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Research Article

Comparison of Virulence, Resistance Genes, and SCCmec Types in CoNS and *Staphylococcus aureus* Strains Isolated from Raw Cow Milk Samples

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Abstract

Although there has been extensive research on the presence of *Staphylococcus aureus (S. aureus)* in raw milk samples, new data on the prevalence and molecular characterization of *Coagulase-negative Staphylococci (CoNS)* are needed. This study aimed to compare the antimicrobial profiles, virulence genes, and *SCCmec* types distribution between *S. aureus* and *CoNS* isolated from one hundred and fifty raw cow milk samples in Istanbul. *Staphylococcus* isolates were identified using VITEK MS following classical culture methods. Phenotypic antibiotic susceptibility was determined using the disc diffusion method. In-house PCR was employed to detect resistance genes, while multiplex PCR and qPCR were utilized for *SCCmec* typing and virulence genes such as *SEs*, respectively. Out of the seventy-five contaminated samples (50%), 32% harbored *S. aureus*, and 68% were *CoNS*. Methicillin resistance was identified in 10.6% of *S. aureus* and 14.6% of *CoNS*. *SCCmec* type IV predominated in both MRSA (50%) and MRCoNS (54.5%). At least one toxin gene was present in 83.3% of *S. aureus* and 22.4% of *CoNS* isolates, with *sei* being the most frequently observed. None of the *S. aureus* isolates tested positive for the *sed, see,* and *pvl* genes. Similarly, the *sea, sed, see, tsst-1, and pvl* genes were not detected in any of the *CoNS* isolates. As a conclusion, *SCCmec* type IV and *sei* gene predominated and community-acquired resistance patterns were prominent in *Staphylococcus* strains, which carried various virulence and resistance genes. Beyond MRSA, the presence of MRCoNS should be considered and monitored as a significant public health concern in raw milk.

Keywords: CoNS, Raw milk, SCCmec types, *Staphylococcus aureus*, Virulence genes

Introduction

Staphylococcus aureus is a highly proficient opportunistic pathogen, implicated in various infectious diseases, ranging from minor skin and soft tissue infections to septicemia and toxic shock syndrome. Moreover, among the diverse diseases caused by *S. aureus*, staphylococcal food poisoning (SFP), a foodborne intoxication, results from the consumption of food contaminated with sufficient amounts of staphylococcal enterotoxins (*SEs*), primarily produced by specific *S. aureus* strains [1].

Coagulase-negative staphylococci *(CoNS)* play the role of opportunistic nosocomial pathogens, often linked to infections associated with foreign bodies and catheters, as well as conditions such as urinary tract infections and endocarditis, among others [2].

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S. aureus exhibits numerous virulence factors and a notable capacity to develop resistance to a broad spectrum of antibiotics. Methicillin resistance is particularly significant within *Staphylococcus* spp., especially in *S. aureus*. The methicillin resistance determinant, mecA, is an integral part of a mobile genetic element known as *SCCmec*. Although mecA is not exclusive to *S. aureus*, its presence has been documented in other Staphylococcal species originating from both human and animal sources. In addition to *S. aureus* and *S. sciuri*, reports indicate the occurrence of mecA in methicillin-resistant strains of *S. pseudintermedius, S. intermedius, S. vitulinus, S. epidermidis, S. haemolyticus, and S. saprophyticus* [3].

Methicillin resistance in *Staphylococci*, especially in *S. aureus* (MRSA), has a global presence, adapting to diverse environmental conditions and modulating its pathogenicity. The prevalence and epidemiology of MRSA, in particular, are continually escalating, attributed to the emergence of new MRSA clones in various geographical regions. Therefore, it is crucial to bear in mind that MRSA can continuously acquire new features and should be closely monitored [3,4].

Monitoring methicillin resistance in food is vital due to the ability of these bacteria to disseminate multiple antimicrobial resistance genes, transforming it into a significant public health concern [5]. While many studies on raw milk have focused on *S. aureus*, especially MRSA, several studies have started to emphasize the importance of CoNS [2,6].

This study aims to compare the antimicrobial profiles, virulence genes, and the distribution of *SCCmec* types between *S. aureus* and *CoNS* isolated from raw cow milk in what there is needed new data.

Material and Methods

Ethical Statement

This study does not require ethical permission.

Sampling and Storage

One hundred and fifty raw cow milk samples offered for sale in various districts of İstanbul were randomly collected between September 2018 to February 2020. None of the milk samples were packed. 500 mL of raw milk samples from each sample were collected in sterile containers and transported to the laboratory under appropriate transport conditions. All samples were kept at +4°C in a refrigerator and taken to the laboratory for processing.

Isolation of *S. aureus* and Other *Staphylococcus spp.*

A volume of 10 µL of each sample was performed on blood agar with 5% sheep blood and chocolate agar (Oxoid, Basingstoke, United Kingdom). Plates were examined after 24 h and 48 h. of aerobic incubation at 37°C. Typical *Staphylococcus* spp. colonies were tested. Gram stain and biochemical identification tests such as catalase test, novobiocin sensitivity, and mannitol fermentation were made for identification. The presence of beta hemolysis, clumping factor, tube coagulase activity, and DNase were also investigated [7].

The strains identified as *Staphylococcus* spp. were subcultured on tryptic soy agar and incubated at 37°C for 24-48 h. The VITEK MS (bioMérieux SA, Marcy l'Etoile, France) Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) (bioMérieux SA, Marcy l'Etoile, France) system was employed for identification following the manufacturer's instructions. Subsequently, the isolates were individually stored in tryptic soy broth medium with 15% glycerol at −80°C for subsequent phenotypic and genotypic analyses [7].

Phenotypic Characterizations

Oxacillin disc diffusion testing: Methicillin resistance was detected by cefoxitin (30 µg) and oxacillin (1 µg) disks (Oxoid, Basingstoke, United Kingdom) according to the EUCAST criteria [7]. *S. aureus* ATCC 25923 (susceptible) and *S. aureus* ATCC 43300 (resistant) were used as control strains.

Antimicrobial susceptibility testing: Antimicrobial susceptibility testing was performed according to the disc diffusion method and the results were interpreted according to the EUCAST-2016 criteria (EUCAST. Version 6.1, 2016). The antimicrobial susceptibility of *Staphylococcus* was assessed for penicillin G, oxacillin, cefoxitin, erythromycin, gentamicin, amikacin, tetracycline, clindamycin, and vancomycin (Oxoid, Basingstoke, United Kingdom) on Mueller-Hinton agar. *S. aureus* ATCC 29213 served as a quality control strain. Isolates demonstrating resistance to at least three different antimicrobial classes were classified as multidrug-resistant [7].

Genotypic Characterisations

DNA Extraction: The High Pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany) was used to perform DNA isolation from the *Staphylococcus* strains according to the manufacturer's instructions.

SCCmec Typing in MRSA and MRCoNS via Multiplex PCR: SCCmec typing was performed via multiplex PCR using specific primer sets (IDT, Coralville, IA, USA) and same protocols used by Kondo et al.[8]. The *SCCmec* typing assignment of the isolates was conducted based on the *ccr* and *mec* gene complexes. Six different multiplex PCR sets were performed on T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) for *SCCmec* typing of each isolate. The protocol involves six M-PCR assays; M-PCR1 was identified the ccr type by targeting specific genes

within the ccr complex. M-PCR2 was determined the mec class by targeting specific regions within the mecA-mecI complex. M-PCR3 was identified the specific ORFs in the J1 region of type I and type IV SCCmec elements. M-PCR4 was identified the specific ORFs in the J1 region of type II, type III, and type V SCCmec elements. M-PCR5 was identified the J2 region of type II or type III SCCmec elements and the presence of ermA and cadB genes. M-PCR6 was identified the integrated plasmids in the J3 region, specifically targeting mecA, ant(4'), and tetK genes. By combining the results of these six M-PCR assays, can be classify *S. aureus* strains based on their SCCmec type. Except M-PCR6, Reaction mixture was included 10 ng DNA, 0.1 µM primers, 200 µM dNTPs, Taq buffer, 2.5U Taq polymerase, 3.2 mM $MgCl_2$, in 50 µL and PCR condition was denaturation for 94°C, 2 min, and 30 cycles annealing of 94°C at 2 min, 57°C at 1 min, 72°C at 2 min, and then final extension 72°C at 2 min. M-PCR1 annealing temperatures was 57°C at 1 min but M-PCR2 to 5 annealing temperatures was 60°C at 1 min. M-PCR6 was a long-range PCR and reaction mixture was included 10 ng DNA, 0.3 µM primers, 200 µM dNTPs, Expand High Fidelity buffer, 1.5 mM $MgCl_2$, 2.6U enzyme mix (Roche Diagnostics GmbH, Mannheim, Germany), in 50 µL. PCR condition of M-PCR6 was; denaturation (94°C, 2 min), 10 cycles (94°C, 15 s, 50°C, 30 s, 68°C, 8 min), 20 cycles (94°C, 15 s, 50°C, 30 s, 68°C, 12 min), and final extension (72°C, 7 min). Agarose gel electrophoresis was performed on mini-sub cell GT horizontal electrophoresis system (Bio-Rad, Hercules, CA) to analyze PCR products and gels were analysis using a Chemi-Doc MP Imaging System (Bio-Rad, Hercules, CA, USA) [8].

Detection of Antimicrobial Resistance Genes via In-house PCRs

Specific primers were used to determine the genotypical antimicrobial susceptibility via the previously described by Demir et al.[9] and Demirci et al.[7]. Fifteen genes were used to find the genotypical resistance. Oxacilline (mecA); penicillin *(blaZ);* aminoglycoside (gentamicin and amikacin) *(aac(6')-aph(2"), aph(3')-IIIa, ant(4')-Ia);* macrolide (erythromycin) *(ermA, ermB, ermC, and msrA),* tetracycline *(tetK, tetM)* [9] and vancomycin *(vanA, vanB, vanC1, and vanC2-C3)* specific genes were analyzed as described by Demirci et al.[7]. The 16S rDNA gene was used as an amplification control. The PCR amplifications were performed in a total of 25 µL volume on T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Each reaction contained 5 µL of template DNA. Agarose gel electrophoresis were performed on mini-sub cell GT horizontal electrophoresis system (Bio-Rad, Hercules, CA) to analyze PCR products and gels were analysis using a Chemi-Doc MP Imaging System (Bio-Rad, Hercules, CA, USA) [9].

Detection of Staphylococcal SEs Genes, tsst -1 Gene, and pvl Gene via qPCR

qPCR was employed to detect virulence genes such as *Staphylococcal Enterotoxins-SEs*, *Panton-Valentine Leukocidin toxin-pvl*, and *toxic shock syndrome toxin 1- tsst-1* using specific primer sets (IDT, Coralville, IA, USA) as previously described by Demirci et al.[7]. The LightCycler 480 Sybr Green Master kit (Roche Diagnostics GmBH, Mannheim, Germany) was utilized with these primers on the LightCycler 480 II instrument (Roche Diagnostics GmBH, Mannheim, Germany) following the manufacturer's instructions.

Positive Controls Included *S. aureus* Strains

ATCC 14458 *(seb),* ATCC 13565 *(sea, sej),* ATCC 19095 *(sec, seh),* ATCC 27664 *(see),* ATCC 23235 *(sed, seg, sei),* ATCC 51650 *(tsst-1)* and, ATCC 25923 *(pvl),* while *S. aureus* ATCC 6538 served as the negative control.

Statistical Analysis

Descriptive statistics were presented with frequency and percentage values. Chi-square analysis was performed for proportional evaluations of *S. aureus* and *CoNS* groups. Bonferroni test was used to determine the groups with differences (a>b). P values less than 0.05 were considered significant in the study. SPSS v25 software (IBM) was used for the statistical analysis.

Results

One hundred and fifty (150) raw milk samples were included in our study. 50% were found to be contaminated with *Staphylococcus* species. *S. aureus* and *CoNS* were found 32% (24/75) and 68% (51/75), respectively *(Table 1).* We found phenotypically eight strains (10.6% - 8 out of 75) and ten strains (13.3% - 10 out of 75) identified as MRSA and *MRCoNS* respectively, but eleven strains of *CoNS* (14.6% - 11 out of 75) carried the *mec*A gene genotypically (One *S. sciuri* strain only carried mecA gene). In addition to *MRSA* and *MRCoNS*, 21.3% (16 out of 75 strains) *MSSA* and 54.6% (41 out of 75 strains) *MSCoNS* were isolated in raw milk samples.

The distribution of *CoNS isolates* was *Staphylococcus chromogenes* 33.3% (17/51), *S. sciuri* 23.5% (12/51), *S. haemolyticus* 13.7% (7/51), *S. saprophyticus* 5.9% (3/51), *S. vitilinus* 5.9% (3/51), *S. warneri* 5.9% (3/51), *S. cohnii*

Table 1. *Microbiological analysis of raw milk samples*

Results	Raw Milk Samples (n: 150)
Negative	75
Positive	75 *(S. aureus – CoNS)* (32% (24/75) – 68% (51/75))

spp. urealyticus 5.9% (3/51), *S. equorum* 3.9% (2/51) and *S. epidermidis* 1.9% (1/51).

Table 2. *The antimicrobial resistance patterns of the Staphylococcus spp. isolated from raw milk samples*

Antibiotics	***S. aureus* (n=24)**	**CoNS (n=51)**	**P****
	n (%)	**n (%)**	
Oxacillin	8 (33.3)[a]	10 (19.6)[b]	0.04*
Cefoxitin	8 (33.3)[a]	10 (19.6)[b]	
Penicillin	11 (45.8)	24 (47.9)	
Gentamicin	5 (20.8)	9 (17.6)	
Amikacin	3 (12.5)[a]	0 (0)[b]	
Tetracycline	8 (33.3)[a]	12 (23.5)[b]	
Erythromycin	5 (20.8)	13 (25.5)	
Clindamycin	4 (16.7)	12 (23.5)	
Multidrug-resistance	5 (20.8)	12 (23.5)	

*** Chi-square test was performed; * Significant difference at the 0.05 level, a>b*

Susceptibility testing was performed using seven different antibiotics for *Staphylococci* isolated from raw milk. The highest phenotypic resistance rate for *S. aureus* was detected in penicillin (45.8%) and tetracycline (33.3%), followed by gentamicin 20.8%, erythromycin 20.8%, and clindamycin 16.7%. The least resistance was observed against amikacin, with a rate of 12.5%. All of the *S. aureus* isolates were susceptible to vancomycin. The highest phenotypic resistance rate for *CoNS* was found at 47.9% for penicillin, 25.5% for erythromycin, and 23.5% for tetracycline, followed by 17.6% for gentamicin. The lowest rate of resistance was found to be against clindamycin 23.5%. All of the *CoNS* isolates were susceptible to amikacin and vancomycin. Methicillin, amikacin, and tetracycline resistance were found to be significantly higher (P<0.05) in *S. aureus* isolates compared to *CoNS* isolates, and similar resistance rates were found in other antibiotics *(Table 2)*.

Distribution of antimicrobial resistance genes; In *S. aureus* isolates were found as *blaZ* 54.2%, *aac(6')-aph(2")* 37.5%, *aph(3')-IIIa* 16.7%, *ant(4')- Ia* 8.3%, *ermA* 4.2%, *ermB* 8.3%, *ermC* 12.5%, *msrA* 20.9%, *tetK* 16.7%, *tetM* 16.7% and *vanA* 0%; In CoNS isolates were found as *blaZ* 47%, aac(6')-aph(2") 21.6 %, *aph(3')-IIIa* 3.9%, *ant(4')- Ia* 1.9%, *ermA* 13.7%, *ermB* 5.9%, *ermC* 29.4%, *msrA* 7.8%, *tetK* 9.8%, *tetM* 19.6% and *vanA* 0%.

Although two *S. aureus* strains carried the *blaZ* gene, phenotypic penicillin resistance was not observed. The *ermC* gene was detected only in *MSSA* isolates. All of the *CoNS* isolates were carrying the *msrA* genes were methicillin-resistant *(MRCoNS)*. The *msrA* gene was

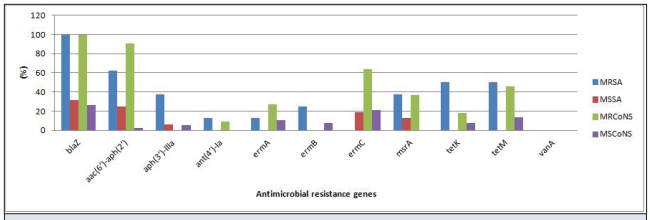

Fig 1. The distrubution of antimicrobial resistance genes between isolates

Table 3. *The distrubution of virulence genes in Staphylococcus spp. isolated from raw milk samples*

Status of Virulence Genes	***S. aureus* (n=24)**	**CoNS (n=51)**	**P****
	n (%)	**n (%)**	
tsst-1	1 (4.8)	0(0)	0,01*
Isolates carrying enterotoxin genome (SEs)	20 (83.3)[a]	11 (22.4)[b]	
Only one group of enterotoxin genome	6 (25.0)[a]	4 (8.2)[b]	
More than one group of enterotoxin genome	13 (54.2)[a]	7 (14.3)[b]	
Isolates non-carrying toxin genome	4 (16.7)[b]	40 (81.2)[a]	

*** Chi-square test was performed.* Significant difference at the 0.05 level, a>b*

significantly higher (P<0.05) in *S. aureus* isolates than in *CoNS* isolates. In addition, the *ermC* gene was significantly higher in *CoNS* isolates compared to *S. aureus* isolates (P<0.05). All phenotypic gentamicin-resistant isolates carried the *aph(3')-IIIa* gene. Although the two strains carried the *tetK* and *tetM* genes, they did not show phenotypic tetracycline resistance *(Fig. 1)*. In our study, a total of 5 (20.8%) isolates in *S. aureus* and a total of 12 (25.3%) isolates in *CoNS* were classified as MDR. *S. sciuri* (28.6%) was the most common species amongst the MDR *CoNS*.

The presence of at least one toxin-gene was found in 83.3% and 22.4% of the isolates for *S. aureus* and *CoNS* respectively. *S. aureus* isolates were carried a significantly higher proportion of toxin genes than *CoNS* (P<0.01) *(Table 3)*.

The sei gene was the most prevalent, and it was identified in 21.3% of all *Staphylococcus* isolates. When the distribution

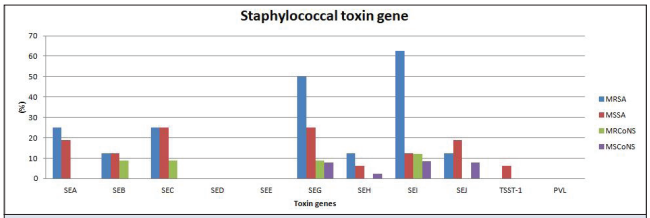

Fig 2. The distrubution of staphylococcal toxin genes between isolates

Table 4. The distrubution of SCCmec type, virulence genes, phenotypical resistance patterns and antimicrobial resistance genes in MRSA (8 strains)

SCCmec	SEs Genes	Phenotypical Resistance	Antimicrobial Resistance Genes
IV	seb+seh+sei	P+M+G+T+E	mecA+blaZ+aac(6')/aph(2") +ant(4')-Ia+ermA+tetK
IV	seg+sei	P+M+A+T+C	mecA+blaZ+aac(6')/aph(2")+msrA+tetK+tetM
IV	sea+seg+sei	P+M+E+T+C	mecA+blaZ+ aac(6')/aph(2")+ermB+msrA+tetK
IV	sea+seg+sei	P+M+A	mecA+blaZ+aac(6')/aph(2")+tetM
III	seg	P+M+T+E+C	mecA+blaZ+aph(3')-IIIa+ermB+tetM
II	ND	P+M+G	mecA+blaZ+aac(6')/aph(2") +ant(4')-Ia+tetK
I	sec+sei	P+M+G+T	mecA+blaZ+aph(3')-IIIa+tetM
NT	sec+sej	P+M	mecA+blaZ+aph(3')-IIIa+msr

P: penicillin; M: methicillin; G: gentamicin; A: amikacin; T: Tetracycline; E: erythromycin; V: vancomycin; MDR: multi drug resistant; NT: Nontypeable; ND: Not Determined SEs gene

Table 5. The distrubution of SCCmec type, virulence genes, phenotypical resistance patterns and antimicrobial resistance genes in MRCoNS (11 strains)

Species	SCCmec	SEs Genes	Phenotypical Resistance	Antimicrobial Resistance Genes
S. chromogenes	IV	seg + sei	P+M+G	mecA + blaz + aac(6')/aph(2")+ermC
S. epidermidis	IV	sei	P+M+G+C	mecA + blaZ + aac(6')/aph(2")+ermC+ tetM
S. haemolyticus	IV	seg +sei	P+M+G+C+T	mecA+blaZ+aac(6')/aph(2")+ermA+msrA+tetK
S. sciuri	IV	ND	P+M+G+E+C	mecA+blaZ+aac(6')/aph(2")+ermC+msrA+tetM
S. sciuri	IV	ND	P	mecA+blaZ+aac(6')/aph(2")
S. warneri	IV	ND	P+M+G+E+T+C	mecA+blaZ+aac(6')/aph(2")+ermC+msrA+tetM
S. chromogenes	II	ND	P+M+G+T	mecA+blaZ+aac(6')/aph(2")+ant(4')-Ia+ermA+tetK
S. haemolyticus	II	sec	P+M+G+T	mecA+blaZ+aac(6')/aph(2")+tetM
S. sciuri	II	ND	P+M+E	mecA+blaZ+ermC+tetK
S. saprophyticus	NT	seb + sej	P+M+G+E+T+C	mecA+blaZ+aac(6')/aph(2")+ermC+msrA+tetM
S. vitilinus	NT	ND	P+M+E+C	mecA+blaZ+aac(6')/aph(2")+ ermA+ermC

P: penicillin; M: methicillin; G: gentamicin; A: amikacin; T: Tetracycline; E: erythromycin; V: vancomycin; MDR: multi drug resistant; NT: Nontypeable; ND: Not Determined SEs gene

of toxin genes in *S. aureus* isolates was examined; the *seg* gene was the most frequent, which was detected in 33.3%, followed by *sei* in 29.2%, *sec* in 25%, *sea* in 20%, *sej* in 16.7%, *seb* in 12.5%, *seh* in 8.3% and *tsst*-1 in 4.6%. The *sed, see,* and *pvl* genes were not detected in any of the *S. aureus* isolates *(Fig. 2).* All MRSA isolates and 75% of MSSA isolates carried the toxin gene (P<0.05) and only one MSSA isolate harbored the *tsst-1* virulence gene. Distribution of toxin genes in CoNS strains; the *sei* gene was the most frequent, which was detected in 18.4% of the *CoNS* isolates, followed by *seg* in 8.2%, *sej* in 6%, *seb* in 2%, *sec* in 2% and, *seh* in 2%. The *sea, sed, see, tsst-1,* and *pvl* genes were not detected in any of the *CoNS* isolates. 45.5% of *MRCoNS* isolates and 18.4% of *MSCoNS* isolates were carried the *SEs* toxin gene (P<0.05).

SCCmec type distribution of *MRSA* isolates in our study; *type IV* (50%), *type I* (12.5%), *type II* (12.5%) and, *type III* (12.5%), *SCCmec type* of one isolate (12.5%) could not be determined (NT). *SCCmec type* distribution of *MRCoNS* isolates were determined as *type IV* (54.5%), and *type II* (27.3%), whereas *SCCmec* type of two isolates (18.2%) could not be determined *(NT). SCCmec type IV* was predominant in both *MRSA* and *MRCoNS* isolates. *SCCmec* type distribution of MRSA and MRCoNS strains are shown in *Table 4* and *Table 5* respectively.

Discussion

Although many studies with raw milk have focused on mainly *S. aureus* (especially MRSA), several studies have begun to highlight the importance of *CoNS* [2,6]. Therefore, in our study, we focused on determining both *S. aureus* and CoNS strains epidemiologically in raw milk samples. The contamination of raw milk by *S. aureus*, particularly harboring multiple antimicrobial resistance genes and carrying virulence genes such as enterotoxin, *tsst-1*, and *pvl*, continues to be a significant public health concern [10-12]. Incidents of outbreaks linked to the consumption of milk and dairy products contaminated with *S. aureus* have been reported in various locations [13]. When reviewing the studies conducted to ascertain the presence of *S. aureus* in

raw milk in Turkiye, the presence of *S. aureus* in the analyzed milk samples were observed to be 61.1% [14], 75% [15], which was quite higher than our results. Whereas, Can et al.[16] detected *S. aureus* in only 12.5% of the milk samples. When examining studies conducted in other countries; It has been reported that *S. aureus* was isolated from raw milk at a rate of 71% in Sweden [17], 46.2% in China [18], and 44% [19] in Egypt. The variation in the frequency of *S. aureus* recovered from milk and its products in different studies may be attributed to differences in sample sizes, origins, and geographic locations. It could reflect the effectiveness of applicable sanitary measures.

CoNS have been traditionally considered only as contaminants or minor pathogens. Nevertheless, their significance has grown as they have become the most frequently isolated group of species from bovine milk in numerous locations [2,19]. In this study, a high diversity of *Staphylococcus* spp among the *CoNS* emerged. Like our result amongst the *CoNS, S. chromogenes* was one of the most prevalent species in previous studies reported ranging between 23.3% and 78.8% of *CoNS* isolates [20-22]. *S. sciuri* is frequently isolated from the skin of humans and animals [22], but in addition to causing bovine mastitis, reported as an opportunistic pathogen [23]. In Australia, *CoNS* was isolated from milk samples, and the dominant strain was reported to be *S. sciuri* [24]. The monitoring of the methicillin resistance trait holds significance for public health and veterinary medicine [17,25,26].

The *mecA* gene conferring methicillin resistance is not only found in *S. aureus* but may be present in other staphylococcal species as well [3]. Limited data are available regarding the prevalence and molecular characterization of *MRCoNS* isolated from food. Some *MRCoNS* are now raising growing concerns in both human and veterinary medicine. A study conducted in Tunisia revealed that 29.4% of *CoNS* isolated from milk exhibited resistance to oxacillin, while 20.6% carried the *mecA* gene [2]. Methicillin resistance among CoNS was comparable to the rates reported in Poland (20%) [27] and China (17.1%) [28]. Khazandi et al.[24] indicated that *S. sciuri* and other *MRCoNS* could serve as a reservoir for gene cassettes containing *mecA* or *mecA* homologs in dairy cattle. Additionally, they may carry additional antimicrobial resistance genes, creating the potential for bidirectional transmission between humans and dairy cattle.

SCCmec is a molecular technique utilized to comprehend the epidemiology and clonal relationships of MRSA strains, particularly in the global occurrence of community-acquired MRSA infections [29]. While *SCCmec* types *I, II,* and *III* were predominant in earlier years, *SCCmec* type IV was initially identified in 2002 in two distinctive MRSA strains and has since become one of the most frequently isolated *SCCmec* types [3]. In the largest epidemiological survey in Türkiye, encompassing 397 MRSA strains collected from 12 hospitals across 11 cities in different geographical regions between 2006 and 2008, the study identified the presence of a hospital Turkish clone *SCCmec III* and a community clone *SCCmec IV* extensively disseminated in Türkiye [30]. Consistent with our findings, other studies conducted in Turkey have reported the dominance of *SCCmec* type IV and *SCCmec* type III clones [31-33].

Antibiotic-resistant staphylococci, which occur as a result of incorrect and unnecessary use of antibiotics in animals, can be transmitted with milk, thus spreading antibiotic-resistance genes, which constitutes an important public health problem [11,34].

When we examined the studies that conducted antimicrobial resistance of *S. aureus* in raw milk in Türkiye, it was seen that antimicrobial resistance is generally higher than our results [16,32,33]. In a study conducted in Switzerland, it was reported that antibiotic resistance rates were found to be quite low in *S. aureus* strains isolated from milk, as in our study [35]. In addition to antimicrobial resistance, the pathogenicity of *Staphylococcus* spp. is closely linked to their ability to produce staphylococcal enterotoxins and staphylococcal enterotoxin-like substances [36]. The presence of *SE* genes in *Staphylococcus* isolates from various food and milk products plays a significant and emerging role. These genes have been identified through screening in numerous surveillance studies, highlighting their potential as a serious public health hazard [5,13,37]. The presence of *SEs* genes were detected in 52.9% of MSSA strains isolated from raw milk in Northern Germany, and the most frequently occurring genes were *seg* and *sei* [38]. Studies showed that more than one *SE* gene encoding different enterotoxins can be found in *S. aureus* [5,37]. For a considerable period, *S. aureus* was regarded as the sole enterotoxigenic species within the genus, while *CoNS* were primarily categorized as contaminants. This classification persisted due to historical controversy surrounding the potential enterotoxigenicity of *CoNS* isolates [37]. Nevertheless, some studies have demonstrated that *CoNS* isolates can indeed harbor enterotoxin genes and, in certain instances, produce toxins at concentrations clinically significant [37,38].

The carriage of *tsst*-1 virulence genes by mobile genetic elements is important in terms of transferring them to people consuming milk and dairy products containing *tsst*-1 positive *S. aureus* [18]. Studies have shown that high *tsst-1* gene carrier rates range from 26.2% to 58.5% [39,40]. As with other virulence genes, the rates of *tsst*-1 gene in *S. aureus* isolates vary according to different geographical regions.

Although our study was not associated with fermented

foods, some *CoNS* species such as *S. carnosus, S. condimenti, S. equorum, S. piscifermentans, S. succinus,* and *S. xylosus* have been associated with fermented foods and have been proposed as very rare opportunistic pathogens [41]. These food-associated CoNS species were also not detected in our study. The CoNS species detected in our study were those considered to be pathogenic, similar to those in other studies [41].

In conclusion, the presence of different *Staphlococcus* species were found in unpackaged raw milk collected in Istanbul. It has been understood that attention should be paid to *MRCoNS* as well as *MRSA*. Most *MRSA* and *MRCoNS* bacteria harbored *SCCmec* type IV which may have assumed their possible community-acquired origins. *S. chromogenes* was the most prevalent species among *CoNS*. *CoNS* isolates also carried various virulence genes. *S. aureus* and *CoNS* isolated from raw milk should be considered a public health problem with both the transmission of resistant pathogens and the horizontal transmission of the carrying genes. In addition to routine control of bacteria in raw milk, these raw milk should be boiled before consumption.

Declarations

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Author Contributions: MMT, MD, SD, İK, AÖ, DP, DSE, BG, SA, OCA, SE and DD conceived and executed the idea, designed experiments, analyzed results and a deep revision of the manuscript. MMT, MD, SD, İK, AÖ, DP, DSE, BG, SA, OCA collected samples, performed experiments, contributed to tand implementation of the research. All authors listed have made a substantial, direct and intellectual contribution to the work and approved it for publication.

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