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*Original article*

# Prevalence, antibiotic resistance, enterotoxin genes, biofilm formation, and agr typing of *Staphylococcus aureus* from raw milk and cheese

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## Abstract

In this study, *Staphylococcus aureus* was detected in 46% of raw milk and 10% of cheese samples collected in Edirne, Türkiye. All isolates carried the sec and seg enterotoxin genes, while 39% harbored sed, 7% seh, and only 4% of isolates carried either sea or sei. A total of 25% of the isolates exhibited multidrug resistance. The highest resistance rate was observed against penicillin (39%), followed by kanamycin (18%), tetracycline (14%), clindamycin (11%), chloramphenicol and rifampin (7%), and trimethoprim-sulfamethoxazole and gentamicin (4%). Methicillin resistance was found in 11%, and mecA was identified in two isolates. All isolates formed biofilms at 22°C and 37°C, and 82% also at 4°C. Agr typing showed that 21% of isolates belonged to group I, 21% to group II, and 11% to group III, while no group IV isolates were detected. These findings demonstrate that enterotoxigenic, antimicrobial-resistant, and biofilm-forming *S. aureus* isolates from dairy products may persist along the food chain and represent a potential public health risk, underscoring the importance of continuous microbiological monitoring and preventive strategies within a One Health framework.

**Keywords:** *Staphylococcus aureus*, antibiotic resistance, biofilm, enterotoxin, agr typing

## Introduction

Milk and dairy products have held an important place in the nutritional chain for thousands of years. In recent years, there has been a global increase in the consumption of raw milk and cheese, largely due to increasing consumer demand for foods that are minimally processed, sustainable, nutritious, and locally sourced. In this context, foodborne pathogens have emerged as a significant food safety concern (Fusco et al. 2020). Although raw milk contains minimal bacterial contamination at the time of extraction from the animal's udder, it can become contaminated through various routes. These include environmental sources, the animal itself, and contact with contaminated equipment. *Staphylococcus aureus* is a key cause of mastitis in dairy cattle and is often found as a contaminant in raw milk and related dairy products (Martin et al. 2023).

*Staphylococcus aureus* possesses numerous toxin-encoding genes. Among its numerous virulence factors, staphylococcal superantigens stand out due to their strong association with specific clinical symptoms and diseases. Although superantigens share structural and functional features, each possesses unique biological characteristics. These toxins contribute to serious clinical outcomes and are frequently associated with the ingestion of food contaminated with *S. aureus* enterotoxins (Azimirad et al. 2017). Initially, five SE serotypes (sea, seb, sec, sed, and see) that are antigenically distinct were identified. Through recombinant DNA techniques, four additional types (seg, seh, sei, and sej) have been characterized. While all SEs from sea to sei have emetic potential, food poisoning outbreaks are mainly attributed to a few serotypes, notably sea, seb, sec, and sed. Among them, sea is the most prevalent, and both seb and sec are also closely associated with toxic shock syndrome (Liu 2015). Enterotoxigenic *S. aureus* remains a major concern for hygienic status of milk and dairy products with respect to microbial risks, as it is responsible for numerous staphylococcal food poisoning incidents.

The threat of antimicrobial resistance persists as a major global public health issue. The emergence of resistant bacterial pathogens has been closely linked to the extensive therapeutic application of antimicrobials, as well as their use as growth promoters in food-producing animals. Among the most alarming threats is the spread of methicillin-resistant *Staphylococcus aureus* (MRSA), poses a serious threat due to its ability to cause infections in healthcare and community environments (Fusco et al. 2020). Multiple *S. aureus* clones have evolved into MRSA strains through the horizontal acquisition of the staphylococcal cassette chromosome mec (SCCmec). This mobile genetic element carries the mecA or mecC genes, which confer resistance to methicillin and consequently render most β-lactam antibiotics ineffective. In addition to β-lactam resistance, MRSA strains frequently exhibit resistance to several other antibiotic categories. Notably, *S. aureus* possesses an extraordinary capacity to develop resistance to nearly all available antibiotics, significantly complicating both current and prospective treatment strategies (Turner et al. 2018).

The formation of biofilms in the dairy industry can lead to economic losses due to reduced product quality and performance, spoilage, food safety concerns, and challenges in effectively cleaning machinery and equipment. Bacteria within biofilms are known to be more resistant (up to 1000 times) to cleaning agents than their planktonic forms (Shi et al. 2009). Microbial biofilms on surfaces in food processing facilities not only compromise the quality and safety of finished products but, more importantly, pose a risk to consumer health if they harbor pathogenic bacteria (Kukhtyn et al. 2017). A major challenge in the dairy industry is minimizing contamination levels in milk and its derivatives to ensure product and consumer safety.

This study investigates the presence of *S. aureus* in raw milk and cheese and comprehensively evaluates the virulence potential of the isolates through antibiotic resistance profiling, agr typing, detection of staphylococcal enterotoxin genes, and assessment of biofilm-forming capacity.

## Materials and Methods

### Sample collection

Raw milk and cheese samples marketed in the city center of Edirne, Türkiye, were collected over a five-month period from October 2024 to February 2025. Edirne is a border city located at the intersection of Türkiye, Bulgaria and Greece. Each sample was aseptically delivered to the laboratory at 4°C and subjected to analysis within 24 hours. The study analyzed 100 samples in total, consisting of 50 raw milk and 50 cheese samples. Milk samples were taken from two supply routes: milk vendors (19) (milkmen distributing raw milk directly from producers) and local vendors (31) (delicatessens offering milk for retail sale). Cheese samples were also collected from delicatessens and local vendors, which were sold unpackaged. In total, six different types of cheese were analyzed: white cheese (13), fresh cheese (10), string cheese (9), curd cheese (6), kashar cheese (5), and tulum cheese (7) (Table 1). Each milk and cheese sample was included in the analysis only once, and no duplicate samples were collected from the same source.

Table 1. Source and strain codes of *Staphylococcus aureus* isolates, M: raw milk isolates; C: cheese isolates.

| Sample Type | Product | Strain code |
|---|---|---|
| Raw milk | Milk vendors | M10, M31, M43, M45 |
| Raw milk | Local vendors | M2, M4, M16, M17, M20, M21, M25, M26, M27, M30, M33, M34, M36, M37, M38, M39, M40, M41, M49 |
| Cheese | Fresh cheese | C6, C7, C22, |
| Cheese | String cheese | C34, |
| Cheese | Curd cheeese | C5 |

### Isolation of *S. aureus*

Aseptically, 10 grams of cheese or 10 milliliters of raw milk were transferred into 90 ml of Giolitti and Cantoni Broth (Sigma-Aldrich), homogenized using a stomacher (Isolab) for 2 minutes. After incubation at 37°C for 24 hours (h), each culture was streaked on Baird-Parker Agar (Sigma - Aldrich) supplemented with egg yolk (Sigma - Aldrich) and telluride (Sigma - Aldrich), and incubated at 37°C for 24-48 h. Black-pigmented colonies showing clear zones were tested for Gram reaction and catalase activity (ISO 6888-3:2003).

### *S. aureus* confirmation and Staphylococcal enterotoxin (SE) detection

The GeneJet Genomic DNA Purification Kit (Thermo Scientific) was employed for the isolation of genomic DNA.

Multiplex PCR was used for the molecular confirmation of *S. aureus* isolates and for the detection of staphylococcal enterotoxin genes (Løseth et al. 2004). Primers targeting the *S. aureus*-specific 16S rRNA gene were also used as an internal control for multiplex PCR. The PCR mixture (50 µL) was prepared to contain 5 µL of template DNA, 5 µL of 1X reaction buffer, 0.4 µL of Taq DNA polymerase (2 U), 10 µL of dNTP mix (400 µM), 8 µL of $MgCl_2$ (4 mM), 2 µL of SE primer mix (300 nM), and 16S rRNA primer mix (60 nM). To detect the SE genes, two separate multiplex PCR reaction mixtures were prepared. Mix 1 included primers for sea, seb-sec, sec, seh, sej, and 16S rRNA, and mix 2 included primers for sed, see, seg, sei, and 16S rRNA. Agarose gel (2%) was used to separate of PCR products. 100 bp DNA marker (Hydra Biotechnology) was employed to calculate the sizes of the PCR bands. The primers spesific for SE genes and PCR conditions are given in Table 2.

### Antimicrobial resistance

The antibiotic resistance of *S. aureus* isolates was determined using the disk diffusion method in accordance with the Clinical and Laboratory Standards Institute guidelines (2018). A total of eleven antibiotics were tested, including erythromycin (E, 15 µg), ciprofloxacin (CIP, 5 µg), chloramphenicol (C, 30 µg), clindamycin (DA, 2 µg), gentamicin (CN, 10 µg), kanamycin (K, 30 µg), penicillin G (P, 10 U), tetracycline (TE, 30 µg), trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 µg), and rifampin (RA, 5 µg). To determine MRSA isolates, a 30 µg cefoxitin (FOX) disk was utilized.

### Multiple antibiotic resistance (MAR) index

The MAR index was determined by dividing the number of antibiotics to which an isolate was resistant (a) by the total number of antibiotics tested in the study (b) [MAR = a/b].

### Biofilm Formation

### Microtiter plate assay

The biofilm-forming ability of *S. aureus* isolates was evaluated using the microtiter plate assay described by Stepanovic et al. (2004), with minor modifications. Overnight cultures were adjusted to an optical density of 0.2 (OD590) and inoculated into microplate wells containing appropriate broth. Wells containing sterile broth served as negative controls. Plates were incubated statically at 4°C, 22°C, and 37°C for 24h. Following incubation, the wells were gently washed to remove non-adherent cells, fixed with methanol, and stained with 0.1% crystal violet. After removal of excess stain, the bound dye was solubilized using 33% acetic acid, and optical density was measured at 590 nm using a Multiskan EX microplate reader (Bio-Rad). Each isolate was tested in triplicate, and the mean OD value was used for analysis. The cut-off OD value (ODc) was defined as the mean OD of negative control wells. Based on the criteria of Stepanovic et al. (2000), isolates were categorized as follows: $OD \leq ODc$ = non-biofilm producer, $ODc < OD \leq 2\times ODc$ = weak biofilm producer, $2\times ODc < OD \leq 4\times ODc$ = moderate biofilm producer, $4\times ODc < OD$ = strong biofilm producer.

### Congo red agar morphology

Slime production of *S. aureus* isolates was phenotypically assessed using Congo Red Agar (CRA) (50 g/L sucrose, 37 g/L BHI broth, and 0.8 g/L Congo

Table 2. Primer sequences and characteristics.

| Primers | Primer sequence (5'-3') | Product size (bp) | PCR Conditions | References |
|---|---|---|---|---|
| sea | GCAGGGAACAGCTTTAGGC<br>GTTCTGTAGAAGTATGAAACACG | 521 | 94°C-10 min, 31 cycles of 94°C-1 min, 60°C-1 min, 72°C-1 min and 72°C-10 min. | Løseth et al. 2004 |
| seb–sec | ACATGTAAT TTTGATATTCGCACTG<br>TGCAGGCATCATATCATACCAA | 667 | | |
| sec | CTTGTATGTATGGAGGAATAACAA<br>TGCAGGCATCATATCATACCAA | 284 | | |
| sed | GTGGTGAAATAGATAGGACTGC<br>ATATGAAGGTGCTCTGTGG | 385 | | |
| see | TACCAATTAACTTGTGGATAGAC<br>CTCTTTGCACCTTACCGC | 171 | | |
| seg | CGTCTCCACCTGTTGAAGG<br>CCAAGTGATTGTCTATTGTCG | 328 | | |
| seh | CAACTGCTGATTTAGCTCAG<br>GTCGAATGAGTAATCTCTAGG | 359 | | |
| sei | CAACTCGAATTTTCAACAGGTACC<br>CAGGCAGTCCATCTCCTG | 466 | | |
| sej | CATCAGAACTGTTGTTCCGCTAG<br>CTGAATTTTACCATCAAAGGTAC | 142 | | |
| 16S rRNA | GTAGGTGGCAAGCGTTATCC<br>CGCACATCAGCGTCAG | 228 | | |
| mecA | GTAGAAATGACTGAACGTCCGATAA<br>CCAATTCCACATTGTTTCGGTCTAA | 310 | 94°C-10 min; 30 cycles of 94°C-30 sec, 60°C-30 sec, and 72°C-1 min, and 72°C-10 min. | Ryffel et al. 1990 |
| pan-agr | ATGCACATGGTGCACATGC | | | |
| agrI | GTCACAAGTACTATAAGCTGCGAT | 440 | 94°C-10 min; 25 cycles of 94°C-1 min, 59°C for agrI and agrII or 58°C for agr III or 57°C for agr IV for 1 min, and 72°C-1 min; and 72°C-10 min. | Shopsin et al. 2003 |
| agrII | GTATTACTAATTGAAAAGTGCCATAGC | 572 | | |
| agarIII | CTGTTGAAAAAGTCAACTAAAAGCTC | 406 | | |
| agrIV | CGATAATGCCGTAATAC CCG | 588 | | |

red dye) according to Freeman et al. (1989). Bacterial cultures were streaked onto CRA plates and incubated at 37°C for 24 h. Colonies exhibiting a black or dark bordeaux coloration with a rough, dry, or crystalline morphology were interpreted as slime-positive. In contrast, colonies showing a smooth, shiny surface with a red to bordeaux color were classified as slime-negative.

### Detection of mecA gene

The PCR mixture (50 µL) was prepared to contain 5 µL of template DNA, 5 µL of 1X reaction buffer, 0.25 µL of Taq DNA polymerase (1.25 U), 4 µL of $MgCl_2$, (2 Mm), 2 µL of dNTP mix (0.2 Mm), 1 µL of primers (each). Agarose gel (1%) was used to separate PCR products. 100 bp DNA marker (Hydra Biotechnology) was employed to calculate the sizes of the PCR bands. The primers specific for mecA and PCR conditions are given in Table 2.

### Agr typing

The agr typing of the isolates was conducted following the protocol described by Shopsin et al. (2003). The pan-agr primer, targeting conserved regions of the agrB gene, was included in all reactions. The PCR mixture (50 µL) was prepared to contain 5 µL of template DNA, 5 µL of 1X reaction buffer, 0.25 µL of Taq DNA polymerase (1.25 U), 4 µL of $MgCl_2$ (2 Mm), 2 µL of dNTP mix (0.2 Mm), 1µL of primers (each). The primers specific for agr genes and PCR conditions are given in Table 2. Agarose gel (1%) was used to separate of PCR products. 100 bp DNA marker (Hydra Biotechnology) was employed to calculate the sizes of the PCR bands.

### Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). One-way ANOVA was used to compare the

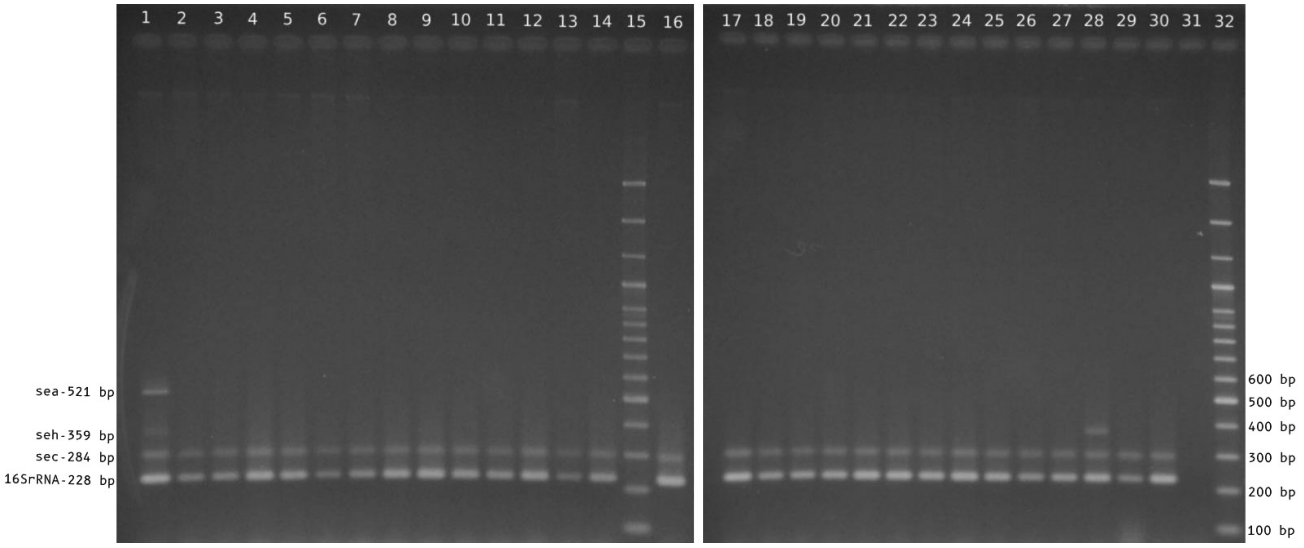


Fig. 1. Agarose gel image of multiplex PCR reaction 1 of *S. aureus* strains. 1-14: M2-M34, 15:100 bp DNA Marker, 16: *S. aureus* ATCC29213, 17-30: M36-C34, 31: negative control, 32:100 bp DNA Marker.

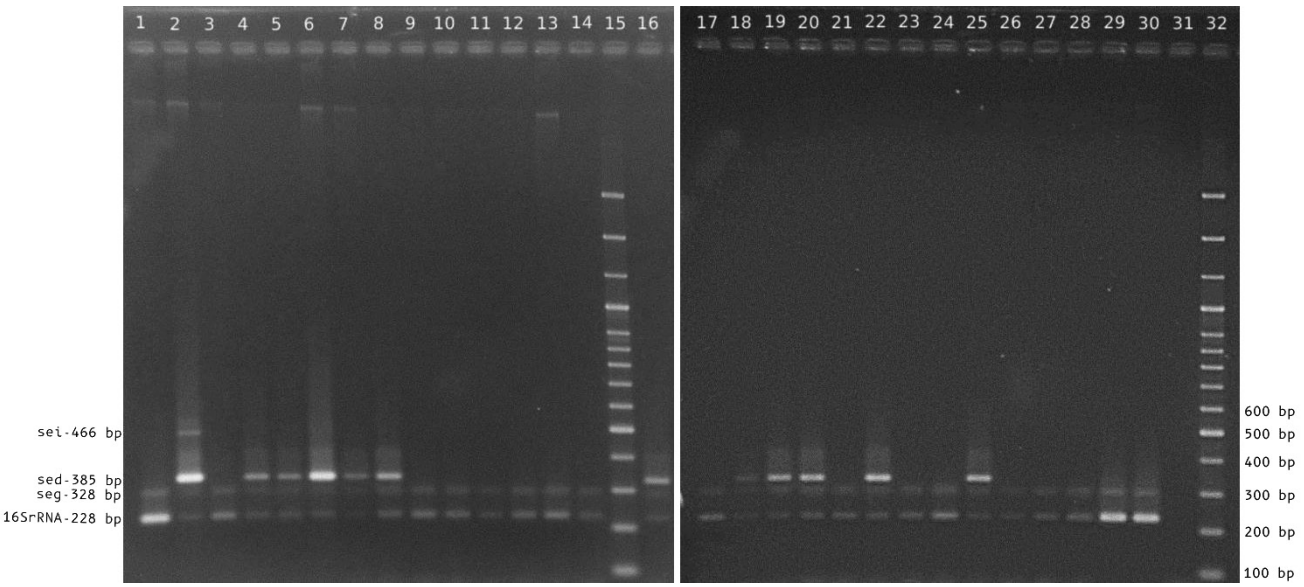


Fig. 2. Agarose gel image of multiplex PCR reaction 2 of *S. aureus* strains. 1-14: M2-M34, 15:100 bp DNA Marker, 16: *S. aureus* ATCC29213, 17-30: M36-C34, 31: negative control, 32:100 bp DNA Marker.

biofilm formation abilities of the isolates at different temperatures ($p<0.05$). Welch's t-test was used to calculate the significance of the differences between the biofilm formation abilities of the isolates from different sources ($p<0.05$).

## Results

### *S. aureus* in dairy products

*S. aureus* was present in 28% of the samples collected from raw milk and cheese (Table 1). From each *S. aureus* positive sample, only a single isolate was selected for inclusion. Among raw milk samples only, the prevalence was 46%. All 28 *S. aureus* isolates (100%) were found to carry the species-specific 16S rRNA gene region (Fig. 1, 2).

### Antibiotic resistance

The resistance profiles of *S. aureus* strains against a total of 11 antibiotics were determined. At least one strain exhibited resistance to each antibiotic tested, except for ciprofloxacin. A total of 19 isolates (67%) were resistant to at least one of the antibiotics used (Fig. 3).

Moreover, 25% of the isolates (7/28) showed multidrug resistance (MDR), with MAR index values above 0.2. The MAR index values for *S. aureus* isolates varied between 0.09 and 0.36 (Table 3).

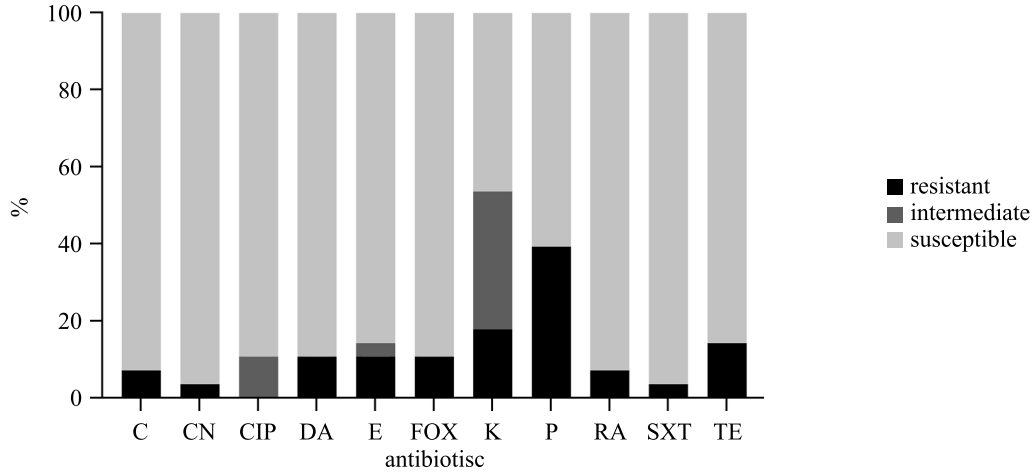


Fig. 3. Antimicrobial resistance of *S. aureus* isolates.

Table 3. Antibiotic resistance patterns and multiple antibiotic resistance (MAR) indexes of *S. aureus* isolates.

| Antibiotic resistance pattern | MAR index | Strain code |
|---|---|---|
| RA, P, DA, FOX | 0.36 | M33 |
| SXT, TE, DA, E | 0.36 | M32 |
| P, E, FOX | 0.27 | M34, M36 |
| P, TE, K | 0.27 | M2, M10 |
| RA, P, DA | 0.27 | M4, |
| P, K | 0.18 | M20 |
| P, C | 0.18 | C5 |
| CN, K | 0.18 | C6 |
| P, TE | 0.18 | C22 |
| K | 0.09 | M21, M37, M38, M39, M49 |
| P | 0.09 | M43, C34 |
| C | 0.09 | M27 |

Penicillin showed the highest resistance rate with 39%, followed by kanamycin (18%), tetracycline (14%), clindamycin (11%), chloramphenicol and rifampin (7% each), and trimethoprim-sulfamethoxazole and gentamicin (4% each). Additionally, the highest rate of intermediate resistance was detected against kanamycin (36%). Among the isolates, strains exhibiting intermediate resistance to ciprofloxacin (7%) and erythromycin (4%) were also identified.

The methicillin resistance rate was found to be 11% (3/28). MRSA isolates were recovered from raw milk samples sold in local vendors, and all three exhibited multidrug resistances. Two of the MRSA isolates (M34 and M36) had a MAR index of 0.27, while the third isolate (M33) had a MAR index of 0.36. MRSA isolates exhibited two distinct antibiotic resistance profiles. One MRSA strain was resistant to rifampin, penicillin, and clindamycin, and exhibited intermediate resistance to ciprofloxacin. The other two MRSA isolates were resistant to erythromycin and penicillin, and showed intermediate resistance to kanamycin. Only two of the MRSA isolates (M34 and M36) harboured mecA gene (Fig. 4).

The antibiotic resistance profiles of *S. aureus* isolates showed considerable diversity. Twelve different resistance patterns were detected in the 19 isolates (Table 3).

## Toxin gene profile

All isolates (100%) were found to carry the sec and seg enterotoxin genes. The second most frequently observed enterotoxin gene was sed, which was identified in 11 isolates (39%). Only two isolates (7%) harboured the seh gene, and one isolate each (4%) carried the sea and sei genes (Fig. 1, 2). Five different SE gene patterns were identified among the *S. aureus* isolates (Table 4).

## Biofilm formation

At both 22°C and 37°C, all isolates (100%) were capable of forming biofilms. At 22°C, 43% of the iso-

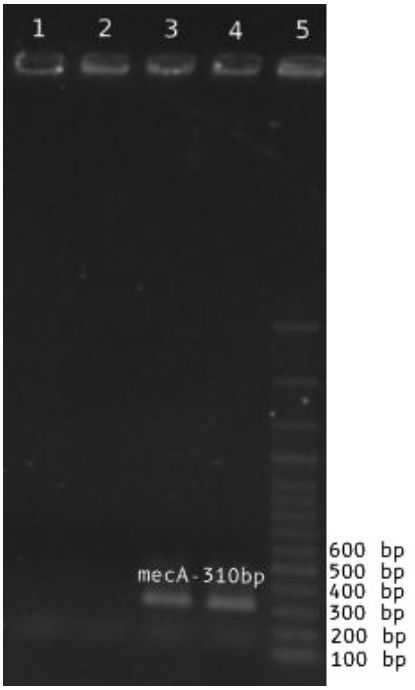


Fig. 4. Agarose Gel Image of mecA gene of *S. aureus* strains. 1: negative control, 2: *S. aureus* ATCC29213 (mecA negative conrol), 3: M34, 4: M36, 5: 100 bp DNA Marker.

Table 4. Staphylococcal enterotoxin gene patterns of *S. aureus* isolates.

| Enterotoxin gene patterns | Strain code |
|---|---|
| sea, sec, sed, seg, seh | M2 |
| sec, sed, seg, sei | M4 |
| sec, sed, seg, | M16, M17, M20, M21, M25, M37, M38, M39, M41, M49, |
| sec, seg, seh | C7 |
| sec, seg | M10, M26, M27, M30, M32, M33, M34, M36, M40, M43, M45, C5, C6, C22, C34 |

lates (12/28) formed weak biofilms, 39% (11/28) formed strong biofilms, and 18% (5/28) formed moderate biofilms. At 37°C, 86% of the isolates (24/28) formed strong biofilms, while 7% formed moderate and 7% formed weak biofilms. At 4°C, 82% of the isolates (23/28) formed weak biofilms, and 18% (5/28) did not form biofilms (Fig. 5).

Biofilm assays at 4°C, 22°C, and 37°C showed significantly different mean OD590 values, indicating temperature-dependent variation in biofilm formation capacity ($p<0.05$, one-way ANOVA). The highest biofilm formation capacity was recorded at 37°C. The lowest OD590 value (0.122±0.002) was obtained at 4°C, while the highest OD590 value (5.993±0.012) was observed at 37°C.

Comparison of OD590 values between raw milk and cheese isolates at 4°C, 22°C, and 37°C, revealed no significant differences in their biofilm formation capacity (Welch's t-test, $p<0.05$).

No significant difference in biofilm formation capacity at 4°C was detected when OD590 values of *S. aureus* strains from raw milk samples taken from local and milk vendors were compared at all three temperatures. However, significant differences were observed at 22°C and 37°C (Welch's t-test, $p<0.05$) (Fig. 6). Higher biofilm-forming capacity was observed in raw milk isolates sourced from local vendors compared to those obtained from milk vendors.

Slime production of *S. aureus* isolates was phenotypically evaluated using CRA. Based on CRA morphology, 71% of the isolates (20/28) were classified as positive, while 29% (8/28) were classified as negative.

## Agr typing

Among the *S. aureus* isolates, 21% (6/28) were classified into the agr group I, 21% (6/28) to agr group II, and 11% (3/28) to agr group III, while 47% of the isolates were determined as agr-negative (Fig. 7).

Of the isolates classified in agr group I, two (M34 and M36) were obtained from raw milk samples collected from local vendors, one (M43) from a raw milk sample obtained from an milk vendors, and three (C5, C6, C22) from cheese samples. All isolates in agr

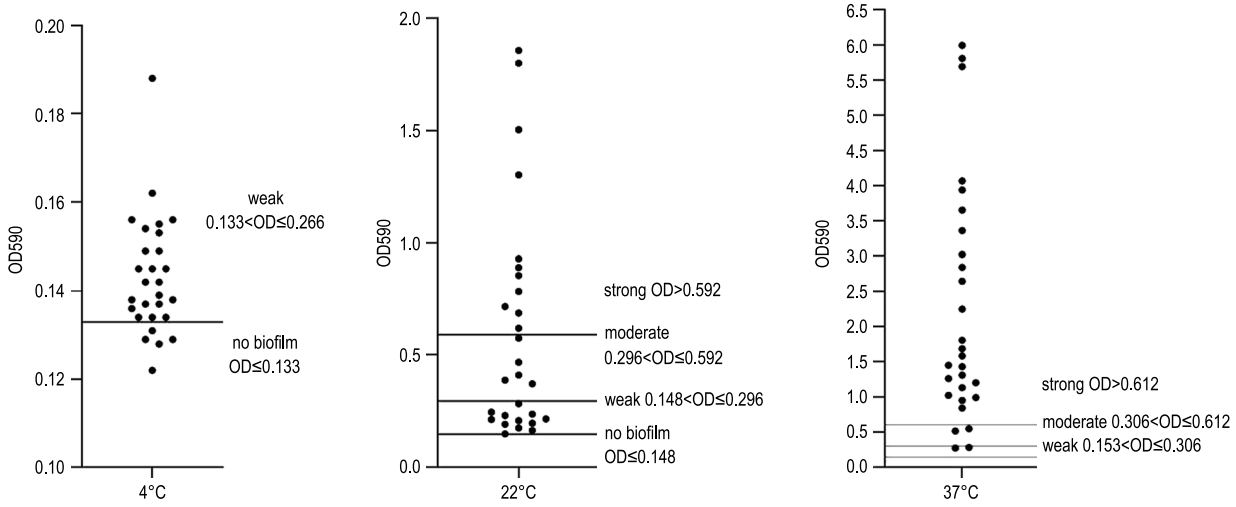


Fig. 5. Biofilm forming abilities of *S. aureus* isolates.

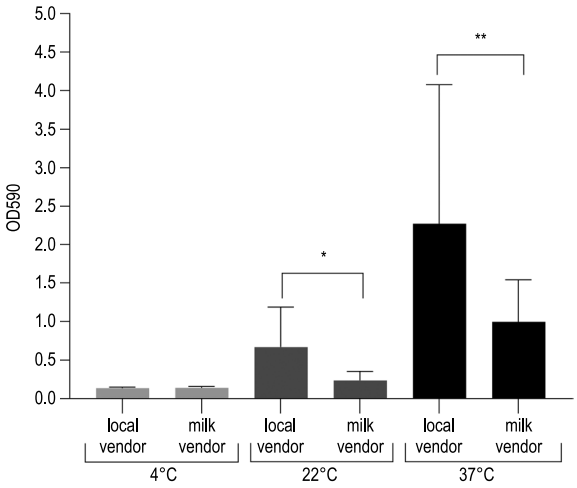


Fig. 6. Comparison of biofilm formation capacities (mean of OD590 values ± standard deviation) of *S. aureus* raw milk isolates obtained from local vendors and milk vendors at 4°C, 22°C, and 37°C. * and ** indicates the significant differences between biofilm formation of isolates from local vendors and milk vendors ($p<0.05$).

group II originated from raw milk samples taken from local vendors. The agr group III isolates were obtained from local vendor (M2), a milk vendor (M10), and a cheese sample (C7). Both of the mecA-positive isolates were classified into agr group I. In contrast, the isolate (M33) that was classified as methicillin-resistant through the disk diffusion test but did not carry the mecA gene was found to be agr-negative.

## Discussion

The nutrient-rich composition of milk and dairy products creates ideal conditions for the growth of diverse bacteria, including pathogens. Among these pathogens, *S. aureus* stands out as one of the most important. The presence of *S. aureus* in raw milk poses a significant threat to the dairy industry (Gajewska et al. 2023). Meta-analyses conducted globally report an average *S. aureus* prevalence of approximately 32-33% in raw milk products (Zhang et al. 2022, Gajewska et al. 2023). Moreover, Gajewska et al. (2023) noted a decrease in *S. aureus* contamination in raw milk in studies conducted over the past decade. In the present study, the *S. aureus* detection rate in raw milk samples was found to be 46%, which exceeds global averages. Farm animals are considered one of the primary sources of *S. aureus* contamination in raw milk. However, factors such as milk transporters, equipment, storage conditions, and retail practices also play significant roles in contamination pathways (Bastam et al. 2021). In this study, the elevated detection rate was primarily observed in raw milk samples collected from local vendors (delicatessens offering milk for retail sale), where *S. aureus* was detected at a rate of 61%. In contrast, the rate was significantly lower (21%) in raw milk samples obtained from a milkman who distributes directly from producers. In the case of local vendors,

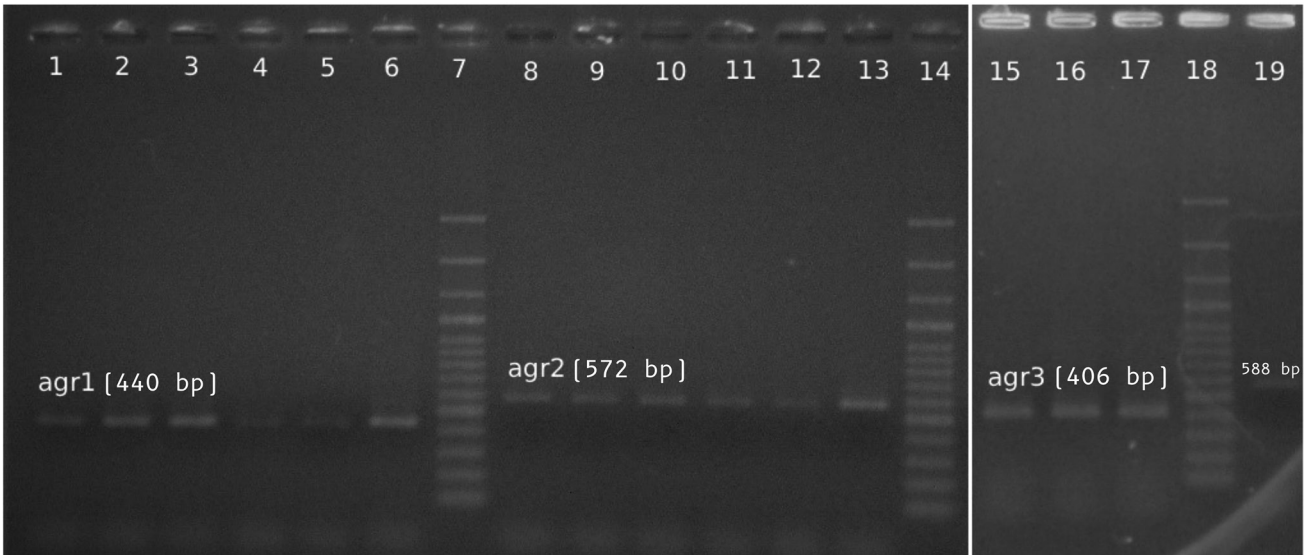


Fig. 7. Agarose gel image of agr typing of S. *aureus* strains. 1:M34, 2:M36, 3:M43, 4:C5, 5:C6, 6:C22, 7:100 bp DNA Marker, 8:M4, 9:M16, 10:M25, 11:M39, 12:M41, 13:M49, 14:100 bp DNA Marker, 15:M2, 16:M10, 17:C7, 18: 100 bp DNA Marker, *19: S. aureus* ATCC29213 (agr IV positive control).

milk is collected in bulk tanks located in rural areas and undergoes various storage and transport stages before reaching the consumer. This discrepancy highlights the critical role these intermediary processes play in bacterial contamination. In cheese samples, the detection rate was 10%. The *S. aureus*-positive cheese samples included fresh cheese (3), curd cheese (1), and string cheese (çeçil) (1). A common characteristic of these cheese types was their low salt content. The fresh and curd cheese samples were obtained from local farmers' markets where producers sell their own products, while the string cheese sample was obtained from a delicatessen. Globally, meta-analyses report a *S. aureus* prevalence of 42.8% in raw milk cheeses. However, prevalence rates in traditional cheeses vary widely by country. For example, in China, the detection rate is reported at 15% (Cai et al. 2021), in Brazil 33% (Ferreira et al. 2016), and up to 80% in a study conducted in Italy (Johler et al. 2018). These variations may be attributed to the diversity and frequency of contamination routes. Food contaminated with *S. aureus* can serve as a pathway for the transfer of enterotoxigenic strains to humans. This contamination can arise from various sources such as food contact surfaces, handlers, processing equipment, food-producing animals, as well as environmental factors like air and dust. Among these, the most significant route is direct contact or respiratory droplets from food handlers who harbor enterotoxin-producing *S. aureus* in their nasal passages or on their hands (Chaalal et al. 2018).

Antimicrobial resistance poses a serious worldwide threat impacting human, animal, and environmental health, and requires a comprehensive response guided by the One Health approach. A particularly concerning element of this challenge is the swift emergence and dissemination of multidrug-resistant bacteria, which can lead to infections that current antimicrobial treatments can no longer effectively manage (Velazquez-Meza el al. 2022). In this study, MDR was determined in 25% of the isolates, all of which were exclusively recovered from raw milk samples. Among these, five strains were isolated from milk obtained from local vendors, while the remaining two originated from samples purchased from milk vendors. No multidrug-resistant *S. aureus* strains were detected among the cheese isolates. In the present study, penicillin exhibited the highest resistance rate in both raw milk (39%) and cheese isolates (60%), followed by kanamycin, with resistance rates of 35% in milk isolates and 20% in cheese isolates. Globally, penicillin has been reported as the antibiotic with the highest resistance rate (73.85%) among *S. aureus* isolates from milk and dairy products (Zhang et al. 2022). The resistance rates determined in the study were lower than the global prevalence for both sample types. The widespread use of antimicrobials in dairy farms, particularly penicillin and kanamycin for both therapeutic and prophylactic purposes may account for the elevated resistance levels (Molineri et al. 2021). Another notable finding in this study was the high level of resistance to tetracycline, observed in 13% of milk isolates and 20% of cheese isolates. Supporting this, Ouabdesselam et al. (2021) detected penicillin and/or tetracycline residues in 75% of milk samples. Frequent antibiotic use in veterinary medicine, coupled with antimicrobial residues present even in milk, promotes the selection and spread of resistant bacterial strains. Nevertheless, the low levels of penicillin resistance reported in some Nordic

European countries, where penicillin is still used to treat *S. aureus* mastitis, underscore the importance of prudent and limited antimicrobial use (Molineri et al. 2021).

Raw milk and dairy products may serve as a reservoir for MRSA, posing a potential risk for the transmission of this pathogen through the food chain. In a comprehensive study by González-Machado et al. (2024) covering 19 countries including Türkiye, the prevalence of MRSA in raw milk and dairy products was reported to be under 5%, with 30% of those MRSA isolates exhibiting multidrug resistance. The MRSA detection rate in this study (11%) aligns with those reported in earlier research. All MRSA strains (3) were isolated from raw milk samples and showed resistance to multiple antibiotics. One of the most significant factors in staphylococcal food poisoning is the presence of staphylococcal enterotoxin genes, which are commonly found in milk and dairy products. A global meta-analysis reported that the overall prevalence of classical enterotoxin genes in milk and dairy products is approximately 39.31%, with sec and sea being the most frequently detected genes (Zhang et al. 2022). In recent years, studies conducted in Korea and Poland that included both classical and newly identified enterotoxin genes reported the presence of at least one enterotoxin gene in 43% and 92% of isolates, respectively. In contrast, a study in China that only focused on classical enterotoxin genes found a lower prevalence of 12.9% (Kou et al. 2021, Jung and Lee 2022, Szczuka et al. 2022). Toxin gene profiles also show considerable variation. In the study by Jung and Lee (2022), see and seg were the most frequently detected enterotoxin genes, while Kou et al. (2020) identified see, sea, and sec, and Szczuka et al. (2022) reported seo and sek as the predominant genes. In this study, all of the *S. aureus* isolates (100%) obtained from raw milk and cheese samples exhibited enterotoxigenic characteristics. At least two enterotoxin genes (sec and seg) were detected in all isolates. These two genes are consistently reported among the most prevalent enterotoxins globally. Notably, the isolate carrying the highest number of enterotoxin genes was also derived from a raw milk sample and exhibited the sea, sec, sed, seg, seh profile. Evaluating the results of this study with the literature, it becomes evident that both the presence of enterotoxin genes and the types of commonly detected toxin genes in raw milk and cheese isolates vary considerably. These prevalence rates vary depending on the number of enterotoxin genes investigated and regional differences. This finding highlights the potential for highly toxigenic *S. aureus* in retail raw milk and cheese, which may pose a greater risk for foodborne illness.

Biofilm-forming capacity is an important virulence factor that enhances the persistence of *S. aureus* under stressful environmental conditions (Yin et al. 2019). In the present study, the biofilm-forming abilities of *S. aureus* isolates were evaluated at storage temperature (4°C), room temperature (22°C), and optimal growth temperature (37°C), which reflect the storage conditions of raw milk and cheese products. All isolates exhibited biofilm formation at 22°C and 37°C to varying degrees, and a considerable proportion (82%) retained the ability to form biofilms, although weakly, even at 4°C. This finding suggests that these isolates may persist in dairy industry environments, potentially compromising the effectiveness of routine cleaning procedures. Notably, *S. aureus* strains isolated from raw milk sold by local vendors exhibited higher biofilm-forming capacities compared to those recovered from samples obtained from milk vendors, which may further support this concern. Carvalho et al. (2021) demonstrated that *S. aureus* is capable of forming biofilms on low-density polyethylene packaging in the presence of Minas cheese whey. In the present study, 80% of cheese isolates and 83% of raw milk isolates exhibited weak biofilm formation at 4°C. These findings suggest that *S. aureus* may persist even under refrigeration conditions in commercially available dairy products. Such persistence could increase the risk of cross-contamination during storage and handling.

The elevated biofilm formation rate observed in the *S. aureus* isolates in this study aligns with findings reported in the literature. Deepak et al. (2024) reported that 93.25% of *S. aureus* strains isolated from raw milk samples were capable of forming biofilms. However, when assessed based on CRA morphology, the positivity rate was lower, at 70%. Similarly, in the present study, a lower proportion of biofilm-forming isolates was identified using CRA morphology, with 71% of *S. aureus* isolates showing positive results.

In *S. aureus*, the expression of most virulence factors is regulated by the agr locus, which has been shown to play a critical role in adhesion, biofilm formation, overall virulence, and the expression of staphylococcal enterotoxins (SEs) (Touaitia et al. 2025). In this study, 47% of the isolates were determined as agr-negative. Eidaroos et al. (2025) reported no detection of agr-negative isolates among *S. aureus* strains isolated from raw milk samples, whereas Pineda et al. (2025) found a 27% prevalence of agr-negative isolates in raw milk cheese samples. In the present study, the proportion of agr-negative isolates (47%) was notably higher than those reported in the literature. Gor et al. (2019) demonstrated that serial passaging of *S. aureus* can result in the emergence of agr-negative variants. In this study, most of the agr-negative isolates (12 out of 13) originated from raw milk samples. Considering their bio-

film-forming capacity, it is plausible that these strains may have escaped disinfection procedures during food processing and persisted within the production environment. Under such stress conditions, they may have transitioned to an agr-negative phenotype. In this study, 21% of the *S. aureus* isolates belonged to agr group I and II, while 11% were classified as agr group III. No isolates were assigned to agr group IV. These results agree with the distribution of agr types reported in the literature for dairy product isolates. Consistent with the present findings, the majority of isolates in these studies also belonged to agr groups I and II, while agr group IV was not detected in any of the isolates (Eidaroos et al. 2025, Pineda et al. 2025). The findings of this study are consistent with those of Rimi et al. (2024), who reported that none of the hospital isolates belonging to agr group IV were capable of forming biofilms. They also found a statistically significant correlation between the biofilm-forming abilities of the isolates and their agr group distribution. This relationship between agr groups and biofilm production may be the reason for the high biofilm forming capacity of dairy isolates and the absence of agr group IV in this study.

In recent years, the growing preference for minimally processed foods has led to an increased consumption of raw milk and dairy products. However, raw milk and its derivatives can serve as reservoirs for *S. aureus*, posing significant risks to public health. In conclusion, this study has demonstrated that enterotoxin genes, antimicrobial resistance, and biofilm-forming capacity may coexist in *S. aureus* strains isolated from raw milk and cheese samples. The ability of all isolates to form biofilms at 22°C and 37°C, together with the observation that the majority of isolates were strong biofilm producers indicates a substantial capacity of these microorganisms to adhere to surfaces and persist under both optimal growth conditions and food-processing environments. Although biofilm formation was more limited at low temperature (4°C), a considerable proportion of isolates retained the ability to form biofilms, suggesting that cold storage conditions may reduce but not eliminate the risk of contamination. The detection of sec and seg enterotoxin genes in all isolates, evaluated together with their biofilm-forming ability, points to an increased potential for persistence and cross-contamination in milk processing and storage environments. Furthermore, the identification of methicillin-resistant and multidrug-resistant isolates in raw milk samples indicates that dairy products may serve as a potential reservoir for the dissemination of resistant *S. aureus* strains along the food chain. Overall, these findings emphasize the interplay between virulence factors, antimicrobial resistance, and biofilm formation, highlighting the public health significance of dairy-associated *S. aureus* isolates and supporting the need for effective hygiene practices and routine microbiological monitoring throughout the dairy production chain.

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## Author Declarations

### Ethics approval

The study did not require approval from an ethics committee.

### Use of generative artificial intelligence

No artificial intelligence-assisted software or tools were used at any stage of this study.

### Conflict of interest

Author has declared no conflict of interest.

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