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Prevalence, antimicrobial susceptibility and characterization of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* isolated from dairy industries in north-central and north-eastern Greece

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ABSTRACT

Staphylococcus aureus is an important cause of food intoxication, whereas methicillin-resistant *S. aureus* (MRSA) constitutes a serious public-health concern due to its ability to colonize and infect humans and animals. *S. aureus* and MRSA have often been isolated from milk and dairy products. The objectives of this study were to estimate the prevalence and the antimicrobial resistance of *S. aureus* and MRSA in four Greek dairy industries, to identify virulence factors of MRSA isolates and to describe their genetic diversity, in order to identify possible epidemiological links and evaluate the risk of MRSA dissemination to the community. *S. aureus* was isolated from 67 out of 305 (22.0%) dairy industry samples (bulk-tank milk, dairy products, employee nasal swabs and equipment/surface swabs). Almost all (99%) of the 227 corresponding *S. aureus* isolates (approximately 4 isolates per positive sample) were resistant to at least one antimicrobial and 22% were multi-drug resistant (MDR). MRSA were isolated from 11 different samples (3.6%) originating from three of the dairy plants. All MRSA isolates were capable of forming biofilms, while staphylococcal enterotoxin (SE) genes were detected in 91% of the MRSA isolates, with *sec* being the most frequent. All of the MRSA isolates harbored the *mec*A gene but the *mec*C and Pandon-Valentine leucocidin (PVL) genes were not detected. Pulse-Field Gel Electrophoresis (PFGE) analysis showed genetic diversity among the MRSA isolates and indicated clonal circulation in one of the dairy plants. Seven *spa* types were identified among the MRSA isolates with the most prevalent (t065) isolated only in one dairy plant. Certain *spa* types (t065, t337 and t3536) were isolated for the first time in Greece. The presence of MDR, biofilm-forming and enterotoxigenic MRSA strains in dairy plant facilities may lead to their dissemination to the community, but also to staphylococcal food poisoning, when conditions are favorable. The study's findings highlight the need for continuous monitoring of the dairy production chain, the need for re-evaluating the implemented cleaning and sanitizing processes and the adoption of preventive strategies in order to minimize public-health risks.

1. Introduction

Staphylococcus aureus (*S. aureus*) is a versatile opportunistic pathogen (Sergelidis and Angelidis, 2017) which can cause a variety of infections, from superficial skin infections to severe or even fatal invasive diseases (Aires-de-Sousa et al., 2006). *S. aureus* has often been isolated from raw milk and the dairy processing environment (equipment, surfaces and food handlers) (Johler et al., 2018). *S. aureus* is

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capable of acquiring antibiotic resistance determinants and, consequently, *S. aureus* isolates often exhibit resistance to multiple classes of antimicrobial agents (Rybak and LaPlante, 2005). Methicillin-resistant *S. aureus* (MRSA) are *S. aureus* strains that have acquired the *mec*A gene, which encodes for the penicillin-binding protein 2a (PBP2a), mediating resistance to methicillin and all other *β*-lactam antibiotics (Fishovitz et al., 2014). MRSA constitutes a serious public-health concern due to its ability to colonize and infect humans and animals (Petinaki and Spiliopoulou, 2012).

MRSA was initially detected in the hospital setting (i.e., as a hospital-associated pathogen; HA-MRSA), where it remained confined until the 1990s. However, being a highly versatile pathogen, it established new reservoirs. Currently, it has been disseminated to the community (referred to as community-associated MRSA; CA-MRSA) and frequently colonizes animals, especially livestock (livestock-associated MRSA; LA-MRSA) (Aires-de-Sousa et al., 2006). Interestingly, CA-MRSA clones often carry the gene encoding for the Panton-Valentine leukocidin (PVL) cytotoxin (EFSA, 2009), a pore-forming toxin involved in the pathophysiology of skin infections (Hodille et al., 2016). Recently, MRSA, including LA-strains, have been isolated from milk and dairy products worldwide (Basanisi et al., 2016; Papadopoulos et al., 2018). It has also been demonstrated that the handling and consumption of MRSA-contaminated food of animal origin can act as a potential vehicle for MRSA transmission to humans (EFSA, 2009; Feingold et al., 2012) and MRSA foodborne outbreaks have been reported (Sergelidis and Angelidis, 2017).

Upon multiplication in contaminated foods under permissive storage-temperature conditions, *S. aureus* can produce staphylococcal enterotoxins (SEs) and cause staphylococcal food poisoning (SFP). Being resistant to heat, proteolytic enzymes and low pH, SEs are highly stable and remain active in the digestive tract, following ingestion (Le Loir et al., 2003). Many types of SEs have been identified but the most important are SEA, SEB, SEC, SED and SEE (Omoe et al., 2003; Orwin et al., 2003), which cause 95% of SFP (Gutierrez et al., 2012). Studies report that SEs in cheese were involved in 6.4% (2013) to 20% (2012) of SFP outbreaks for all food categories (Asselt et al., 2017). In Europe, milk and dairy products have being incriminated in 5% of all staphylococcal foodborne outbreaks (Bianchi et al., 2014) and are considered as the main source of enterotoxigenic MRSA (Oniciuc et al., 2017).

S. aureus forms biofilms on biotic and abiotic surfaces, including processing surfaces and equipment in dairy plants (Gutierrez et al., 2012). Biofilm formation is increasingly recognized as an important virulence property of *S. aureus*, leading to persistent contamination or infection because the cells within the biofilm are very resistant to sanitation procedures and to the action of both the immune system and antimicrobial agents (Song et al., 2017). Biofilm formation on food-processing surfaces is a significant source of food cross-contamination. In particular, MRSA biofilms not only enable bacteria to tolerate sanitation processes but also promote horizontal spread of antibiotic-resistance determinants (Savage et al., 2013). Data on biofilm formation by food-related *S. aureus* isolates (isolates from food, environment of food-processing facilities and food handlers) are still limited (Di Ciccio et al., 2015).

Therefore, the objectives of this study were: (i) to estimate the prevalence and the antimicrobial resistance of *S. aureus* and MRSA in four Greek dairy industries, (ii) to identify virulence factors among the corresponding MRSA isolates and (iii) to assess the genetic diversity of MRSA isolates in order to identify possible epidemiological links and evaluate the risk for their dissemination to the community.

2. Materials and methods

2.1. Sample collection

Between December of 2016 and May of 2017, 305 samples were collected from four dairy plants (designated as plant A, B, C and D) located in north-central and north-eastern Greece. Plants A and B are located in the region of Thrace and plants C and D are located in the region of Macedonia. Plants A and C are 'large-scale', with 50,000 l of milk processed per day; plant B is 'medium-scale' (10,000 l per day) and D is 'small-scale' (2000 l per day). The collected samples consisted of 18 raw bulk-tank milk samples (9 bovine, 7 ovine and 2 caprine; the milk was stirred before sampling and 50 ml were aseptically collected into sterile tubes), 74 samples of dairy products (pre-packaged soft and hard cheese, yogurt and butter), 56 nasal swab samples from employees (taken from both anterior nares by means of cotton-tipped swabs) and 157 swab-samples from equipment surfaces (door handles, hosepipes, cheese cutters, knives, spoons, knobs, tanks). The swabs were immediately placed into 10 ml Tryptone Soy broth (TSB; LAB M, Lancashire, United Kingdom) with 6.5% w/v NaCl (Merck, Darmstadt, Germany) and 0.3% yeast extract (LAB M).

All human participants gave oral informed consent to participate in the study and all reported not receiving any recent (within the preceding 3 months) antimicrobial treatment. All collected samples were transported to the laboratory under refrigeration (ca. 2 °C) within a maximum of 5 h and were processed immediately.

2.2. Isolation and identification of S. aureus

Isolation (all samples) and enumeration (milk and dairy product samples) of staphylococci was carried out on Baird-Parker agar supplemented with egg-yolk tellurite (LAB M) after aerobic incubation for 24–48 h at 35 °C as previously described (Papadopoulos et al., 2018). For each sample, up to four well-isolated typical *S. aureus* colonies (black with or without opaque halo) were selected for purification (24–48 h at 35 °C) and further characterization on non-selective medium (Tryptone Soya Yeast Extract Agar; TSYEA, LAB M). Preliminary identification was based upon morphological and cultural characteristics and simple microscopic and biochemical assays (Gram-staining, catalase reaction, mannitol fermentation, coagulase test). *S. aureus* strains were stored at −80 °C in TSB as glycerol (20%) stocks. A sample (raw milk, swab or dairy product) was defined as positive if it contained at least one *S. aureus* isolate.

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility towards 14 antimicrobials (penicillin, P; oxacillin, Ox; amoxicillin/clavulanic acid, Amc; tetracycline, T; erythromycin, E; vancomycin, V; chloramphenicol, C; ciprofloxacin, Cp; trimethoprim/sulfamethoxazole, Sxt; trimethoprim, Tm; gentamicin, G; amikacin, Ak; kanamycin, K; rifampicin, R) was determined by the agar-dilution method in Mueller-Hinton agar (Merck) according to the Clinical and Laboratory Standard Institute (CLSI) guidelines (CLSI, 2009) and the results were evaluated after incubation at 35 °C for 24 h. Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) minimum inhibitory concentration (MIC) breakpoints (EUCAST, 2018). Multidrug-resistance was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos et al., 2012). *S. aureus* ATTC 25923, *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as control strains.

2.4. Detection, molecular confirmation and genetic characterization of MRSA

2.4.1. Detection of the coa, nuc, mecA and mecC ($mecA_{LGA251}$) genes

Presumptive *S. aureus* (Gram-, catalase-, mannitol- and coagulase-positive cocci) and MRSA (oxacillin-resistant) strains were submitted to further molecular characterization. The DNA purification protocol for Gram-positive bacteria (Pure Link Genomic DNA kit, Invitrogen, Carlsbad, CA) was used for genomic DNA extraction. In order to confirm the identification of *S. aureus* isolates and verify their methicillin-

Table 1
PCR primers used in this study.

Gene target	Primer	Sequence 5′-3′	Product (bp)	Reference
coa	F	ATAGAGATGCTGGTACAGG	500–650	(Hookey et al., 1998)
	R	GCTTCCGATTGTTCGATGC		
nuc	F	GGCAATTGTTTCAATATTAC	416	(Sudagıdan and Aydın, 2009)
	R	TTTTATTTGCATTTTCTACC		
*mec*A	F	AAAATCGATGGTAAAGGTTGGC	533	(Murakami et al., 1991)
	R	AGTTCTGCAGTACCGGATTTGC		
*mec*C	$mecA_{LGA251}$MultiF	GAAAAAAAGGCTTAGAACGCCTC	138	(Stegger et al., 2012)
	$mecA_{LGA251}$MultiR	GAAGATCTTTTCCGTTTTCAGC		
sea	SEA-1	GAAAAAAGTCTGAATTGCAGGGAACA	560	(Jarraud et al., 2002)
	SEA-2	CAAATAAATCGTAATTAACCGAAGGTTC		
seb	SEB-1	ATTCTATTAAGGACACTAAGTTAGGGA	404	(Jarraud et al., 2002)
	SEB-2	ATCCCGTTTCATAAGGCGAGT		
sec	SEC-1	GTAAAGTTACAGGTGGCAAAACTTG	297	(Jarraud et al., 2002)
	SEC-2	CATATCATACCAAAAAGTATTGCCGT		
sed	SED-1	GAATTAAGTAGTACCGCGCTAAATAATATG	492	(Jarraud et al., 2002)
	SED-2	GCTGTATTTTTCCTCCGAGAGT		
see	SEE-1	CAAAGAAATGCTTTAAGCAATCTTAGGC	482	(Jarraud et al., 2002)
	SEE-2	CACCTTACCGCCAAAGCTG		
PVL	pvl-F	GCTGGACAAAACTTCTTGGAATAT	83	(Stegger et al., 2012)
	pvl-R	GATAGGACACCAATAAATTCTGGATTG		

resistance phenotype, previously described PCR conditions (Zdragas et al., 2015) and primer sets (listed in Table 1) were employed for the detection of the *coa*, *nuc*, *mec*A and *mec*C genes. The PCR amplicons were separated in 1.5% agarose gels stained with ethidium bromide and visualized under UV illumination (TEX-20 M, Life Technologies, Gibco BRL System).

2.4.2. Detection of staphylococcal enterotoxin genes, PVL gene and biofilm formation ability

All confirmed MRSA isolates were screened for the presence of the five genes encoding for the classical staphylococcal enterotoxins (*sea*, *seb*, *sec*, *sed*, *see*) using specific primer sets described in Table 1. Amplifications were performed as single PCR assays as follows: an initial DNA denaturation at 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension of 5 min at 72 °C. The PCR method described by Stegger et al. (2012) was employed for the detection of the PVL gene (Table 1).

Biofilm production by MRSA strains was determined using a semi-quantitative, microtiter-plate (MTP), adherence-assay as previously described by Wang et al. (2010). The protocol relies on the measurement of the optical density (OD) of adherent biofilms stained with 0.3% (w/v) crystal violet at 570 nm. The cut-off (ODc) was defined as the mean OD value of the negative control (plain broth medium). Based on OD measurements, MRSA strains were classified as either no biofilm producers ($OD < ODc$), weak ($ODc < OD \leq 2 \times ODc$), moderate ($2 \times ODc < OD \leq 4 \times OD_C$) or strong ($4 \times ODc < OD$) biofilm producers (Borges et al., 2012).

2.4.3. Spa and Pulsed Field Gel Electrophoresis (PFGE) typing

Spa typing was conducted according to Aires-de-Sousa et al. (2006) as previously described (Papadopoulos et al., 2018). PFGE analysis was conducted per the PulseNet protocol (McDougal et al., 2003) using the size standard, electrophoretic conditions, dendrogram construction and comparison criteria previously described (Papadopoulos et al., 2018), with the exception that a newer version of the Bionumerics (Applied Maths, Sint-Martens-Latem, Belgium) software was used (ver. 7.5) to analyze restriction patterns.

2.5. Statistical analysis

Comparisons of categorical variables were performed using the Fisher's exact test at an alpha of 0.05. Where appropriate, *S. aureus* and MRSA prevalence estimates were also compared via Fisher's exact test.

3. Results and discussion

3.1. Prevalence, genetic characterization and antimicrobial susceptibility of S. aureus and MRSA

Sixty-seven of the 305 samples (22.0%) were *S. aureus*-positive; 7 (38.9%) of the raw-milk samples, 25 (44.6%) of the employee samples, 33 (21.0%) of the equipment samples and 2 (2.7%) of the samples from dairy products. The isolation frequencies per dairy plant and sample type are shown in Table 2. The average *S. aureus* isolation frequency varied significantly ($p < 0.05$) among the four dairy plants: 13.3% in plant A, 20.6% in plant C and 33.3% in plants B and D. A total of 227 *S. aureus* isolates were obtained from the 67 positive samples.

The antimicrobial susceptibility testing results of the *S. aureus* isolates are shown in Table 3. Of the 227 isolates, 224 (98.7%) were found to be resistant to at least one antimicrobial and 50 (22.0%) were multidrug resistant (MDR). Higher resistance frequencies were observed against penicillin (96.9%), amoxicillin/clavulanic acid (93.0%) and erythromycin (18.9%). Thirty-three (14.5%) of the *S. aureus* isolates were identified as presumptive MRSA (based on their phenotypic resistance to oxacillin) and carried the *coa*, *nuc* and *mec*A genes, but the *mec*C gene was not detected. These MRSA strains were isolated from 11 different samples (11/305, 3.6%) originating from three (A, C and D) of the dairy industries (Table 2). The dairy plant-specific MRSA isolation frequencies were significantly ($p < 0.05$) different: 3.8% (A), 0.0% (B), 2.8% (C) and 9.5% (D). One strain per MRSA-positive sample was further analyzed and their exact origin, genotypic and antimicrobial characteristics are shown in Fig. 1.

The presence of *S. aureus* in the incoming raw milk in dairy plant facilities represents a potential source for the introduction of the pathogen into the dairy-food chain, with a resulting risk of food contamination (D'Amico and Donnelly, 2011). Overall, in raw milk, *S. aureus* and MRSA were recovered from 33.3% (mean count of 3.5 log CFU/ml) and 11.1% of the bovine milk samples, and from 57.1% (mean count of 4.4 log CFU/ml) and 14.3% of the ovine milk samples, respectively. These findings are comparable with those of our previous study in the region of Epirus (north-western Greece), in which *S. aureus* and MRSA were isolated from 40% and 10% of the bovine milk samples, and from 63.2% and 0% of the ovine milk samples tested, respectively (Papadopoulos et al., 2018). In two earlier surveys in the region of Thessaly (central Greece), lower isolation frequencies of *S. aureus* and MRSA were reported from raw ovine milk: 24.3% and 0.7% (Pexara et al., 2016) and 22.8% and 0% (Zdragas et al., 2015),

Table 2
Isolation frequency of *S. aureus* and methicillin-resistant *S. aureus* (MRSA) from samples of raw-milk tanks, employees, equipment surfaces and dairy products taken from four dairy plants.

Sample type	Plant A			Plant B			Plant C			Plant D			Total		
	No of samples	*S. aureus* (%)	MRSA (%)	No of samples	*S. aureus* (%)	MRSA (%)	No of samples	*S. aureus* (%)	MRSA (%)	No of samples	*S. aureus* (%)	MRSA (%)	No of samples	*S. aureus* (%)	MRSA (%)
Bovine raw milk	1	0	0	1	0	0	7	3	1	0	–	–	9	3 (33.3)	1 (11.1)
Ovine raw milk	2	2	1	1	1	0	3	1	0	1	0	0	7	4 (57.1)	1 (14.3)
Caprine raw milk	1	0	0	0	–	–	0	–	–	1	0	0	2	0	0
Employees	26	10	2	8	5	0	19	9	2	3	1	0	56	25 (44.6)	4 (7.1)
Equipment	43	2	1	31	9	0	52	9	0	31	13	4	157	33 (21.0)	5 (3.2)
Dairy products	32	0	0	10	2	0	26	0	0	6	0	0	74	2 (2.7)	0
Total	105	14 (13.3)	4 (3.8)	51	17 (33.3)	0	107	22 (20.6)	3 (2.8)	42	14 (33.3)	4 (9.5)	305	67 (22.0)	11 (3.6)

respectively. Interestingly, an earlier survey in the region of Thrace (north-eastern Greece) reported that all of the raw ovine milk samples collected from 21 sheep farms were contaminated with *S. aureus* (Alexopoulos et al., 2011). However, these prevalence comparisons should be interpreted with caution, given the low number of raw-milk samples examined in the present study. The reported prevalence of *S. aureus* and MRSA in ovine and bovine milk in other countries is quite variable. In Italy, the isolation frequencies of *S. aureus* and MRSA in ovine raw milk were 53.5% and 0.7%, respectively (Giacinti et al., 2017). In Iran, *S. aureus* was detected in 15.7% of the bovine and in 9.6% of the ovine raw-milk samples tested (Jamali et al., 2015), whereas in a similar study in the US, the corresponding isolation frequencies were 29% and 47% (D'Amico and Donnelly, 2010). In bovine raw milk, prevalence estimates of 62% (*S. aureus*) and 1.3% (MRSA) have been reported in the US (Haran et al., 2012).

In samples taken from dairy plant employees, the overall *S. aureus* and MRSA isolation frequencies were 44.6% and 7.1%, respectively, while among those taken from equipment surfaces of the dairy processing plants, 21.0% and 3.2%, respectively. In dairy products, *S. aureus* was isolated from 2.7% (two out of 74) of the tested samples (mean count of 4.0 log CFU/ml), while MRSA was not detected. Both positive samples were semi-hard cheeses. Reports on the prevalence of *S. aureus* and MRSA in food handlers and in the environment of dairy plants are very limited. With respect to the MRSA nasal carriage estimate in dairy-plant employees (7.1%), our data suggest a considerably higher MRSA prevalence than that reported a decade ago in healthy humans in Greece (0.94%) (Karapsias et al., 2008); however that study considered a larger sample size (n = 959). In our previous investigation of two dairy plants in Epirus (Papadopoulos et al., 2018), comparable *S. aureus* and MRSA isolation frequencies were noted in personnel (35.5% and 6.5%, respectively) but higher in equipment (41.8% and 7.3%, respectively). In Brazil, a study conducted in a single dairy plant reported similar results in humans (31.5% *S. aureus* and 10.3% MRSA colonization), but higher in dairy products (70.8% *S. aureus* and 1.5% MRSA) (André et al., 2008). A more recent Brazilian study in five dairy plants reported overall *S. aureus* isolation frequencies of 6.6% in surfaces and 7.6% in dairy products, with no MRSA isolation (Dittmann et al., 2017). Similar to our results, a recent study in Italy showed that 19.0% of surface samples (drying worktops) were *S. aureus*-positive (Johler et al., 2018). Other studies on the isolation of *S. aureus* and MRSA from dairy products have reported variable estimates: 6.0% and 1.0% in cheese (Can and Çelik, 2012) in Turkey, 32.6% and 0% in cow-milk cheeses (Traversa et al., 2015) and 20.0% and 1.9% in sheep-milk cheeses (Carfora et al., 2015) in Italy. The differences in the reported *S. aureus* and MRSA prevalence estimates among different studies may be attributed to differences in dairy plants' hygiene levels as well as to several other factors such as the types of tested samples (geographical origin, quantity, manufacturing technology, e.g. use of pasteurized vs. raw milk, sample storage and handling) and/or the sensitivity of the detection methods used (Chen et al., 2010; Sader et al., 2006). The data obtained from our survey show that raw milk and employees are the main sources for the introduction and dissemination of *S. aureus* and MRSA into the dairy plants facilities.

3.2. Virulence-associated genes and ability to form biofilm

With regard to the risk of SFP due to the presence of SE-coding genes, analysis of the 11 MRSA isolates revealed the presence of at least one toxin-gene in 10 (90.9%) of the isolates with a total of four different toxin-gene profiles (Fig. 1). The majority of the SE-gene-positive MRSA isolates (eight out of 10) carried only one enterotoxin gene. The most frequent SE-gene was *sec* (present in 10 isolates), while *sea* and *see* were only detected in one isolate each and only in combination with the *sec* gene (Fig. 1). Similar to our findings, all the MRSA isolates from Turkish cheeses harbored the *sec* gene (Can and Çelik, 2012), whereas an Italian study on food poisoning caused by dairy products reported the *sec* gene

Table 3
Numbers and percentages of *S. aureus* isolates resistant to different antimicrobials according to their isolation source.

Origin of *S. aureus* isolates	No of *S. aureus* isolates	No of *S. aureus* isolates resistant to different antimicrobials[a] (%)												
		P	Ox	Sxt	G	E	Ak	K	T	Tm	Amc	Cp	R	V	C
Bovine raw milk	9	2	1	0	4	0	0	0	4	0	2	0	0	0	0
Ovine raw milk	13	13	2	0	0	0	0	4	0	2	13	0	0	0	0
Equipment	106	106	14	6	32	27	7	11	6	14	97	0	3	8	0
Employees	91	91	16	0	4	16	0	8	8	0	91	0	0	4	8
Dairy products	8	8	0	0	0	0	0	0	0	0	8	0	0	8	0
Total	227	220 (96.9)	33 (14.5)	6 (2.6)	40 (17.6)	43 (18.9)	7 (3.1)	23 (10.1)	18 (7.9)	16 (7.0)	211 (93.0)	0	3 (1.3)	20 (8.8)	8 (3.5)

[a] P, penicillin; Ox, oxacillin; Sxt, trimethoprim/sulfamethoxazole; G, gentamicin; E, erythromycin; Ak, amikacin; K, kanamycin; T, tetracycline; Tm, trimethoprim; Amc, amoxicillin/clavulanic acid; Cp, ciprofloxacin; R, rifampicin; V, vancomycin; C, chloramphenicol.

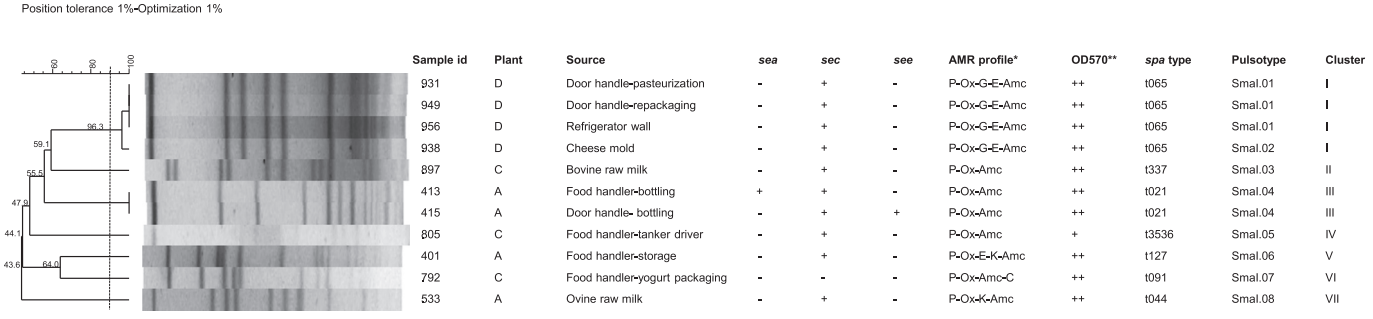
as being the most prevalent (Vitale et al., 2015). Generally, in dairy microbiology, the *sec* gene is often reported as the most widespread SE-coding gene among *S. aureus* isolates (Alibayov et al., 2014; Riva et al., 2015; Valihrach et al., 2013). Notably, the detection of enterotoxigenic MRSA in our study is consistent with previous studies reporting the ability of MRSA to produce enterotoxins and cause staphylococcal foodborne outbreaks (Jones et al., 2002; Kerouanton et al., 2007; Kluytmans et al., 1995). PVL genes were not detected among the 11 MRSA isolates, in agreement with previous studies regarding MRSA isolates from milk and dairy products (Johler et al., 2018; Riva et al., 2015; Traversa et al., 2015).

All MRSA isolates in our study were found capable for biofilm production, with 10 (90.9%) characterized as moderate and one (9.1%) as a weak biofilm-producer. Similar to our results, an Italian study in bulk tank milk reported that all MRSA isolates had the ability to form biofilm (Parisi et al., 2016), while another study in milk and dairy products reported that 97.5% of the MRSA isolates were biofilm-producers (Basanisi et al., 2017). A study in Pakistan reported that 98.3% of 180 foodborne MRSA isolates were able to produce biofilm (Mirani et al., 2013). The ability of MRSA isolates to form biofilm is considered to be a significant virulence property and represents a serious problem for the dairy industry because biofilms can be resistant to the usual sanitation processes (Basanisi et al., 2017) and, once established, biofilms become a source of contamination of dairy products and surfaces (Oliveira, 2014). Hence, the design and implementation of effective cleaning and sanitation protocols in the dairy industry is of utmost importance and requires the careful consideration of risk managers and risk assessors.

3.3. Spa typing and PFGE clusters

Seven different *spa* types were identified among the 11 MRSA isolates. The most prevalent were t065 (n = 4) and t021 (n = 2), while t044, t091, t127, t337 and t3536 were represented only once (Fig. 1). To our knowledge, this is the first time that *spa* types t065, t337 and t3536 were detected in Greece. According to Ridom Spa Server (www.spaserver.ridom.de), the global frequency of occurrence of t065 is moderate and it has been reported in many countries worldwide, both as MSSA and MRSA. Recently, *spa* type t065 has been reported in MSSA isolated from humans (beef-packing workers) in the US (Leibler et al., 2016) and previously in MRSA isolated from human patients in Germany (Cuny et al., 2015). *Spa* type t021 has been previously reported in MRSA isolates from many countries worldwide (Grundmann et al., 2010; Harastani et al., 2014). *Spa* type t337 is frequent in LA-MRSA isolates from pigs and pork meat (Ivbule et al., 2017; Krupa et al., 2015). Interestingly, *spa* type t127 has been previously detected in the country, in a patient hospitalized in the internal medicine ward in a hospital of northern Greece (Kachrimanidou et al., 2014) and also as the dominant *spa* type in our previous study of dairy plants in the region of Epirus (Papadopoulos et al., 2018). *Spa* type t127 represents a prevalent lineage of LA-MRSA also in Italy (Franco et al., 2011) and has been isolated from sheep (Caruso et al., 2016) and goat milk (Cortimiglia et al., 2015), from both humans and animals in dairy sheep-farms (Macori et al., 2017) and from dairy products (Carfora et al., 2015). Furthermore, *spa* type t044 has also been previously detected in Greece, in hospital patients (Kachrimanidou et al., 2014; Vourli et al., 2009) and also in healthy employees of the Hellenic Air Force (Karapsias et al., 2008). According to Ridom Spa Server, the global frequency of occurrence of t091 is high and has been reported recently in MRSA isolates recovered from infants and children in Poland (Ilczyszyn et al., 2016).

Eight different pulsotypes (PT) (designated as *Sma*I.01–SmaI.08) were identified in the 11 MRSA isolates; these were assigned into seven clusters, designated from I to VII (Fig. 1). Each different PT was associated with a specific plant and circulation of common PT among the plants was not observed (Fig. 1). Four MRSA strains were isolated from plant D, all from equipment surfaces. These four isolates grouped in PFGE cluster I, with three sharing the same PT; all four were ascribed to



Sample id	Plant	Source	*sea*	*sec*	*see*	AMR profile*	OD570**	*spa* type	Pulsotype	Cluster
931	D	Door handle-pasteurization	-	+	-	P-Ox-G-E-Amc	++	t065	SmaI.01	I
949	D	Door handle-repackaging	-	+	-	P-Ox-G-E-Amc	++	t065	SmaI.01	I
956	D	Refrigerator wall	-	+	-	P-Ox-G-E-Amc	++	t065	SmaI.01	I
938	D	Cheese mold	-	+	-	P-Ox-G-E-Amc	++	t065	SmaI.02	I
897	C	Bovine raw milk	-	+	-	P-Ox-Amc	++	t337	SmaI.03	II
413	A	Food handler-bottling	+	+	-	P-Ox-Amc	++	t021	SmaI.04	III
415	A	Door handle- bottling	-	+	+	P-Ox-Amc	++	t021	SmaI.04	III
805	C	Food handler-tanker driver	-	+	-	P-Ox-Amc	+	t3536	SmaI.05	IV
401	A	Food handler-storage	-	+	-	P-Ox-E-K-Amc	++	t127	SmaI.06	V
792	C	Food handler-yogurt packaging	-	-	-	P-Ox-Amc-C	++	t091	SmaI.07	VI
533	A	Ovine raw milk	-	+	-	P-Ox-K-Amc	++	t044	SmaI.08	VII

* P, penicillin; Ox, oxacillin; G, gentamicin; E, erythromycin; K, kanamycin; Amc, amoxicillin/clavulanic acid; C, chloramphenicol.
** Moderate biofilm producer (++); weak biofilm producer (+).

Fig. 1. Dendrogram of *Sma*I Pulsed Field Gel Electrophoresis (PFGE) pulsotypes (PT) and characteristics of the 11 MRSA isolates examined in this study. The vertical dashed line indicates the cutoff (90% level of similarity). All 11 MRSA strains tested positive for the presence of the *nuc*, *coa* and *mecA* genes and negative for the presence of the *mecC* and PVL genes.

spa type t065 and displayed identical antimicrobial resistance profiles, being resistant to penicillin, oxacillin, gentamicin, erythromycin and amoxicillin/clavulanic acid (Fig. 1), indicating clonal circulation through the dairy plant. The fact that all four strains were isolated from different areas on the same day strongly suggests that this clone is endemic inside plant D, which can act as a source for transmitting MRSA to dairy products and subsequently to the community. Unlike plant D, dissimilar PFGE patterns of MRSA isolates within the same dairy plant were observed for plants A and C (Fig. 1). Specifically, among the four MRSA strains isolated from dairy plant A, two (one from an employee working at the bottling area and the other from a door handle in the same area) grouped in PFGE cluster III, shared the same PT, were ascribed to *spa* type t021 and displayed identical antimicrobial resistance profiles (being resistant to penicillin, oxacillin and amoxicillin/clavulanic acid), indicating cross-contamination between the employee and equipment surfaces, while the remaining two (one from an employee and the other from raw ovine milk) were assigned in PFGE clusters V and VII and ascribed to *spa* types t127 and t044, respectively. Finally, the three MRSA strains isolated inside plant C (one from raw bovine milk, one from a tank driver and the third from an employee in yogurt packaging) were assigned to PFGE clusters II, IV and VI and ascribed to *spa* types t337, t3536 and t091, respectively (Fig. 1). Both *spa*- and PFGE-typing were able to distinguish all the MRSA isolates of the present study and confirm their high genetic diversity, even within the same dairy plant. Future studies should be directed towards sampling of dairy farms, farmers and animals from the same areas, which will help us determine the exact origin, dissemination and epidemiology of *S. aureus* and MRSA, throughout the dairy production chain in Greece.

4. Conclusion

In this study, an overall prevalence of 22.0% for *S. aureus* and of 3.6% for MRSA was noted in dairy industry samples. Among the 11 MRSA isolates seven different *spa* types were identified. The enterotoxin C gene exhibited the highest prevalence among the MRSA isolates and three *spa* types (t065, t337 and t3536) were isolated for the first time in Greece. In agreement with previous studies (André et al., 2008; Basanisi et al., 2017; Carfora et al., 2015; Vitale et al., 2018), our data suggest multiple sources of MRSA contamination throughout the dairy production chain, including raw milk, processing environment and personnel, and indicate cross-contamination inside dairy plants facilities, albeit no common clones were detected among the different industries. The isolation of MDR and enterotoxigenic MRSA strains from inside the dairy industry facilities and specifically from critical areas such as repackaging and bottling, where the products do not undergo any further decontamination process, represents a serious health risk for consumers, that may result not only to the dissemination of MRSA and MDR staphylococci to the community, but also to staphylococcal food poisoning, when conditions are favorable. This is reinforced by the ability for biofilm formation (presented by all MRSA isolates), which renders them resistant to the usual sanitation processes. Consequently, the results of the present study highlight the need for continuous monitoring of the dairy production chain, re-evaluation of the implemented cleaning and sanitizing processes and the adoption of preventive strategies, in order to minimize public-health risks. This is the first study to document the dissemination of enterotoxigenic and biofilm-producing MRSA inside Greek dairy industry facilities and the reported data should be of great importance to risk assessors.

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Conflict of interest

All authors declare no conflict of interest.

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