Investigation of a Staphylococcal Food Poisoning Outbreak from a Chantilly Cream Dessert, in Umbria (Italy)

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Abstract

On August 28, 2015, a staphylococcal food poisoning outbreak occurred in Umbria, Italy, affecting 24 of the 42 customers who had dinner at a local restaurant. About 3 h after ingesting a variety of foods, the customers manifested gastrointestinal symptoms. Within 24 h of notification from the hospital emergency department, Sanitary Inspectors of the local Public Health Unit performed an epidemiological investigation. A retrospective cohort study was conducted among the customers. Food and environmental samples were collected. Due to the rapid onset of symptoms (vomiting, diarrhea), the food samples were analyzed for the presence of toxigenic bacteria and their toxins; nasopharyngeal swabs were collected from the waiters and cooks. Among the food tested, high levels of coagulase-positive staphylococci (CPS) (3.4×10^8 CFU/g) and staphylococcal enterotoxins (2.12 ng SEA/g) were only detected in the Chantilly cream dessert. CPS were also detected on the surface of a kitchen table (10 CFU/swab), and five food handlers were positive for *Staphylococcus aureus*. In total, five enterotoxigenic *S. aureus* isolates were recovered from three food handlers, a kitchen surface, and the Chantilly cream dessert. These isolates were further characterized by biotyping, pulsed-field gel electrophoresis, and multiplex polymerase chain reaction assays for the detection of eleven enterotoxin encoding genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sep*, and *ser*) and three genes involved in antibiotic resistance (*mecA*, *mecC*, and *mupA*). Three *sea*-positive strains, isolated from the dessert, environment, and one of the cooks, had the same pulsed-field gel electrophoresis profile and belonged to the human biotype, suggesting that the contamination causing the outbreak most likely originated from a food handler. Moreover, improper storage of the dessert, at room temperature for about 5 h, permitted microbial growth and SEA production. This study underlines the importance of both laboratory evidence and epidemiological data for outbreak investigation.

Keywords: outbreak investigation, foodborne outbreaks, food microbiology, pathogen identification and typing methods

Introduction

Foodborne outbreaks (FBOs) due to bacterial toxins are one of the most common diseases connected with food illness.

According to the data published by the European Food Safety Authority and the European Centre for Disease Control (EFSA and ECDC, 2016), bacterial toxins were responsible for 19.5% of all FBOs in 2015 and represented the third foodborne causative agent in the European Union. In 2015, 16 Member States (MS) reported 434 foodborne outbreaks caused by staphylococcal toxins. This represents 9.9% of all outbreaks, a small increase compared with 2014.

Staphylococcal enterotoxins (SEs) are produced by enterotoxinogenic strains of coagulase-positive staphylococci (CPS), mainly by *Staphylococcus aureus*, which, as ubiquitous bacteria, can colonize the human skin and mucosa. To date, 23 SEs have been reported in literature (Ono *et al.*, 2015). The

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presence of a *S. aureus* enterotoxigenic strain in the nasopharyngeal or oropharyngeal tract of a food handler is frequently associated with FBOs (Todd *et al.*, 2008; Todd *et al.*, 2010; Gallina *et al.*, 2013). Food poisoning occurs after the ingestion of food contaminated with enterotoxins produced by *S. aureus*; the onset of symptoms occurs a few hours (2–8) after ingestion of the contaminated food and improper preparation, handling, or storage (Schelin *et al.*, 2011). Nausea, vomiting, and diarrhea are the most relevant symptoms; the disease severity depends on the amount of the ingested toxin and health of the consumer. In most cases after 24 h, there is remission of symptoms; only a few cases of intoxications, ranging from 0.03% to 4.4%, are fatal in children and in the elderly (Doyle *et al.*, 2007).

S. aureus can frequently colonize the skin and mucosa of a high percentage of healthy human beings: about 50% of the healthy adults can spread *S. aureus* throughout their nasal mucosae (Kluytmans and Wertheim, 2005). A study conducted by Lues and Van Tonder (2007) indicated that *S. aureus* was present on 88% of the hands and 48% of the aprons of food handlers working in the delicatessen sections of a retail store. These data indicate that human contamination with enterotoxigenic strains during food preparation and processing plays an important role as a source of food contamination.

In this study, we describe a FBO due to SEs that occurred at a dinner in the restaurant of a sports team and identify the source of contamination as one of the restaurant workers carrying the same enterotoxigenic *S. aureus* strain as that found in the food, positive for SE type A.

Materials and Methods

Background

On August 28, 2015, the local Public Health Unit was notified by the hospital emergency department in Gubbio, Perugia (Italy), of a suspected FBO. On the previous day, 24 members of a sports team were admitted to the emergency room with complaints of abdominal pain, nausea, emesis, and diarrhea; all patients reported to have had dinner at the same local restaurant 3 h before. They did not eat together at any other restaurant on the day they became unwell. Eleven of these patients needed medical care. From 8.30 to 9.30 p.m., a total of 42 meals were served and at 11.30 p.m., the first set of customers started manifesting gastrointestinal symptoms. Microbiological analyses of biological, environmental, and food samples were performed, and an epidemiological investigation was conducted to characterize the outbreak, identify the probable source, and implement control measures.

Epidemiological investigation

After the notification, Sanitary Inspectors of the local Public Health Unit performed an epidemiological investigation of the suspected outbreak. Based on the information obtained from the patients, the Inspectors hypothesized that the outbreak could be associated with the consumption of a specific dish of meal served during the dinner. To test this hypothesis, a retrospective cohort study was conducted. Qualitative exposure variables (food items eaten) were compared among the ill and nonill people who had dinner at the restaurant. All 42 customers were asked to fill in a questionnaire to collect information about the food exposure and the main symptoms. The food specific attack rates (AR) and the risk ratios (RR) with confidence intervals (95%) were calculated. *p*-Values <0.05 were considered significant. Moreover, food handlers who worked at the restaurant were interviewed by the Sanitary Inspectors about the methods of preparing the meals served to customers.

Laboratory investigation

Officials of the local Health Unit performed environmental investigations at the restaurant on the day after the dinner. Detailed information about the food served, preparation procedures, and the food handlers were collected. Waiters and cooks underwent nasopharyngeal swab collection. Moreover, three environmental and four food samples were collected and transported under refrigeration to the Istituto Zooprofilattico Sperimentale of Umbria and Marche (IZSUM), for microbiological analyses.

Food samples consisted of refrigerated leftovers from two different pasta sauces (one made of tomatoes, mushrooms, and meat and one made of sheep milk cheese), a traditional Italian starter (tomato soup with pork and beans), and a dessert (Chantilly cream). No information was available about the possible pasteurization process of the sheep cheese used for the sauce. Due to the rapid onset of symptoms, food samples were analyzed for the presence of toxigenic bacteria and their toxins, which are commonly involved in food poisoning (EFSA and ECDC, 2016).

Bacterial enumeration and identification

– Food specimens were subjected to *Bacillus cereus* and CPS enumeration using standards UNI EN ISO 7932:2005 (UNI, 2005) and UNI EN ISO 6888-2:2004 (UNI, 2004), respectively. Enumeration of *Clostridium perfringens* was performed with an in-house method, using Tryptose Sulfite Cycloserine agar medium (TSC; Oxoid, Basingstoke, United Kingdom) for the anaerobic growth of sulfite-reducing bacteria and the API® systems (bioMérieux) for identifying the isolates.

– The environmental samples included three swabs collected from the surfaces of two kitchen tables and from a cold room shelf, respectively. These samples were investigated only for CPS enumeration according to the UNI EN ISO 6888-2:2004 protocol.

– CPS strains isolated from food and environmental samples were analyzed for the presence of *S. aureus*, by inoculating them onto 5% sheep blood agar plates (Oxoid) and identifying them using API systems (BioMérieux). The human swabs were streaked onto Mannitol Salt Agar plates (MSA; Oxoid). After 18–24 h incubation, typical yellow colonies on MSA were subcultured onto 5% sheep blood agar plates, and presumptive *S. aureus* isolates were identified using the catalase and coagulase tests (Han *et al.*, 2007).

Polymerase chain reaction analysis of *S. aureus* strains

– Isolates of different origins (food, environment, and humans) were confirmed as *S. aureus* using a multiplex polymerase chain reaction (PCR) assay (Zhang *et al.*, 2004) allowing the simultaneous detection of 16S rRNA (genus

specific) and *nuc* (species specific) genes. Two additional genes are targeted in this assay, that is, *mecA* (methicillin resistance) and *mupA* (mupirocin resistance).

– All the *S. aureus* isolated strains were tested for the genes encoding SEs, using two multiplex PCR assays, according to the EURL for the CPS method (De Buyser *et al.*, 2009a; De Buyser *et al.*, 2009b). Moreover, these strains were also characterized for the presence of *mecA*, *mecC* (Zhang *et al.*, 2005), and the Panton–Valentine leucocidin (PVL) genes lukS-PV/lukF-PV (Lina *et al.*, 1999) and were biotyped (Devriese, 1984; Kérouanton *et al.*, 2007).

Pulsed-field gel electrophoresis

Further molecular characterization of *S. aureus* isolates was performed with pulsed-field gel electrophoresis (PFGE), according to the EURL for CPS method (Marault *et al.*, 2011). Restriction fragments were separated using the CHEF-Mapper® electrophoresis system (Bio-Rad Laboratories, Hercules, CA). The gel was stained with SYBR® Safe DNA (Invitrogen, Carlsbad, CA) stain, and the DNA bands were visualized with the UVIsave® transilluminator (UVItec, Cambridge, United Kingdom). Gel images were sent to the Italian National Reference Laboratory for CPS (It-NLR, Turin) and analyzed with the BioNumerics software (Applied-Maths, Sint-Martens-Latem, Belgium).

Qualitative assay (European screening method) for SE detection

SEs were detected only in the Chantilly cream where in high levels of CPS were detected using the official European Screening Method (ESM) (Ostyn *et al.*, 2016). Briefly, the sample was first subjected to a protein extraction step followed by dialysis concentration. Detection was performed from the protein extract using the validated kits, VIDAS SET2 (bioMérieux®, Marcy l'Étoile, France) and RIDASCREEN SET Total (R-Biopharm® AG, Darmstadt, Germany) (Ostyn *et al.*, 2011), which can simultaneously detect SEA to SEE in food matrices without differentiating the five SEs (Hennekinne *et al.*, 2007; Nia *et al.*, 2016).

Quantitative enzyme-linked immunosorbent assay for SE quantification

Identification and quantification of the enterotoxin type present in the Chantilly cream were performed using an in-house quantitative enzyme-linked immunosorbent assay (ELISA). Briefly, quantification of SEs was performed using a quantitative indirect sandwich-type ELISA. A single sandwich type was used for SEB, whereas double sandwich ELISA types were used for SEA, SEC, and SED. Specific commercially available antibodies (Toxin Technology, Sarasota, FL) were used as coating (ref SLAI101, SLBI 202, SLCI 111, SLDI 303) and probing antibodies (ref LAI101, LBC 202, LCI 111, and LDI 303). The presence of enterotoxins was revealed by immunoglobulins coupled to horseradish peroxidase (goat-anti rabbit antibodies coupled to peroxidase) and determined by a colorimetric measurement at 405/630 nm after addition of the substrate, ABTS–H$_2$O$_2$ (KPL).

The calibration curves were generated from SE stock solutions purchased from Toxin Technology (batch 120794A

TABLE 1. FOOD-SPECIFIC ATTACK RATES AND RISK RATIOS

Food	Exposed (ate the specific food) N			Attack rate (%) Symptomatic exposed/total exposed	Not exposed (did not eat the specific food) N			Attack rate (%) Symptomatic not exposed/total not exposed	Risk ratio	p	Confidence interval (95%)	
	Symptomatic	Asymptomatic	Total		Symptomatic	Asymptomatic	Total				Lower	Upper
Italian starter buffet (pasta and beans, pork and beans, and ham and melon)	8	5	13	62	16	13	29	55	1.12	0.7	0.65	1.92
Pasta alla boscaiola (pasta and sauce with tomatoes, mushrooms, and meat)	15	7	22	68	9	11	20	45	1.52	0.13	0.86	2.66
Pasta with sheep cheese sauce	7	7	14	50	17	11	28	61	0.82	0.51	0.45	1.5
Pork skewers	6	6	12	50	18	12	30	60	0.83	0.55	0.44	1.58
Beef steak	17	9	26	65	7	9	16	44	1.49	0.17	0.8	2.78
Raw and cooked vegetables	14	13	27	52	10	5	15	67	0.78	0.35	0.47	1.3
Chantilly cream dessert	22	13	35	63	2	5	7	29	2.2	0.12	0.66	7.29
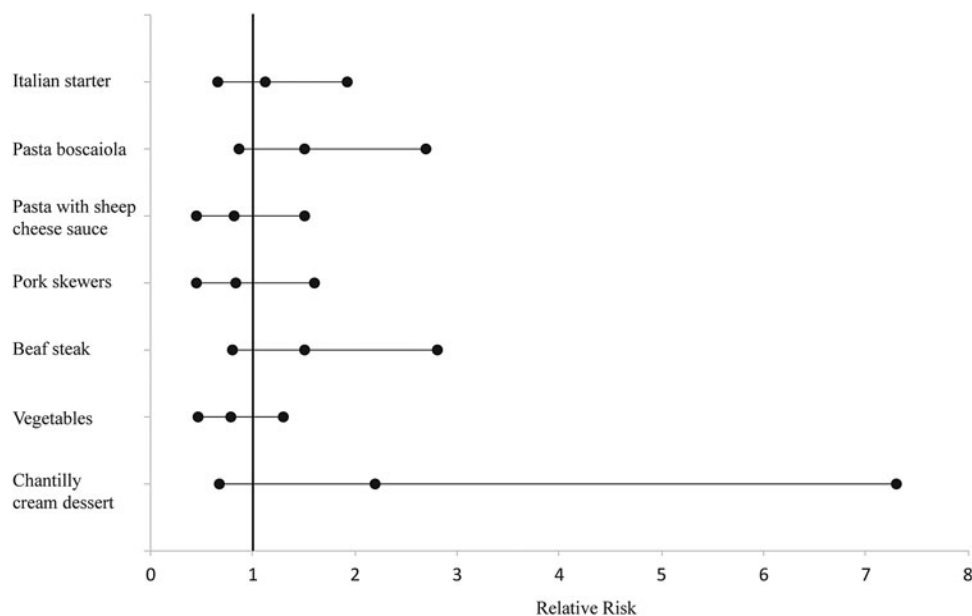
FIG. 1. Forest plot of the food items. The point represents the relative risk of each food; the width of the lines shows the confidence intervals (95%). The confidence intervals of all food items cross the vertical line of the value 1, showing that there is no statistical significance.

for SEA, 61499B1 for SEB, 113094C2 for SEC, and 12802D for SED) and were constructed from the raw absorbance values (AU) obtained for the range of standards tested in duplicate. For each analysis, internal positive and negative controls were prepared from milk extract and analyzed in the same manner as that of the sample.

Limits of quantification (LOQs) were estimated as follows: 0.048 ng/mL for SEA, 0.204 ng/mL for SEB, 0.044 ng/mL for SEC, and 0.374 ng/mL for SED. This method was used in the context of several studies on detection of SEs in foods.

Results

Epidemiological investigation

All 42 restaurant customers who attended the dinner filled in the questionnaire (response rate, 100%). The results from the retrospective cohort study (Table 1) implicated the Chantilly cream as the most likely vehicle of food poisoning. The AR of the customers who ate the dessert were 63% and the RR were 2.2. Although the confidence intervals demon-

strated that RR values were not statistically significant for any of the served food, these data are still suggestive of the central role of the dessert in the food poisoning (Fig. 1). The Chantilly cream was not a commercially prepared food product, but it was made by the restaurant cooks and served as dessert only to the customers. The information obtained from the interview with the kitchen workers revealed improper food storage; in particular, the Chantilly cream was not refrigerated after preparation, but was kept at room temperature (which rose above 25°C) for about 5 h, thus permitting microbial growth and SE production. Moreover, food safety inspection at the restaurant revealed poor application of basic hygiene practices: inappropriate storage of raw and cooked foodstuffs in the refrigerators and the absence of separation between the dirty and clean kitchen areas, which could lead to microbial cross-contamination.

Laboratory investigation

The microbiological results on foodstuffs and environmental swabs are shown in Table 2. Among the food samples

TABLE 2. LABORATORY ANALYSIS RESULTS

	CPS enumeration	Staphylococcal enterotoxin detection	Clostridium perfringens enumeration	Bacillus cereus enumeration
Italian starter (pork and beans)	<1000 CFU/g	Not detected	<1000 CFU/g	<1000 CFU/g
Pasta sauce (with tomatoes, mushrooms, and meat)				
Pasta sauce (with sheep milk cheese)				
Dessert (Chantilly cream)	3.4×10^8 CFU/g	Detected	<1000 CFU/g	<1000 CFU/g
Swab table 1	<10 CFU/swab	Not done	Not done	Not done
Swab table 2	10 CFU/swab			
Swab cold room	<10 CFU/swab			

CFU, colony forming unit; CPS, coagulase-positive staphylococci.

tested, CPS contamination was only detected in the Chantilly cream dessert (3.4×10^8 CFU/g). Furthermore, positive results were obtained using qualitative immunoassay detection (both VIDAS SET2 and RIDASCREEN SET Total assays) indicating the presence of, at least, one of the five following toxins: SEA, SEB, SEC, SED, and SEE (Table 3). The use of the quantitative ELISA method based on antibodies specific for SEs confirmed the sample contamination (absence of cross-reactions or interference effect) and the presence of SEA at a concentration of 2.12 ng/g. CPS were also detectable on the surface of a kitchen table (10 CFU/swab).

In total, seven *S. aureus* strains were isolated: one from the dessert sample, one from a table surface, and five from the nasopharyngeal mucosa of healthy food handlers. The nasopharyngeal swabs of five food handlers tested positive for CPS and were confirmed as *S. aureus*. Genes encoding SEs were detected in three of five human isolates: one strain harbored *sea*, *seg*, and *sei* genes, one strain carried *seg*, *seh*, and *sei*, and one was only *sea* positive. Environmental and food isolates were also positive only for the *sea* gene. Moreover, these *sea*-positive strains obtained from the human, environmental, and food samples belonged to the human biotype. None of the strains harbored the *pvl*, *mecA*, *mupA*, and *mecC* genes (Table 4).

The five *S. aureus* strains carrying the enterotoxin genes were analyzed by PFGE and all of them showed clear bands that allowed the comparison of PFGE profiles. The three strains carrying only *sea* demonstrated the same PFGE profile (100% similarity coefficient) (Fig. 2).

Discussion

This study underlines the importance of the combined use of microbiological, immunological, and molecular analyses to support the data obtained from an epidemiological investigation. In particular, in our study, laboratory analyses permitted the identification of the definite source of food poisoning, even though the epidemiological data from cohort study lacked strong statistical significance. Indeed, microbiological analyses showed the presence of CPS in the Chantilly cream, the use of PCR allowed the identification of *S. aureus* strains carrying the *sea* gene, and the immunoenzymatic assays showed the toxinogenic capacity of the strains in food and confirmed the presence of the toxin type SEA. The microbiological, molecular, and immunoenzymatic analyses were in good agreement and the Chantilly cream and SEA toxin could therefore be incriminated in this FBO. It
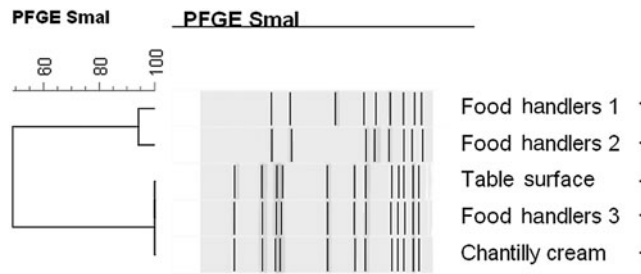
TABLE 3. VALUES OF STAPHYLOCOCCAL ENTEROTOXIN DETECTION

	TV[a]	RFV[b]
VIDAS®	4.00	15,357
VIDAS® after heat treatment	4.13	15,846
	Cutoff	Absorbance value
RIDASCREEN®	0.187	4.577

[a]TV (test value) = is the ratio of RFV and the standard.
[b]RFV (relative fluorescence value) = is the final reading of the test sample minus the background reading.

TABLE 4. CHARACTERISTICS OF *STAPHYLOCOCCUS AUREUS* ISOLATES

Origin	Type of sample	sea	seb	sec	sed	see	seg	seh	sei	sej	sep	ser	SEnG profile	Biotyping	pvl	mecA	mecC	mupA
Chantilly cream	Food isolate	+	–	–	–	–	–	–	–	–	–	–	1	Human	neg	neg	neg	neg
Table surface swab	Environmental isolate	+	–	–	–	–	–	–	–	–	–	–	1	Human	neg	neg	neg	neg
Restaurant worker's swab	Human isolate	+	–	–	–	–	–	–	–	–	–	–	1	Human	neg	neg	neg	neg
Restaurant worker's swab	Human isolate	–	–	–	–	–	–	–	–	–	–	–	2	NHS6	neg	neg	neg	neg
Restaurant worker's swab	Human isolate	–	–	–	–	–	–	–	–	–	–	–	2	Human	neg	neg	neg	neg
Restaurant worker's swab	Human isolate	–	–	–	–	–	+	+	+	–	–	–	3	Human	neg	neg	neg	neg
Restaurant worker's swab	Human isolate	+	–	–	–	–	+	–	+	–	–	–	4	Human	neg	neg	neg	neg

FIG. 2. Dendrogram of enterotoxigenic *Staphylococcus aureus* isolates. Isolates from Chantilly cream dessert, table surface swab, and food handler 3 show the same PFGE profile. PFGE, pulsed-field gel electrophoresis.

should be highlighted that the use of both the SEs' qualitative detection assay (ESM) and the in-house quantitative ELISA (specific ELISA) allowed to discard any doubt about the presence of interference effect.

In the literature, the predominance of SEA is well documented in different countries. A study of 359 staphylococcal FBOs, which occurred in the United Kingdom between 1969 and 1990, and 31 staphylococcal FBOs, which occurred between 1981 and 2002 in France, revealed that >70% of the *S. aureus* isolated strains produced SEA (Kérouanton *et al.*, 2007; Argudín *et al.*, 2010). Nevertheless, during the investigation of staphylococcal FBOs, SE concentrations were rarely measured, thereby hindering the establishment of a dose response. In this study, a concentration of the contaminating enterotoxin (i.e., SEA at 2.12 ng/g) could be estimated using quantitative ELISA method.

Using a multiparametric approach for laboratory investigation, the identification of the causative agent with strong evidence could be obtained. In fact, the EFSA and ECDC data reveal the difficulty in obtaining strong epidemiological evidence of outbreaks caused by SEs. In 2015, 16 MS reported 434 foodborne outbreaks caused by staphylococcal toxins. This represents 9.9% of all outbreaks, a small increase compared with 2014. In 95 outbreaks (6 strong-evidence and 89 weak-evidence outbreaks), an "infected food handler" was reported as a contributory factor (EFSA and ECDC, 2016).

Studies of FBOs in which enterotoxigenic strains were isolated from patients, food, and food handlers are rare. The lack of complete data about the epidemiology of SE outbreaks is potentially linked to multiple factors that can occur during sample collection or laboratory analyses (Hennekinne *et al.*, 2012; Mossong *et al.*, 2015).

Although the present investigation also lacked the collection of stool and emesis samples from the patients, because of the rapid symptomatic relief after the medical treatment, the molecular characterization of *S. aureus* isolates from different samples highlighted the relationship among human carriers, the environment, and food contamination. Moreover, detailed information collected during the inspection and the laboratory analyses demonstrates how staphylococcal contamination of the dessert occurred and when SEA was produced. Considering that three *S. aureus* isolates from different samples showed the same human biotype, the same *se* gene pattern, and the same pulsotype, it can be assumed that the human carrier represented the most likely source of this FBO. Food contamination occurred either directly during food processing or indirectly through

the table surface. Improper food storage in an unrefrigerated condition was the most probable cause of CPS proliferation and enterotoxin production.

Conclusions

The results of this study indicate that both epidemiological data and laboratory investigation play an important role in the elucidation of FBOs. Immediate and accurate epidemiological investigation of a FBO represents a useful tool for a prompt association between illness reports and the consumption of a specific food, leading to targeted laboratory analyses. Conducting these analyses is of high importance for the identification of the microbial contaminants and their source, as illustrated in this study. In this study, information was also provided on the SE concentration in the contaminated food. Such data remain scarce in the literature and are needed to determine toxic doses of SEs causing staphylococcal food poisoning.

Furthermore, this outbreak underlines the usefulness of complying with the fundamental principles of Good Hygienic Practices and Good Manufacturing Practices guidelines for preventing FBOs due to SEs (Gallina *et al.*, 2013). Food handlers should be properly trained in food hygiene procedures, to understand the importance of sanitation and cleanliness of kitchen equipment and the maintenance of the cold chain during food production and storage (Schmid *et al.*, 2007).

Disclosure Statement

No competing financial interests exist.

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