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Enterotoxigenic *Staphylococcus aureus* in brined cheese from weekly street markets in Ankara, Turkey

Enterotoxinbildende Staphylococcus aureus in Salzlakenkäse aus dem mobilen Straßenverkauf in der Provinz Ankara, Türkei

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Summary

A total of 63 brined cheeses (30 white pickled cheeses, 33 Tulum cheeses) were randomly collected from informal market places such as weekly street markets in the province Ankara during a seven-month period, and quantitatively analysed for *Staphylococcus (S.) aureus. S. aureus* isolates obtained from these samples were analysed for staphylococcal enterotoxin (SE) genes by PCR, selected isolates were tested for SEA-SEE production by enzyme immunoassay. *S. aureus* was found in eleven (17.0 %) of cheese samples at levels between 9.5 × 10^2 and 5.0 × 10^6 cfu/g. All 22 *S. aureus* isolates were positive by PCR for one or more toxin genes *(sea, sed, seg, sei, selj, sem, sen, seo, sep, ser* and *selu),* forming three distinct toxin profiles. SEA or SED production was found for isolates from five samples, by enzyme immunoassay. Further characterization of isolates by macrorestriction analysis yielded three different pulsed-field gel electrophoresis (PFGE) profiles which corresponded well with SE gene profiles. Identical PFGE profiles were obtained for isolates from several alleged unique cheeses, purchased from different vendors in different markets, indicating a common source of production and disproving the claimed originality. These findings highlight the existence of health hazards related to consumption of traditional cheeses originating from such underregulated markets and the need to implement more intensive hygiene control measures.

Keywords: Brined cheese, Tulum cheese, white pickled cheese, *S. aureus,* Enterotoxin

Zusammenfassung

Traditionell hergestellte Salzlakenkäse (n=63), darunter 30 Salzlaken-Weißkäse sowie 33 „Tulum“-Käse wurden im informellen Straßenverkauf (Straßenmärkte etc.) in der Provinz Ankara gekauft und quantitativ auf ihren Gehalt an *Staphylococcus (S.) aureus* untersucht. *S. aureus*-Isolate aus diesen Proben wurden mittels PCR auf das Vorhandensein von Staphylokokken-Enterotoxin (SE)-Genen geprüft, ausgewählte Isolate wurden mittels Enzymimmunoassays auf Produktion von SEA-SEE getestet. In elf (17,0 %) Käseproben wurde *S. aureus* in einem Keimzahlbereich von 9,5 × 10^2 KbE/g bis 5,0 × 10^6 KbE/g nachgewiesen. Alle geprüften 22 *S. aureus*-Isolate wiesen Gene für ein oder mehrere Enterotoxine auf *(sea, sed, seg, sei, selj, sem, sen, seo, sep, ser* und *selu),* wobei drei verschiedene Toxinprofile festgestellt wurden. Für Isolate aus fünf Proben konnte in Flüssigkultur die Bildung von SEA oder SED nachgewiesen werden. Eine weitere Charakterisierung der Isolate mittels Makrorestriktionsanalyse ergab drei verschiedene Pulsfeld-Gelelektrophorese (PFGE)-Profile, die mit denjenigen der SE-Genprofile übereinstimmten. Identische PFGE-Profile wurden für Isolate aus Käseproben erhalten, die von verschiedenen Verkaufsstellen und von verschiedenen Verkäufern bezogen worden waren, was auf einen gemeinsamen Ursprung der Kontamination hinweist und damit gleichzeitig die von den Verkäufern behauptete Originalität der Produkte in Frage stellte. Die Ergebnisse deuten darauf hin, dass Gesundheitsrisiken im Zusammenhang mit dem Konsum traditionell hergestellter Salzlakenkäse aus dem weitgehend unreglementierten Straßenverkauf existieren und zeigen die Notwendigkeit intensiverer Hygienekontrollmaßnahmen an.

Schlüsselwörter: Tulum Käse, Salzlaken-Weißkäse, Straßenverkauf, *S. aureus,* Enterotoxin

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Introduction

Staphylococcus (S.) aureus is a common commensal of the skin and mucosal membranes of both humans and animals, and is also a frequent cause of bovine mastitis (Cremonesi et al., 2007; Normanno et al., 2007). Therefore, manual (artisanal) cheese-making implies the risk that cheeses may be contaminated with *S. aureus,* which is a food safety issue in case of enterotoxin-producing strains (de Buyser et al., 2001; Jorgensen et al., 2005; Rall et al., 2008; Argudín et al., 2010; El-Shenawy et al., 2013; Nunes et al., 2016).

Traditionally manufactured brined cheeses, such as white pickled cheese and Tulum cheese, are very popular in Turkey. White pickled cheese is a brined cheese made from thermized or raw cow's milk with a soft or semi-hard texture with a salty and acidic taste (Hayaloglu et al., 2002). Tulum cheese is traditionally produced from heat-treated or raw ewe's milk and ripened in goats' skins (tulums) or plastic containers with a semi-hard crumbly texture and a natural mouldy taste and flavour (Yilmaz et al., 2005; Hayaloglu et al., 2007). The small-scale production of brined cheese requires extensive manual processing of the curd by the cheese maker, usually under poor hygienic conditions (Hayaloglu et al., 2002). Such environments very much favour the growth of coagulase-positive staphylococci, including enterotoxigenic *S. aureus* (de Buyser et al., 2001; Cremonesi et al., 2007; El-Shenawy et al., 2013). Weekly street markets are an important channel of distribution of such products, which are commonly advertised as being very natural and organic products. The vendors usually buy brined cheeses directly from small dairy plants or from farmhouse producers from different regions of Turkey. Unfortunately, this form of marketing is often associated with poor hygienic conditions. This includes open, non-refrigerated display of products without preventive measures to avoid contamination by direct or indirect contact with environmental bacteria, for example during slicing and weighing. The general education level of the vendors, specifically their knowledge about food hygiene and foodborne pathogens, frequently is very low.

Irrespective of the apparent hygienic problems related to traditional marketing of brined cheeses, in particular for Tulum cheeses (Küplülü et al., 2004), few studies have been conducted in recent years in Turkey with regard to enterotoxigenic *S. aureus* in such products, and the results were inconsistent. For white cheese and Tulum cheese from weekly markets only in the Istanbul region, Bingöl et al. (2012) reported extraordinarily high frequencies (32–52 %) *S. aureus* positive samples, enterotoxins (SEA-SED) were found in 32–40 % of the cheeses. In a similar study on white cheese from the Black Sea region of Turkey, Gücükoglu et al. (2012) found 37.5 % *S. aureus*-positive samples and several enterotoxigenic isolates *(sea, sec, sed)*. Likewise, Aydin et al. (2011) reported that the majority of dairy products sold in the Marmara Region were positive for enterotoxigenic *S. aureus,* but they did not specify type and source of food products, cheeses were not specifically addressed. In contrast, Can and Celik (2012) reported much lower frequencies for *S. aureus* (6 %) in packaged white pickled and Tulum cheeses purchased from the formal markets, and only a few isolates were found to be enterotoxigenic (SEC and SED). To our knowledge, this is the first study reporting enterotoxigenic *S. aureus* contamination and their enterotoxin production in unpackaged brined cheeses sold as in weekly street markets in the capital city of Turkey. Therefore the objectives of this study were (i) to estimate and update the risk for the consumer from enterotoxigenic *S. aureus* in traditionally distributed brined cheeses in the Ankara region of Turkey, (ii) to characterize the spectrum of enterotoxins potentially produced by these bacteria, and (iii) to check the genetic diversity of *S. aureus* isolates from different cheeses to identify possible common sources of contamination.

Materials and Methods

Samples collection

A total of 30 white pickled and 33 Tulum unpackaged cheese samples were randomly collected between October 2014 and April 2015 from 18 different weekly street markets in the capital city Ankara, Turkey (Table 1). In each case, information on milk type (cow, sheep) or milk processing (raw milk, heat treated milk) was requested from the vendors. According to this information, all Tulum cheeses were from ewe's milk, while white pickled cheeses were made from either cow's milk or mixed from ewe's and cow's milk. Concerning heat treatment, either no reliable information or no information at all could be obtained from the vendors. At each location, white pickled and Tulum cheeses from different local areas were purchased. The fact that cheese vendors were not regularly on the weekly street markets limited the number of collected unpackaged cheese samples from the vendors. All cheese samples were placed in an ice-cooled box and then transported to the laboratory where the analyses started immediately.

Analysis of cheese

A 10 g test portion of each cheese sample was quantitatively analysed for coagulase-positive staphylococci according to ISO 6888-1 (ISO 1999). In brief, 10 g of each cheese were added to 90 ml of sterile 1/4 strength Ringer's solution (Merck, Darmstadt, Germany) and homogenized for 90 s at maximum speed in a Stomacher 400 (Seeward, West Sussex, UK). Then, decimal serial dilutions were made with 1/4 strength Ringer's solution, and 0.1 ml of each dilution

TABLE 1: *Origin of S. aureus positive cheese samples under study.*

Sampling date	Location	Vendors	Cheese sample	No. of isolates	Isolate designation
November 2014	A	A1	Wp3	1	Wp3-22
	B	B1	Tu4	4	Tu4-3, Tu4-8, Tu4-16, Tu4-17
		B2	Tu9	1	Tu9-18
		B3	Tu10	4	Tu10-1, Tu10-5, Tu10-15, Tu10-24
December 2014	C	C1	Tu15	1	Tu15-6
	D	D1	Wp22	5	Wp22-12, Wp22-13, Wp22-20, Wp22-23, Wp22-26
	E	E1	Tu27	1	Tu27-19
January 2015	B	B3	Tu29	1	Tu29-27
			Tu34	1	Tu34-2
March 2015	C	C2	Wp51	2	Wp51-7, Wp51-11
			Tu56	1	Tu56-14

Tu: Tulum cheese, Wp: White pickled cheese

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was streaked onto Baird Parker agar supplemented with egg yolk tellurite emulsion (Merck). The plates were incubated at 37 °C for 24–48 h, and typical colonies were counted after 24 and 48 h. Up to five characteristic colonies per plate were picked and then identified using Gram stain, catalase test, clumping factor/protein A (latex agglutination, Staphaurex-Plus, Remel, Lenexa, USA), and coagulase tube test using EDTA rabbit plasma (Merck). Hemolysin production was determined as described by Skalka et al. (1979). On the basis of the phenotypic characterization, all coagulase positive isolates were further tested using the API® Staph (bioMérieux Marcy l'Etoile, France). Colonies were identified as *S. aureus* by species specific PCR detection of the thermonuclease *(nucA)* gene (Brakstad et al., 1992). All confirmed *S. aureus* isolates were stored at –80 °C in brain heart infusion (BHI) broth (Merck) containing 25 % glycerol until further characterization of the enterotoxinogenic potential.

For three cheese samples (Wp22, Wp51, Tu56), the number of coagulase positive staphylococci exceeded a level of 10^5 cfu/g. According to regulations given by Turkish Food Codex (TFC) (similar to European Union commission regulation (EC) No 2073/2005), these samples were therefore analysed for SEA-SEE using a commercial enzyme immunoassay kit (VIDAS™ SET2, bioMérieux, France). Extraction of SEs was performed using the dialysis concentration protocol of Vernozy-Rozand et al. (2004). In brief, a 20 g sample was homogenized with 40 ml of distilled water. The pH was adjusted to 4 using 5 mol/l HCl, and then the homogenate was centrifuged at 2.000 × g for 15 min at 4 °C. The supernatant was adjusted to pH 6–8, centrifuged again and then filtered. The extract was concentrated by dialysis against 30 % Polyethylene Glycol (Mol wt 20000, Merck) with a cellulose dialysis bag retaining molecules >6000–8000 Da (24006; Visking R dialysis bag, Poly Labo, Strasbourg, France) at 4 ± 2 °C overnight. After washing in cold water, the bag was placed in phosphate-buffered saline (PBS; 0.01 M) at a pH of 7.2 until the concentrated extract was reconstituted to about 1 ml, and was then analysed by enzyme immunoassay according to manufacturers' instructions.

PCR analysis of enterotoxin genes

For DNA extraction, bacterial cells were harvested from BHI culture by centrifugation at 10.000 × g for 5 min, resuspended in 180 µl TE buffer (10 mM of Tris HCl, 1 mmol/l of EDTA, pH 8.0) containing 5 µl of lysostaphin (1.8 U/µl; Sigma-Aldrich Chemie, Steinheim, Germany), and incubated at 37°C for 1 h. Then the total genomic DNA was isolated by using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturers' recommendations. PCR for 18 *S. aureus* enterotoxin genes *(sea, seb, sec, sed, see, seg, seh, sei, selj, sek, sel, sem, sen, seo, sep, seq, ser* and *selu)* was performed as described by Akineden et al. (2008). Enterotoxin genes *ses, set* (Ono et al., 2008), and *sev* (Thomas et al., 2006) were not included because positive reference strains were not available in this study. The oligonucleotide primer sequences and the corresponding sequence locations of SEs, as well as PCR conditions are listed in Table 2. All amplification reactions were performed as uniplex. The PCR reaction (total volume 30 µl) was performed in a 0.2 ml reaction vial and consisted of 3 µl GeneAmp 10x PCR Gold Buffer (150 mmol/l Tris-HCl, 500 mmol/l KCl; pH 8.0) (Applied Biosystem, Darmstadt, Germany), 1.8 µl $MgCl_2$ (25 mmol/l) (Applied Biosystem), 1.0 µl of each primer (10 pmol/µl),

TABLE 2: *Oligonucleotide primers used for PCR analysis of staphylococcal enterotoxins (SEs).*

Target genes	Primer	Oligonocleotides sequence (5′ to 3′)	Amplicon size (bp)	Programme[a]	Reference
sea	SEA-1 SEA-2	AAAGTCCCGATCAATTTATGGCTA GTAATTAACCGAAGGTTCTGTAGA	219	1	Tsen and Chen (1992)
seb	SEB-1 SEB-2	TCGCATCAAACTGACAAACG GCAGGTACTCTATAAGTGCC	478	4	Johnson et al. (1991)
sec	SEC-1 SEC-2	GACATAAAAGCTAGGAATTT AAATCGGATTAACATTATCC	257	5	Johnson et al. (1991)
sed	SED-1 SED-2	CTAGTTTGGTAATATCTCCT TAATGCTATATCTTATAGGG	317	4	Johnson et al. (1991)
see	SEE-1 SEE-2	TAGATAAAGTTAAAACAAGC TAACTTACCGTGGACCCTTC	170	4	Johnson et al. (1991)
seg	SEG-1 SEG-2	AATTATGTGAATGCTCAACCCGATC AAACTTATATGGAACAAAAGGTACTAGTTC	642	3	Jarraud et al. (1999)
seh	SEH-1 SEH-2	CAATCACATCATATGCGAAAGCAG CATCTACCCAAACATTAGCACC	375	2	Jarraud et al. (1999)
sei	SEI-1 SEI-2	CTCAAGGTGATATTGGTGTAGG AAAAAACTTACAGGCAGTCCATCTC	576	3	Jarraud et al. (1999)
selj	SEJ-1 SEJ-2	CATCAGAACTGTTGTTCCGCTAG CTGAATTTTACCATCAAAGGTAC	142	3	Monday and Bohach (1999)
sek	SEK-1 SEK-2	CACAGCTACTAACGAATATC TGGAATTTCTCAGACTCTAC	378	5	Chiang et al. (2006)
sel	SEL-1 SEL-2	CATACAGTCTTATCTAACGG TTTTCTGCTTTAGTAACACC	275	5	Chiang et al. (2006)
sem	SEM-1 SEM-2	TCTTAGGAACTATTATGGTAGC CCTGCATTAAATCCAGAA	471	6	Akineden et al. (2008)
sen	SEN-1 SEN-2	GGAGTTACGATACATGATGG ACTCTGCTCCCACTGAAC	292	4	Akineden et al. (2008)
seo	SEO-1 SEO-2	TGATGATTATATATAAATAATCGATTTACG ATATGTACAGGCAGTATCC	249	4	Akineden et al. (2008)
sep	Sep 01 Sep 02	ATCATAACCAACCGAATCAC AGAAGTAACTGTTCAGGAGCTA	148	4	Chiang et al. (2008)
seq	SEQ-1 SEQ-2	TCAGGTCTTTGTAATACAAAA TCTGCTTGACCAGTTCCGGT	359	4	Chiang et al. (2008)
ser	SER-1 SER-2	AGATGTGTTTGGAATACCCTAT CTATCAGCTGTGGAGTGCAT	123	4	Chiang et al. (2008)
selu	SEU-1 SEU-2	ATTTGCTTTTATCTTCAT GGACTTTAATGTTTGTTTCTGAT	167	7	Chiang et al. (2008)

[a] Thermal cycler program: 30 cycles, initial denaturation 94 °C for 30 s, annealing (1: 57 °C, 2: 58 °C, 3: 55 °C, 4: 50 °C, 5: 48 °C, 6: 49 °C, 7: 45 °C) for 30 s, elongation 72 °C for 60 s.

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TABLE 3: *Characteristics of 22 S. aureus isolates obtained from brined cheeses.*

Sample	*S. aureus* cfu/g	Presence of SE[a]	No. of isolates obtained from sample	Isolate designation	Clumping factor	Hemolysin	Staphaurex latex agglutination	Api Staph ID%	SE detected in culture supernatants[b]	SE genes detected by PCR	PFGE profile
Wp3	5.0×10^4	n.a.	1	Wp3-22	+	α	+	99.9	n.d.	*seg, sei, sem, sen, seo, sep, selu*	I
Tu4	$2,7 \times 10^4$	n.a.	4	Tu4-3	+	α	+	99.5	n.a.	*seg, sei, sem, sen, seo, sep, selu*	I
				Tu4-8	+	α	+	99.5	n.d.	*seg, sei, sem, sen, seo, sep, selu*	I
				Tu4-16	+	α	+	99.5	n.a.	*seg, sei, sem, sen, seo, sep, selu*	I
				Tu4-17	+	α	+	99.5	n.a.	*seg, sei, sem, sen, seo, sep, selu*	I
Tu9	$3,7 \times 10^2$	n.a.	1	Tu9-18	+	α & β	+	99.9	SED	*sed, selj, ser*	II
Tu10	$3,7 \times 10^3$	n.a.	4	Tu10-1	+	α	+	99.9	SEA	*sea*	III
				Tu10-5	+	α	+	99.9	SEA	*sea*	III
				Tu10-15	+	α	+	99.9	SEA	*sea*	III
				Tu10-24	+	α	+	99.9	SEA	*sea*	III
Tu15	3.4×10^4	n.a.	1	Tu15-6	+	α & β	+	98.4	SED	*sed, selj, ser*	II
Wp22	3.4×10^5	n.d.	5	Wp22-12	+	α	+	99.5	n.a.	*seg, sei, sem, sen, seo, sep, selu*	I
				Wp22-13	+	α	+	99.5	n.a.	*seg, sei, sem, sen, seo, sep, selu*	I
				Wp22-20	+	α	+	98.4	n.a.	*seg, sei, sem, sen, seo, sep, selu*	I
				Wp22-23	+	α	+	98.4	n.a.	*seg, sei, sem, sen, seo, sep, selu*	I
				Wp22-26	+	α	+	97.6	n.a.	*seg, sei, sem, sen, seo, sep, selu*	I
Tu27	3.0×10^3	n.a.	1	Tu27-19	+	α	+	98.4	n.a.	*seg, sei, sem, sen, seo, sep, selu*	I
Tu29	9.5×10^2	n.a.	1	Tu29-27	+	α	+	99.9	SEA	*sea*	III
Tu34	3.0×10^3	n.a.	1	Tu34-2	+	α	+	99.9	SEA	*sea*	III
Wp51	5.0×10^6	n.d.	2	Wp51-7	+	α	+	98.4	n.d.	*seg, sei, sem, sen, seo, sep, selu*	I
				Wp51-11	+	α	+	99.5	n.a.	*seg, sei, sem, sen, seo, sep, selu*	I
Tu56	1.3×10^5	n.d.	1	Tu56-14	+	α	+	98.4	n.a.	*seg, sei, sem, sen, seo, sep, selu*	I

Tu: Tulum cheese, Wp: White pickled cheese; [a] VIDAS™ SET2; [b] Ridascreen SET; n.a.: not analysed; n.d.: none detected; +: positive.

0.6 µl dNTP-mix (10 mmol/l) (MBI Fermentas, St Leon-Rot, Germany), 0.2 µl AmpliTaq Gold® polymerase (5 U/µl, Applied Biosystem), 19.9 µl sterile aqua dest., and 2.5 µl DNA template. All the amplifications were performed with an *i*Cycler (BioRad, Munich, Germany) and PCR products were determined by electrophoresis of 12 µl of the reaction product in a 2 % agarose gel (Biozym, Hessisch-Oldendorf, Germany) at 120 Volt in 1x Tris-acetate-electrophoresis buffer (TAE) [(0.04 mol/l Tris, 0.001 mol/l EDTA; pH 7.8)] and a Gene-Ruler™ 50 and 100 bp DNA ladder (MBI Fermentas) as molecular markers followed by staining with 5 µl/ml ethidium bromide solution (Sigma, St. Louis, the USA) for 5 minutes. Finally, the amplicons were visualized under a UV transilluminator using the Geldoc system (BioRad).

Enzyme immunoassay analysis of enterotoxin production

All *S. aureus* isolates which were found positive by PCR for genes encoding one of the classical SEs (SEA-SEE) were also tested for toxin expression by enzyme immunoassay (Ridascreen SET, R-Biopharm, Darmstadt, Germany). Each isolate was inoculated in 5 ml BHI broth and incubated at 37 °C for 24 h. The broth was centrifuged at 5.000 × g for 5 min at 4 °C then a portion of each supernatant was filtered through a 0.2 µm filter (FP30/0.2CA-S, Schleicher and Schuell). The analysis of the filtrate for the five enterotoxins by enzyme immunoassay were also tested according to the manufacturers' instructions.

Analysis of chromosomal DNA restriction patterns by PFGE

All isolates were characterized after digestion of their chromosomal DNAs with the restriction enzyme *Sma*I and subsequent separation of the fragments by pulsed-field gel electrophoresis (PFGE) using the Chef-Dr II pulsed-field electrophoresis system (BioRad). The preparation of the whole bacterial DNA in agarose gel plugs, subsequent digestion of the bacterial DNA with the *Sma*I restriction enzyme and PFGE conditions was done as described previously (Akineden et al., 2001). A dendrogram of the restriction patterns was created using the Bionumerics software package version 6.6 (Applied Maths NV, Sint-Martens-Latem, Belgium).

Results and Discussion

Frequency and levels of *S. aureus* in brined cheeses

Coagulase-positive staphylococci were isolated from three out of 30 samples of white pikkled cheeses and from eight out of 33 samples of Tulum cheeses, the quantitative contamination ranged from 3.4×10^5 cfu/g to 5.0×10^6

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cfu/g (white pickled cheese), and from 9.5 × 10^2 to 1.3 × 10^5 cfu/g (Tulum cheese), respectively (Table 3). Widely differing results concerning frequency and levels of *S. aureus* have been reported for traditional cheeses in various studies from Turkey for example, 40 % of white pickled cheeses sold in Ankara were positive at 10^3 cfu/g (Koluman et al., 2011), and 37.5 % of white pickled cheeses from north Turkey were positive at levels between 10^2 and 10^6 cfu/g (Gücükoglu et al., 2012), which are quite higher than our findings. In contrast, much lower frequency of *S. aureus* than found in our study was reported from white pickled and Tulum cheeses from the Ankara province (6 % positives, mean level 10^4 cfu/g, Can and Çelik, 2012) and for white cheese samples from the Izmir province (8 % positives, mean level 10^1 cfu/g; Turantaş et al., 1989). Although no clear pattern could be derived from published data, sampling bias may explain some of the discrepancies. In this study, the dedicated focus on cheese products sold in weekly markets introduced some selection bias, because prepacked products of industrial produce as available from retail shops were not included. However, with the exception that all products were from street markets, no further specific criteria were applied during sample collection.

A total of 22 isolates which were coagulase-positive, clumping factor positive and Staphaurex positive were further analysed by API® Staph, and all were identified as *S. aureus* (ID 98.4 % to 99.9 %). PCR analysis showed that all isolates possessed the *nuc*A gene and were therefore *S. aureus.* After cultivation on sheep blood agar, two of these isolates showed both alpha- and beta-hemolysins, 20 isolates were positive for alpha-hemolysin only (Table 3). Each three samples of white pickled cheeses (Wp3, Wp22, and Wp51) and of Tulum cheese (Tu4, Tu15, and Tu56) exceeded the maximum tolerable limit (10^3 cfu/g) for *S. aureus* in cheese as set by TFC for microbiological criteria (Turkish Food Codex, 2011). Three of these samples (Wp22, Wp51, Tu56) even exceeded the upper limit of 10^5 cfu/g of *S. aureus* per gram set by European commission regulation (EC) No 2073/2005 (EC Commission, 2005), which requires enterotoxin analysis as a food safety criterion. These samples were analysed for SEA-SEE by VIDAS® SET2 assay (bioMérieux), but all yielded negative results.

Enterotoxigenic properties of *S. aureus* isolates

All 22 isolates obtained from eleven cheese samples were positive for either one or more enterotoxin genes (Table 3). Isolates from the three samples with the highest *S. aureus* contamination (Wp22, Wp51, Tu56) were found to be negative for the genes encoding for the classical toxins SEA-SEE, which is in agreement with the negative enzyme immunoassay results for these cheeses. However, these isolates had identical gene profiles concerning presence of the newer enterotoxins and enterotoxin like compounds *(seg, sei, sem, sen, seo, sep* and *selu).*

Isolates from three samples of Tulum cheeses (Tu10, Tu29 and Tu34), purchased from the same vendor but at different times, were positive for SEA both by enzyme immunoassay and by PCR. Isolates from two other Tulum cheeses (two different vendors) were positive for SED by enzyme immunoassay, while PCR detected genes encoding for *sed, selj* and *ser.* SEA is predominantly associated with human isolates of *S. aureus* (Larsen et al., 2000) while SED production and a PCR pattern *sed-selj-ser* has been reported to occur preferably in ovine (Boerema et al., 2006) or bovine (Hummerjohann et al., 2014; Rola et al., 2016) strains. Therefore it is reasonable to assume that inoculation of cheeses with the SEA-positive isolates resulted from a manual contamination during cheese-making, while the SED positive isolates probably originated from ewe's milk and survived the cheese-making process. Although all cheeses containing these SEA/SED producers had – at the time

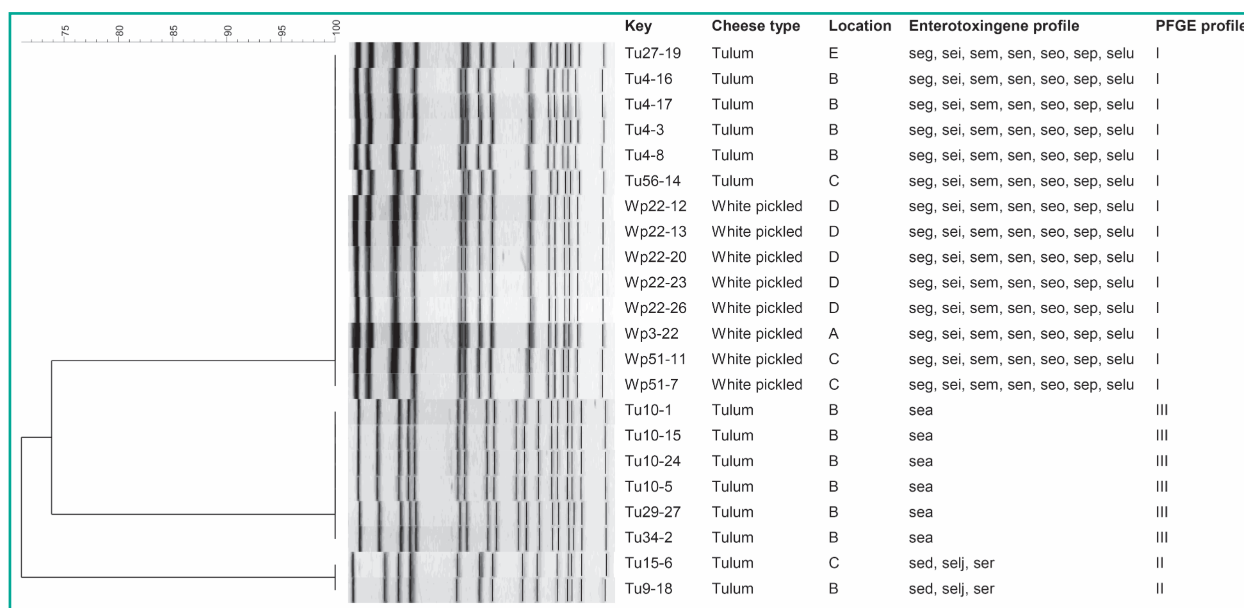

FIGURE 1: *Pulsed field gel electrophoresis (PFGE) patterns of 22 S. aureus isolates obtained from unpackaged cheese samples of white pickled and Tulum obtained from weekly street markets digested with the SmaI restriction enzyme. The dendrogram was constructed with the BioNumerics software 6.6 (Applied Maths NV, Sint-Martens-Latem, Belgium) choosing the Dice coefficient setting both tolerance and optimization at 1 %. The horizontal scale on the left side (100 to 70) indicates the level of similarity in percent among fingerprints. Details given in the 3rd through 7th column from the left are the isolate designation, cheese type, purchased location of samples, enterotoxin gene and PFGE profile for each isolate.*

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of sampling – contamination levels below 10^5 cfu/g, *S. aureus* survives well in brined white cheese (Bintsis and Papademas, 2002), and storage at ambient temperature may result in critical numbers at the time of consumption.

Identical gene profiles *(seg, sei, sem, sen, seo, sep* and *selu)* for the newer enterotoxins were detected in 14 isolates from each three samples of white pickled cheese (Wp3, Wp22, Wp51) and Tulum cheese (Tu4, Tu27, Tu56). Similar to the present study, the coexistence of the genes *seg, sei, sem, sen* and *seo* has been reported in *S. aureus* isolates from dairy product samples, while the *sea* gene was occurring solitary (Akineden et al., 2008; Aydin et al., 2011). Nevertheless, the frequency of identical SE gene profiles in brined cheeses over various locations, vendors and sampling times was striking. It was also noticeable that all isolates could be grouped into only three clusters according to their PCR results (Table 3). Therefore, genetic diversity was investigated for these isolates by using pulse-field gel electrophoresis (PFGE).

PFGE analysis

Macrorestriction analysis of the chromosomal DNAs with the restriction enzyme *Sma*I yielded three distinct PFGE profiles I to III, which were in full accordance with the three enterotoxin gene clusters (Fig. 1). In our opinion, the only reasonable explanation for these findings is that all isolates within one cluster are clones, which suggests that all cheeses within one cluster have the same origin. Although the corresponding cheeses had been purchased from different vendors at different times and locations of Ankara, the claimed uniqueness of several products was questionable. For example, PFGE profile I was found in isolates from three white pickled cheeses and two Tulum cheeses samples (Wp3, Tu4, Wp51, Tu56, Wp22 and Tu27) purchased at five different locations (A, B, C, D and E) and vendors (Table 1). Although no tracing of the products back to the production sites was feasible within this study, it seems to be possible that the cheeses could have been contaminated with distinct strains displaying specific enterotoxin gene and PFGE profiles during early processing stages (Walcher et al., 2014). Very speculatively, the genetic characteristics of contaminating bacteria may also be used for fingerprinting of different brands of cheese. If evidence for a common production site or distribution source could be obtained by comparing PFGE profiles, this could then could be useful to identify incorrect labelling practices.

In conclusion, this study demonstrated that with eleven positive samples (17.5 %) out of 63 brined cheeses samples, the frequency of *S. aureus* was only moderate, but all isolates obtained from positive samples were enterotoxigenic. The fact that many samples were obviously from the same source, contrary to the label information, also indicates an intrinsic problem of representative sampling for surveys in such informal weekly street market structures. All results should be checked for salient and abnormal similarities between unrelated samples, to avoid over- or underestimation of the true frequency of toxinogenic bacteria due to multiple analysis of ultimately the same sample. Although our results on enterotoxigenic *S. aureus* in brined cheese are less alarming than those reported in some previous studies, they still indicate the need to enhance control measures of these under regulated weekly street markets and to aim at improving awareness of cheese-makers and vendors for hygiene issues in the production environments and environmental sources at the weekly street markets.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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