride or sodium hypochlorite solutions) were employed following the Sanitary Standard Operating Procedure (SSOP) of each dairy industry.

Isolation and identification of *S. aureus*. Briefly, each sample was spread onto Baird Parker agar plates supplemented with an egg yolk tellurite emulsion and were incubated at 37°C for 48 h. The *S. aureus* strains were identified by the methods described in the Bacteriological Analytical Manual (FDA, 2016). In general, strains were cultured in TSB broth and BPA (BD Diagnostic Systems) and then subjected to Gram staining, the coagulase and Voges-Proskauer tests, tests for catalase and thermostable nuclease, glucose and mannitol utilization tests (FDA, 2016), and finally, PCR for confirmation. DNA extraction from *S. aureus* was performed using the protocol described by Pu *et al.* (2012). Specific primers for PCR detection of *S. aureus* were used as described by Straub *et al.* (1999). The first primer pair (staur4: 5′ ACGGAGTTACAAAGGACGAC 3′ and staur6: 5′ AGCTCAGCCTTAACGAGTAC 3′) (Invitrogen, Carlsbad, CA, USA) was used to target the 23S ribosomal rDNA. Amplification conditions were as follows: 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 58°C, and 75 s at 72°C; and a final extension at 72°C for 5 min. Reactions were carried out in a Thermal Cycler (Techne, Bibby Scientific Ltd, U.K.). A 1,250 base pair (bp) PCR fragment was expected. PCR products were electrophoresed through a 1% agarose gel containing ethidium bromide (0.5 μg mL^{-1}; Sigma-Aldrich) and visualized by transillumination under ultraviolet (UV) light (UVP, Upland, CA, USA). *S. aureus* ATCC 25923 was used as positive control.

Detection of enterotoxin genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, and *sej*). Genomic DNA extraction and detection of enterotoxin genes in *S. aureus* was performed using the protocol described by Pu *et al.* (2011). The detection of nine enterotoxin genes was performed using two multiplex PCRs. Primer set A allows for the detection of the genes *sea*, *seb*, *sec*, *sed*, *see*, and *seg*, while primer set B allows for the detection of the genes *seh*, *sei*, and *sej* (Table 1) (Monday and Bohach, 1999). Each PCR reaction had a total volume of 25 μL and consisted of the following: 1X PCR buffer; 0.2 mM aliquots of each deoxynucleotide triphosphate; 2.5 mM $MgCl_2$; 0.625 U of Taq polymerase (Taq Platinum; Invitrogen); 0.3 μM aliquots of each primer (Invitrogen, Carlsbad, CA, USA); and DNA template. The following conditions were employed: 5 min at 95°C; 30 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C; and a final extension of 7 min at 72°C. *S. aureus* ATCC 25923, 6538, 13565 (SEA), 14458 (SEB), 19095 (SEC), 23235 (SED), 27664 (SEE), FRI472 (SED, SEG, SEI, SEJ) and 51811 (SEH) were used as positive controls for the indicated SE genes. PCR products were electrophoresed through a 1.5% agarose gel containing ethidium bromide (0.5 μg mL^{-1}; Sigma-Aldrich) and visualized by transillumination under UV light.

Detection of the *nuc* gen in isolations of *S. aureus*. PCR targeted the *S. aureus*-specific *nuc* gen according to

Table 1. Sequences of primers used for PCR in this study.
Tabla 1. Secuencia de los cebadores usados en la PCR en este estudio.

Target gene	Sequences (5′ - 3′)	Product (bp)*
sea	GCA GGG AAC AGC TTT AGG C GTT CTG TAG AAG TAT GAA ACA CG	521*
seb	ATG TAA TTT TGA TAT TCG CAG TG TGC AGG CAT CAT ATC ATA CCA	665*
sec	CTT GTA TGT ATG GAG GAA TAA CAA TGC AGG CAT CAT ATC ATA CCA	284*
sed	GTG GTG AAA TAG ATA GGA CTG C ATA TGA AGG TGC TCT GTG G	385*
see	TAC CAA TTA ACT TGT GGA TAG AC CTC TTT GCA CCT TAC CGC	171*
seg	CGT CTC CAC CTG TTG AAG G CCA AGT GAT TGT CTA TTG TCG	328*
seh	CAA CTG CTG ATT TAG CTC AG GTC GAA TGA GTA ATC TCT AGG	359*
sei	CAA CTC GAA TTT TCA ACA GGT AC CAG GCA GTC CAT CTC CTG	466*
sej	CAT CAG AAC TGT TGT TCC GCT AG CTG AAT TTT ACC ATC AAA GGT AC	142*
nuc	GCG ATT GAT GGT GAT ACG GTT AGC CAA GCC TTG ACG AAC TAA AGC	280**

* Monday and Bohach (1999), ** Brakstad *et al.* (1992).

Brakstad *et al.* (1992) (Table 1). Amplification conditions were the following: 2 min at 94ºC; 37 cycles, of 1 min at 94ºC, 30 sec at 55ºC, and 30 sec at 72ºC, and a final extension of 5 min at 72ºC. A 280-bp PCR fragment was expected. PCR products were electrophoresed through a 2% agarose gel containing ethidium bromide (0.5 µg mL^{-1}; Sigma-Aldrich) and visualized by transillumination under UV light. *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 35984 were employed as positive and negative controls, respectively**.**

RESULTS

It is very important to emphasize that the food handler and the contact surfaces play an important role as sources of pollution in the dairy industry. In the present study, the *S. aureus* strains were identified by the conventional methods and the biochemical profile was as follows: Gram staining "positive", the coagulase, Voges-Proskauer, catalase and thermostable nuclease tests "positive", glucose and mannitol utilization tests "positive". Likewise, the *S. aureus* strains were confirmed by PCR. The isolation and identification of *S. aureus* showed that 16.4% (48/293) of the total studied samples were contaminated by *S. aureus*. A total of 84 *S. aureus* strains [SA1-SA84, FCS-polypropylene (26 strains) and FCS-stainless steel (58 strains)] were recovered from 16.4% food contact surfaces of the Jalisco dairy industries (México) (Figure 1 and Table 2).

The detection of enterotoxin genes was carried out by multiplex PCR. The results of the multiplex PCR analysis for all 84 *S. aureus* isolates are presented in Figure 2 and Table 2. The enterotoxigenic *S. aureus* strains were isolated with higher frequency of FCS-stainless steel. Overall, 30 (35.7%) of the 84 *S. aureus* isolates contained at least one enterotoxin gene, 6 (6 of 26, 23.1%) strains were isolated from FCS-polypropylene and 24 (24 of 58, 41.4%) strains from FCS-stainless steel. The most frequently detected genes were: *sej* (11 isolates, 13%), *sed* (4 isolates, 4.7%), *sed* + *seg* (2 isolates, 2.3%), and *sed* + *seg* + *sej* (2 isolates, 2.3%). The genotypes detected less frequently (1 isolates, 1.1%) were: *seh*, *sei*, *sej*, *sea* + *sej*, *sec* + *sej*, *sei* + *sej*, *seh* + *sej*, *sei* + *sej*, *sed* + *seg* + *sei*, *sec* + *sed* + *seh* + *sej*, and *sec* + *sed* + *seg* + *sej*; while the *see* gene was not detected.

Subsequently, 70 (83.3%) of the 84 *S. aureus* isolates were positive for amplification of the *nuc* gene, the 280-bp specific band (Figure 3), with a relatively low positive correlation (p = 0.22-0.25) with the enterotoxin genes.

DISCUSSION

Studies conducted in many countries have detected the presence of enterotoxigenic *S. aureus* in various food products and in food handlers, which are regarded as a primary contamination source leading to SFP (Seo and Bohach, 2007). A study of small-scale dairy systems located in the highlands of Central Mexico detected the presence of 102 enterotoxigenic *S. aureus* isolates (84 from cows; 18 from farmers), 78.9% of which harbored at least one enterotoxin gene (*sea-sed*, *seg*, and *sei*) (Salgado-Ruiz *et al.*, 2015). Similar findings were reported in 59.8% of meat and dairy products in Italy (Normanno *et al.*, 2007) and in 5.8% of dairy products in Iran, whereas 18% harbored at least one enterotoxin encoding gene [*seg+sei* (20%), *sej* (15%), *sec+sej* (15%), *sei+seh* (10%), *sea+sed+sej* (5%), *sea+sej+sei* (5%) and *sea+sec+seh* (5%)] (Rahimi, 2013).

It is important to emphasize the role that FCS play as sources of contamination in the dairy industry. Gutiérrez *et al.* (2012) reported the frequencies of *S. aureus* on inert surfaces in the dairy (2.7-5.8%), meat (3.2%), and shrimp (9.9%) industries. Djekic *et al.* (2016) detected *S. aureus* on 0.9% of FCS–stainless steel and 1.3% on FCS-plastic in take-out food establishments and reported on the impact of climatic con-

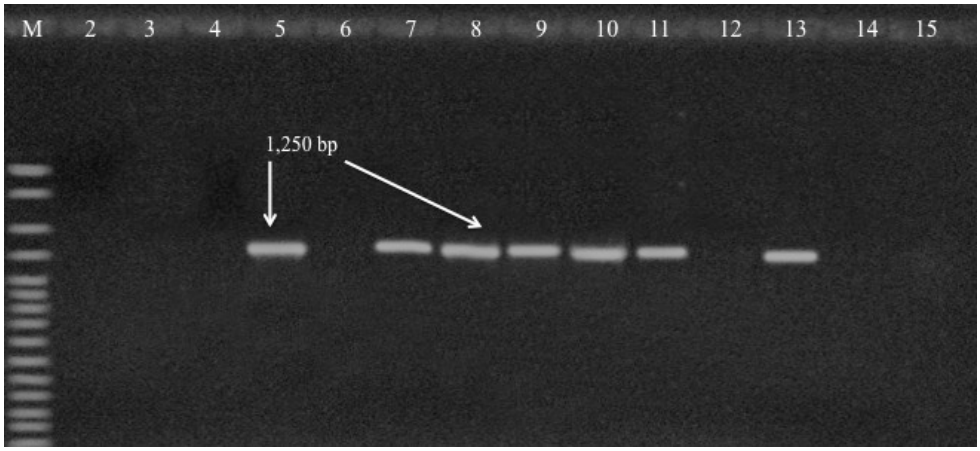

Figure 1. Identification of *S. aureus*. Lane 1 (M): 100 bp DNA ladder (Invitrogen); lane 2: negative control (no DNA); Lane 5: *S. aureus* ATCC 25923 (positive control) *23S* (1,250 bp); Lanes 7-11 and 13: *S. aureus* isolated from surfaces (1,250 bp); and Lanes 3-4, 6, 12, and 14-15 = other isolates of *Staphylococcus* spp.
Figura 1. Identificación de *S. aureus*. Carril 1 (M): marcador de peso molecular de 100 pb (Invitrogen); carril 2: control negativo (no contiene DNA); carril 5: *S. aureus* ATCC 25923 (control positivo) *23S* (1,250 pb); carriles 7-11 y 13: *S. aureus* aislados de las superficies (1,250 pb); y carriles 3-4, 6, 12, y 14-15 = otros aislados de *Staphylococcus* spp.

Table 2. *Se* genes distribution among *S. aureus* isolates.
Tabla 2. Distribución de los genes *se* en los aislados de *S. aureus*.

No. (%) of Strains (SA1-SA84)			
Genotype Total (n= 84)	**FCS-polypropylene n=26 (100)**	**FCS-stainless steel n=58 (100)**	**Total 84 (100)**
se negative	20 (76.9)	34 (58.6)	54 (64.3)
se positive	6 (23.1)	24 (41.4)	30 (35.7)
sed	1 (3.8)	3 (5.1)	4 (4.7)
seh		1 (1.7)	1 (1.1)
sei	1(3.8)		1 (1.1)
sej	1(3.8)	10 (17.2)	11 (13)
sea + *sej*		1(1.7)	1 (1.1)
sed + *seg*		2 (3.4)	2 (2.3)
sec + *sej*		1(1.7)	1 (1.1)
sei + *sej*		1(1.7)	1 (1.1)
seh + *sej*		1(1.7)	1 (1.1)
sei + *sej*		1(1.7)	1 (1.1)
sed + *seg*+ *sej*	2 (7.6)		2 (2.3)
sed + *seg* + *sei*	1(3.8)		1(1.1)
seb + *sed* + *sei*		1(1.7)	1 (1.1)
sec + *sed* + *seh* + *sej*		1(1.7)	1 (1.1)
sec + *sed* + *seg* + *sej*		1(1.7)	1 (1.1)

n = *S. aureus* strains.

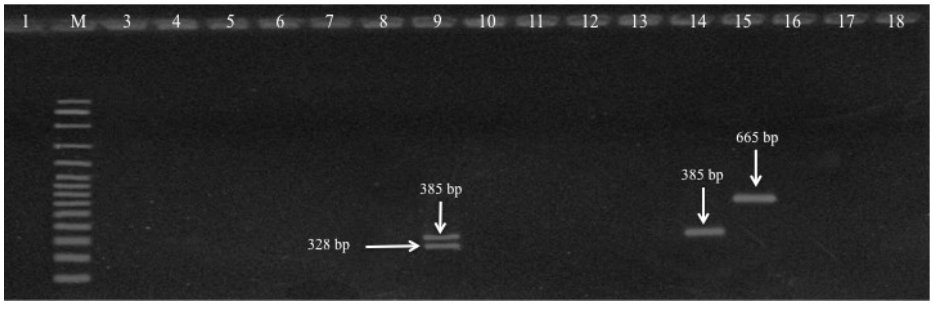

Figure 2. Identification of enterotoxin genes. Lane 1: control negative (no DNA); Lane 2 (M): 100 bp DNA ladder (Invitrogen); Lane 9: 328 and 385 bp amplicons of respective *sed* and *seg* genes of *S. aureus* isolated from surfaces; Lane 14: 385 bp amplicon of the *seg* gene of *S. aureus* isolated from surfaces; Lane 15: 665 bp amplicon of the *seb* gene of *S. aureus* ATCC 14458 (positive control), and Lanes 3-8, 10-13, and 16-18 = other isolates of non-enterotoxigenic *S. aureus*.
Figura 2. Identificación de genes de enterotoxinas. Carril 1: control negativo (no contiene DNA); carril 2 (M): marcador de peso molecular de 100 pb (Invitrogen); carril 9: fragmentos amplificados de 328 and 385 pb de los genes *sed* y *seg* de *S. aureus* aislados de superficies; carril 14: fragmento amplificado de 385 pb del gen *seg* de *S. aureus* aislado de superficie; carril 15: fragmento amplificado de 665 pb del gen *seb* de *S. aureus* ATCC 14458 (control positivo); y carriles 3-8, 10-13, and 16-18 = otros aislados de *S. aureus* no enterotoxigénico.

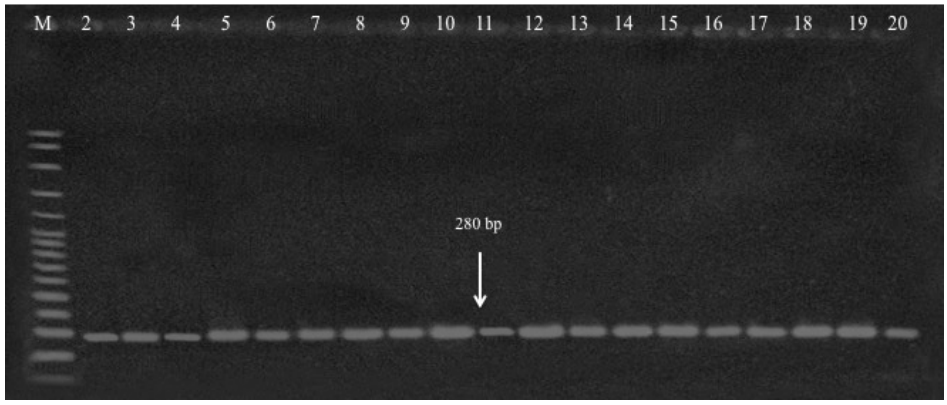

Figure 3. Identification of *nuc* gen. Lane 1 (M): 100 bp DNA ladder (Invitrogen); Lane 2: 280 bp amplicon of the *nuc* gene for *S. aureus* ATCC 25923 (positive control); Lanes 3-20: 280 bp amplicons of the *nuc* gene of *S. aureus* isolated from surfaces.
Figura 3. Identificación del gen *nuc*. Carril 1 (M): marcador de peso molecular de 100 pb (Invitrogen); carril 2: fragmento amplificado de 280 pb del gen *nuc* de *S. aureus* ATCC 25923 (control positivo); carriles 3-20: fragmentos amplificados de 280 pb del gen *nuc* de *S. aureus* aislados de superficies.

ditions. Sospedra *et al.* (2012) detected *S. aureus* on 5.8% of stainless-steel tables used by food handlers and food service establishments in Spain. Marino *et al.* (2011) detected *Staphylococcus* species in isolates from food (15.4%), on food contact surfaces (21.1%) and on the gloves of the catering staff (46.3%). The most predominant species of all of the isolates was *S. aureus,* which accounted for 26.8% of all strains.

In this study, the majority of the isolated *S. aureus* (69%; 58/84) was recovered from FCS-stainless steel due to the high surface-free-energy of stainless steel, which renders a hydrophilic surface, whereby bacterial adhesion is favored when the bacterial surface energy is greater (Brooks and Flint, 2008; Marino *et al.*, 2011). Similarly, surface roughness, hydrophobicity, and vulnerability-to-wear, which are all factors to which such materials are prone, should be taken into consideration (Van Houdt and Michiels, 2010; Schlisselberg and Yaron, 2013). Differences between FCS identified in this research can be linked to several factors, including the quality of management systems, food safety practices established by the industries, the validation and verification of SSOP, food handlers' good hygiene practices, good agricultural practices, and preventive controls (cooling temperatures, etc.), as well as the material surfaces sampled or the accumulation of organic and inorganic wastes generated during food processing (Lindsay and von Holy, 2006).

Our study is consistent with studies reporting on inert dairy-industry FSC, which indicate that the presence of *S. aureus* (21.1-58%) is related to improper food handling or poor cleaning and disinfection of surfaces (Moore and Griffith, 2002; Marino *et al.*, 2011; Soares *et al.*, 2011; Antoci *et al.*, 2013). An inadequately cleaned surface can contaminate any food with which it comes into contact with, which can increase the microbial load of the food and decrease its shelf-life (Moore and Griffith, 2002). Chen *et al.* (2001) detected a high frequency of *S. aureus* in Ready-To-Eat foods, representing a potential health hazard to consumers and indicating improper handling and cross-contamination. Foodborne diseases originate from bacterial cross-contamination of FCS and subsequent cross-contamination to food products (Shi and Zhu, 2009).

These data are consistent with research conducted in other countries, which found a high percentage of new enterotoxin genes and a low abundance of classical enterotoxin genes (*see*) (Pu *et al.*, 2011; Tang *et al.*, 2011; Gutiérrez *et al.*, 2012; Rahimi, 2013). It is noteworthy that previous studies have revealed that SE are encoded by mobile genetic elements, such as plasmids, bacteriophages or pathogenicity islands. These elements facilitate horizontal gene transfer among bacterial populations and can allow strains to acquire multiple copies of the same enterotoxin gene (Zouharova and Rysanek, 2008; Argudín *et al.*, 2010). For example, the enterotoxin gene cluster (*egc*) contains several SE or SEl genes (*seg*, *sei*, *sem*, *sen*, *seo*, and *seu*) and is a possible source of new SE genes (Jarraud *et al.*, 2001; Argudín *et al.*, 2010; Sato'o *et al.*, 2014; Rong *et al.*, 2017).

In this study, the prevalence of *nuc* gene was 83.3% in the 84 *S. aureus* isolates, which had a relatively low positive correlation with the enterotoxigenic genes. Similar results were obtained by Moghassem-Hamidi *et al.* (2015); these authors reported that the prevalence of the enterotoxin A gene was 14% (human samples: 22%; food samples: 16.6%), but no correlation was demonstrated between the presence of *coa* and *nuc*. Although thermonuclease and coagulase tests are valuable for identifying *S. aureus*, these tests do not necessarily imply enterotoxigenic strains (Borelli *et al.*, 2011; Moghassem-Hamidi *et al.*, 2015).

Differences in the presence of classical and new *S. aureus* enterotoxin types in these studies indicates a geographic variation in the distribution of enterotoxigenic *S. aureus* strains. This variation may reflect the distinct ecological reservoirs present in different countries or may simply result from differences in the sensitivity and specificity of the techniques employed to detect the enterotoxins.

CONCLUSIONS

In conclusion, this study is, to our knowledge, the first study conducted in dairies in Jalisco state, Mexico, for the detection of enterotoxigenic *S. aureus* from FCS. In addition, this study provides evidence suggesting an increased risk of staphylococcal poisoning associated with the consumption of dairy products, where FCS play an important role as sources of food contamination. Therefore, vigilant food safety practices need to be implemented regarding FCS to prevent foodborne infections and intoxications due to *S. aureus* contamination.

Conflict of interest: The authors declare that they have no conflict of interest.

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