Food Research International 245 (2026) 120793

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Genomic diversity, farm-level persistence, and food safety risk traits of *Staphylococcus aureus* in small-ruminant raw milk dairy chains in central Italy

David Savelli [a], Claudia Gabucci [a,1], Vesna Milanović [b,*], Di Lullo Stefania [a,**], Angelo Romano [c], Andrea Osimani [b], Federica Cardinali [b], Francesca Romana Massacci [a], Sara Primavilla [a], Lucilla Cucco [a], Marta Paniccià [a], Cinzia Lorenzetti [a], Eleonora Scoccia [a], Giuliana Blasi [a], Piermario Mangili [a], Fabio Zuccon [c], Annalisa Petruzzelli [a]

[a] *Istituto Zooprofilattico Sperimentale Dell'umbria e Delle Marche "Togo Rosati", Via G Salvemini, Perugia 06126, Italy*
[b] *Dipartimento Di Scienze Agrarie, Alimentari ed Ambientali, Università Politecnica Delle Marche, Via Brecce Bianche, Ancona 60131, Italy*
[c] *Laboratorio Nazionale Di Riferimento (LNR) Per Gli Stafilococchi Coagulasi Positivi, Incluso S. Aureus, Istituto Zooprofilattico Sperimentale Del Piemonte e Della Valle D'Aosta, Via Bologna, Torino 10154, Italy*

ARTICLE INFO

Keywords:
Staphylococcus aureus
Raw milk cheese
Small ruminants
Antimicrobial resistance
Staphylococcal enterotoxins
Surface adhesion
Whole-genome sequencing

ABSTRACT

Staphylococcus aureus is a major food safety and One Health concern in raw milk dairy chains. This study investigated its occurrence, contamination levels, antimicrobial resistance (AMR), enterotoxigenic potential, surface-adhesion phenotype, and genomic relatedness across 15 small-scale ovine–caprine dairy farms in the Umbria and Marche regions of Central Italy. Overall, 425 food, environmental, and operator hand swab samples were analysed. *S. aureus* was detected in 208/425 samples (48.9%), with the highest occurrence in food matrices (62.1%) and marked farm-level heterogeneity. One finished cheese product tested positive for staphylococcal enterotoxins (SEs). A total of 1662 colonies from 208 positive samples were confirmed as *S. aureus*, and a representative subset, including the MRSA isolates, underwent phenotypic characterization and whole-genome sequencing (WGS). No isolate carried the mupirocin resistance gene *mupA*. WGS revealed diverse sequence types, mainly ST133, ST700, and ST522. Phenotypic resistance was observed mainly to sulfisoxazole, tetracycline, and penicillin, whereas the most frequent AMR genes were *blaZ*, *tet(M)*, and *tet(K)*. Classical SE genes were detected in a subset of sequenced isolates, and all sequenced isolates carried at least one SE or SE-like gene. All tested isolates adhered to polystyrene, mostly moderately or strongly, and all sequenced isolates carried the biofilm-associated *icaA*, *icaB*, *icaC*, *icaD*, and *icaR* genes. Phylogenetic analyses indicated farm-specific clustering and persistence or recurrence of genetically related isolates across matrices and seasons. These findings support farm-specific hygiene interventions and integrated phenotypic–genomic surveillance to control AMR and potentially toxigenic *S. aureus* in artisanal small-ruminant raw milk dairy production.

1. Introduction

Staphylococcus aureus is a ubiquitous commensal bacterium that colonizes the skin and mucosal surfaces of both humans and animals. However, it also represents a highly adaptable opportunistic pathogen capable of causing a broad spectrum of diseases, ranging from localized infections to severe, life-threatening systemic conditions (Pal et al., 2023).

A key determinant of the epidemiological success of *S. aureus* is its remarkable capacity to develop antimicrobial resistance (AMR) against multiple classes of antibiotics (Beshiru et al., 2024). This capability is driven by both the selective pressure exerted by the widespread use of antimicrobials in human and veterinary medicine and the ability of *S. aureus* to acquire and disseminate mobile genetic elements carrying

* Corresponding author at: Dipartimento Di Scienze Agrarie, Alimentari Ed Ambientali, Università Politecnica Delle Marche, Brecce Bianche, 60131 Ancona, Italy.
** Corresponding author.
E-mail address: v.milanovic@staff.univpm.it (V. Milanović).
[1] *Co-first Author*

https://doi.org/10.1016/j.foodres.2026.120793
Received 9 July 2026; Received in revised form 8 September 2026; Accepted 9 September 2026
Available online 11 September 2026
0963-9969/© 2026 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

AMR determinants (Kalayu et al., 2020). Methicillin-resistant *S. aureus* (MRSA) is of particular concern because it is resistant to most beta-lactam antibiotics, which are among the main therapeutic options for staphylococcal infections. In the 2024 World Health Organization (WHO) bacterial priority pathogens list, MRSA is classified as a high-priority pathogen because of its substantial public-health burden and the therapeutic challenges associated with resistant infections (World Health Organization, 2024a). MRSA has been increasingly reported in both human and animal populations, with evidence of bidirectional transmission between them (Kalayu et al., 2020; Magro et al., 2018).

The dairy production sector represents a critical animal–environment–human interface for the circulation of *S. aureus*. At the farm level, the pathogen may disseminate among animals, workers, milk, equipment, and the farm environment, favoured by mastitis in dairy animals, asymptomatic human carriage, and potential shortcomings in good hygienic and manufacturing practices. These reservoirs can facilitate the spread of *S. aureus* from the farm environment to processing facilities and final products (Giacinti et al., 2011). Genetically related MRSA isolates have been recovered from animal, human, and environmental sources on dairy farms in Italy and other countries, supporting the potential for both animal-to-human and human-to-animal exchange, although the direction of transmission cannot always be established from cross-sectional data (Carfora et al., 2016; Feßler et al., 2012; Lienen et al., 2021; Lim et al., 2013; Macori et al., 2017). Such cross-host dissemination may increase occupational exposure, maintain resistant lineages within farms, and introduce clinically relevant *S. aureus* strains into raw-milk dairy chains.

In addition to its clinical relevance, *S. aureus* poses a significant food safety hazard because of its ability to produce heat-stable staphylococcal enterotoxins (SEs) in food matrices, which are responsible for food poisoning outbreaks worldwide (Cieza et al., 2024). In small artisanal dairies, these risks may be amplified by close animal–operator contact, manual milking or product handling, raw-milk processing without a pasteurization step, and heterogeneous or incompletely standardized cleaning and disinfection procedures.

Moreover, the ability of *S. aureus* to attach to surfaces and subsequently develop biofilms under suitable conditions may favour recurrent contamination and reduce the effectiveness of sanitation procedures (Dawan et al., 2025). These challenges are particularly relevant in the ovine–caprine dairy sector of Central Italy (Umbria and Marche regions), which is largely characterized by small- to medium-scale farms adopting traditional, often non-standardized production practices, frequently involving raw milk cheesemaking (Mezher et al., 2023).

Italian surveys have reported enterotoxigenic *S. aureus* in raw sheep milk and documented the occurrence of *S. aureus* and occasional MRSA isolates in bulk-tank milk from dairy sheep and goat farms (Cortimiglia et al., 2015; Giacinti et al., 2011). Molecular studies have also characterized MRSA and methicillin-susceptible *S. aureus* from ovine dairy chains and farm-associated humans and have demonstrated substantial genetic diversity and frequent SE gene carriage among isolates from goat milk (Macori et al., 2017; Romanò et al., 2020). These findings confirm the regional relevance of *S. aureus* but have primarily addressed bulk-tank milk, selected farms, specific dairy-chain compartments, or particular isolate characteristics.

Integrated evidence remains limited from multi-farm studies that repeatedly examine raw materials, intermediate and finished products, processing surfaces, and operators while linking quantitative contamination data with phenotypic AMR, enterotoxigenic potential, surface adhesion, and whole-genome relatedness. Consequently, the relationships among farm-level contamination patterns, recurrence across production-chain compartments, and the phenotypic and genomic characteristics of the recovered isolates remain incompletely defined.

Within this context, the present study aimed to assess the occurrence, contamination levels, AMR, enterotoxigenic potential, surface-adhesion phenotype, and genomic relatedness of *S. aureus* along the ovine–caprine raw milk dairy supply chain in the Marche and Umbria regions of Central Italy. Food matrices, environmental surfaces, and operator hand swabs were analysed to determine sample-level contamination, while recovered isolates were molecularly confirmed and screened for *mecA* and *mupA* to identify MRSA and mupirocin resistance determinants. A representative subset of isolates was further characterized by phenotypic antimicrobial susceptibility testing, assessment of surface-associated adherent biomass using the crystal-violet microtiter plate assay, and whole-genome sequencing (WGS). WGS-based analyses included sequence typing, detection of AMR genes and their putative localization, characterization of SE and SE-like gene profiles, screening of genes associated with adhesion and biofilm development, and assessment of phylogenetic relatedness. By integrating chain-level microbiological data with isolate-level phenotypic and genomic characterization within a multi-farm framework, the study sought to identify critical contamination points, farm-associated circulation or recurrence patterns, and AMR or toxigenic traits relevant to One Health-oriented control strategies. The specific contribution of the study therefore lies in the epidemiological information obtained by linking these datasets, rather than in the individual analytical methods themselves.

2. Materials and methods

2.1. Sampling procedure

A stratified sampling approach was applied to select small-scale dairy farms with on-site processing of raw ovine and caprine milk located in the Marche and Umbria regions (Central Italy). Based on a preliminary mapping of eligible farms, a statistically driven sampling plan was developed to define the number of farms to be included, the types and number of samples to be collected, and the sampling frequency. The initial sampling plan aimed to include 32 farms from an eligible population of 60 farms. However, because of limited farm participation and operational constraints, the final study population included 15 farms overall, of which 11 were located in the Marche region and 4 in the Umbria region. The distribution and approximate geographical location of the sampled farms are shown in Fig. 1. Additional farm-level information, including herd size and animal type, is provided in Supplementary Table 1.

Each farm was visited twice, once during the cold season (September–February) and once during the warm season (March–August), whenever animals in lactation were available. Operators were notified in advance to ensure that sampling aligned with production days. Within each farm, sampling targeted matrices considered at higher risk of contamination by MRSA, based on field inspections and prior sector-specific expertise. Overall, 425 samples were collected and analysed, of which 101 originated from four farms located in Umbria and 324 from eleven farms located in the Marche region. Samples were categorized into three main groups: i) food, ii) environmental, and iii) human samples, and further subdivided according to matrix type or sampled surface. Food samples ($n = 103$) included bulk tank milk ($n = 29$), intermediate products ($n = 40$) consisting of curd ($n = 28$) and cheeses during ripening ($n = 12$), and finished products ($n = 34$), defined as ready-to-eat cheeses with a ripening time of less than 30 days. Environmental samples ($n = 292$) comprised both food-contact and non-food-contact surfaces. Food-contact surfaces ($n = 176$) included milking liners ($n = 37$), multipurpose equipment and containers used for milk and curd ($n = 27$), molds ($n = 33$), processing tables and work surfaces ($n = 43$), and ripening and drying surfaces, as well as cold rooms and refrigerators ($n = 36$). Non-food-contact surfaces ($n = 116$) included multipurpose equipment control panels ($n = 14$), aprons ($n = 16$), handles ($n = 31$), and sinks ($n = 55$). Human samples ($n = 30$) consisted of swabs collected from the hands of dairy operators.

Bulk milk samples were collected as single sampling units directly from storage tanks in accordance with Regulation (EC) No. 853/2004 (European Parliament and Council of the European Union, 2004). Intermediate products were sampled as single units at stages of the

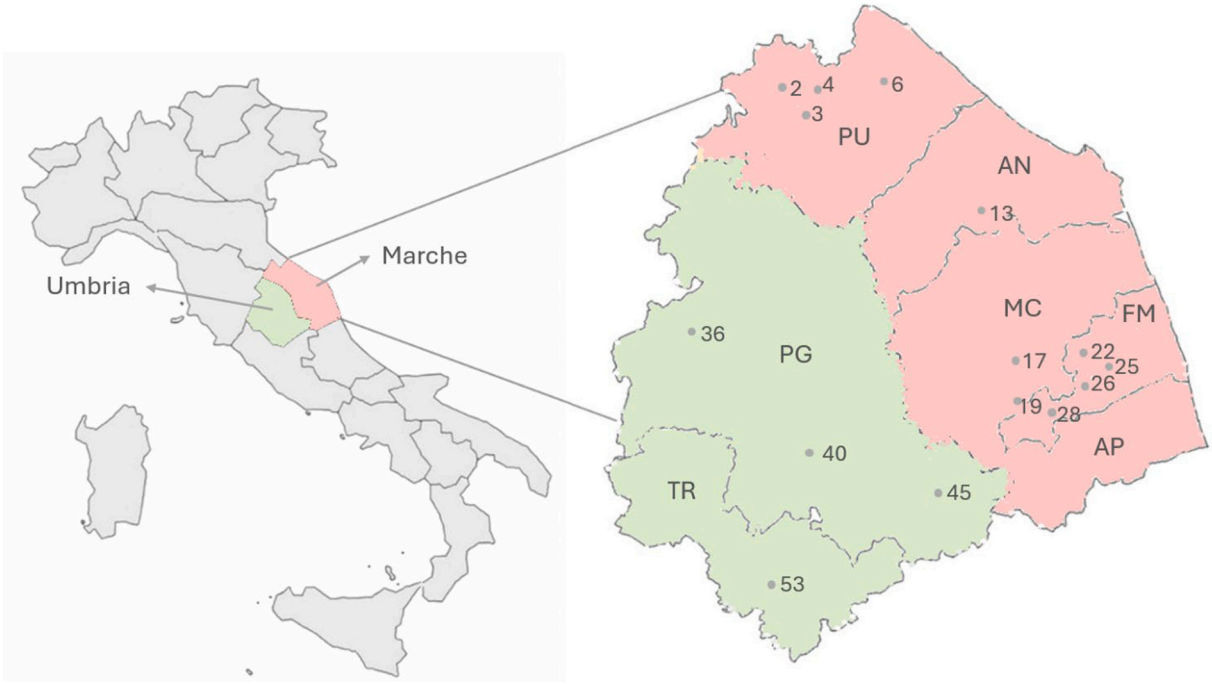


Fig. 1. Geographical distribution of the sampled farms in the Marche and Umbria regions of Central Italy. The enlarged map shows the approximate location of each farm, identified by its numerical code, within the respective provinces. Province abbreviations: PU, Pesaro and Urbino; AN, Ancona; MC, Macerata; FM, Fermo; AP, Ascoli Piceno; PG, Perugia; TR, Terni.

production process expected to favour staphylococcal growth (2–5 days). Finished products were sampled in five units per batch following Regulation (EC) No. 2073/2005. Environmental and human samples were collected from surfaces and sites with a higher likelihood of contamination. Swab samples (single or pooled) were taken from milking equipment and operators' hands using swabs premoistened with a neutralizing solution (monopotassium phosphate, 0.0425 g/L; sodium thiosulfate, 0.16 g/L; and aryl sulfonate complex, 5 g/L) to neutralize any chemical residues from cleaning and disinfection procedures. Other surfaces were sampled using sponges pre-moistened with 10 mL buffered peptone water (BPW).

The overall sampling strategy was designed assuming an expected MRSA prevalence of 20%, with a 90% confidence level and 15% precision. Written informed consent was obtained from operators before hand-swab sampling, and farm owners were requested to temporarily withhold sampled product batches from sale until microbiological analyses were completed.

2.2. Enumeration, isolation and identification of Staphylococcus aureus

All collected samples were subjected to enumeration of coagulase-positive staphylococci, including *S. aureus*, using an in-house method (PRT.PGMICALI.009; Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati", Perugia, Italy), accredited according to ISO/IEC 17025:2018 (International Organization for Standardization, 2017a). Raw materials, intermediate products, and finished products were diluted 1:10 in BPW (10 g sample + 90 mL BPW) and homogenized using a Stomacher apparatus (400 Circulator, International PBI, Milan, Italy). Pre-moistened sponges used for surface sampling were supplemented with 90 mL BPW to obtain a 1:10 dilution and homogenized using a Stomacher apparatus. Swab samples were collected in 10 mL of neutralizing solution, and the eluate used for subsequent analysis was obtained after vortex mixing for at least 30 s. All samples were subsequently subjected to serial tenfold dilutions in maximum recovery diluent (sodium chloride 8.50 g/L, peptone 1 g/L, *w/v*).

Enumeration was performed by pour plating 1 mL of the initial suspension or appropriate decimal dilutions onto Baird-Parker agar (BPA) supplemented with rabbit plasma fibrinogen (BP-RPF). Plates were incubated at 37 °C for 48 h, after which typical black or grey colonies surrounded by a clear precipitation halo were counted. From plates showing growth, up to five typical colonies were isolated on BP-RPF and subsequently subcultured onto tryptic soy agar (TSA). After incubation at 37 °C for 24 h, a loopful from each culture was inoculated into methyl red/Voges–Proskauer broth (MR/VP) and incubated at 37 °C for 24 h. The Voges–Proskauer (VP) test was performed to assess acetoin production. Briefly, 1 mL of each MR/VP culture was mixed with 0.6 mL of 5% (*w/v*) α-naphthol solution in ethanol and 0.2 mL of 40% (*w/v*) potassium hydroxide solution in water. Tubes were shaken to ensure oxygenation and left at room temperature. Cultures developing a red colour within 15 min were considered VP-positive (Barritt, 1936; MacFaddin, 2000). In case of negative results, MR/VP broths were further incubated at 37 °C for 24 h and the test repeated. Quality control was performed in each analytical session using a VP-positive reference strain (*Listeria innocua* ATCC 33090, American Type Culture Collection, Manassas, VA, USA) and a VP-negative strain (*Escherichia coli* ATCC 25922). All VP-positive isolates were subsequently subjected to molecular identification for confirmation of *S. aureus*. Based on the proportion of confirmed *S. aureus* colonies, the final enumeration for each sample was calculated according to UNI EN ISO 7218:2024 (International Organization for Standardization, 2024).

2.3. Detection of staphylococcal enterotoxins

Finished products were tested for SEs A, B, C, D, and E. For each batch, five units were analysed as a pooled sample by combining 25 g from each unit, resulting in a total analytical portion of 125 g. Extraction and concentration were performed according to UNI EN ISO 19020:2017 (International Organization for Standardization, 2017b), and the resulting extracts were analysed using an enzyme-linked fluorescent assay (ELFA) on the VIDAS automated immunoassay system (Vitek Immuno Diagnostic Assay System; bioMérieux, Marcy-l'Étoile, France) with the VIDAS Staph Enterotoxin II (SET2) kit, following the

manufacturer's instructions.

2.4. DNA extraction and multiplex PCR for detection of Staphylococcus aureus, MRSA and mupirocin resistance genes

To identify *S. aureus* at molecular level and screen for methicillin resistance (MRSA) and mupirocin resistance, VP-positive isolates were further analysed by endpoint multiplex PCR targeting the 16S rRNA (*Staphylococcus* genus-specific), *nuc* (*S. aureus* species-specific), *mecA* (methicillin resistance), and *mupA* (mupirocin resistance) genes.

For this purpose, isolates previously grown on TSA were subcultured onto 5% sheep blood agar (SBA) and incubated at 37 °C for 24 h before DNA extraction. DNA was extracted by suspending each colony in 100 µL of 6% Chelex® 100 solution (Bio-Rad Laboratories, Hercules, CA, USA), followed by incubation at 56 °C for 1 h and 95 °C for 45 min, and centrifugation at 20,000 ×*g* for 10 min (Mikro 20, Hettich, Tuttlingen, Germany). The supernatant was retained and used as template DNA for multiplex PCR. *S. aureus* ATCC BAA-1708 strain was included as positive control, and appropriate extraction and amplification negative controls were included in each analytical session. Amplification was performed using the primers previously reported by Zhang et al. (2004). Each reaction was carried out in a final volume of 25 µL containing 12.5 µL of 2× Multiplex PCR Master Mix (Qiagen, Hilden, Germany), primers at optimized final concentrations (0.1 µM for Nuc1 and Nuc2; 0.2 µM for MecA1, MecA2, MupA and MupB; and 0.12 µM for Staph756 16S F and Staph750 16S R), 4.8 µL of molecular biology-grade water, and 5 µL of DNA template. Thermal cycling conditions were as follows: initial denaturation at 95 °C for 15 min; 10 cycles of 94 °C for 40 s, 68 °C for 40 s and 72 °C for 1 min; 25 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min; and a final extension at 72 °C for 10 min. PCR products were resolved by electrophoresis on a 2.5% agarose gel in 1× Tris-borate-EDTA buffer and visualized under UV light. Amplicon sizes were determined by comparison with a molecular-weight marker (Applied Biological Materials Inc., Richmond, Canada).

2.5. Phenotypic antimicrobial susceptibility assessment

Two *S. aureus* isolates were selected, when available, from each sampling session for phenotypic antimicrobial susceptibility testing; one from a food matrix and one from an environmental surface. Selection was prioritised according to MRSA detection and/or the highest *S. aureus* enumeration values in the corresponding matrices.

Disc diffusion testing was performed on 90-mm Mueller–Hinton (MH) agar plates (pH 7.3) using fresh cultures (<24 h). Bacterial suspensions were adjusted to 0.5 McFarland in sterile saline solution (NaCl, 8.5 g/L, *w/v*) and inoculated onto the agar surface using sterile swabs. The antimicrobial discs tested were norfloxacin (NOR; 10 µg), linezolid (LNZ; 30 µg), gentamicin (CN; 10 µg), erythromycin (E; 15 µg), tetracycline (TE; 30 µg), rifampicin (RD; 5 µg), clindamycin (DA; 2 µg), cefoxitin (FOX; 30 µg), sulfamethoxazole/trimethoprim (SXT; 23.75/1.25 µg), levofloxacin (LEV; 5 µg), and ceftaroline (CPT; 30 µg). A maximum of four discs was applied to each plate. Plates were incubated at 35 °C for 18 h, and inhibition zones were measured with a digital calliper, rounded to the nearest millimetre, and interpreted according to Clinical and Laboratory Standards Institute guidelines ((Clinical and Laboratory Standards Institute, 2025)). *S. aureus* ATCC 25923 was included as the quality-control strain in each analytical session. In isolates classified as erythromycin-resistant and clindamycin-susceptible, inducible clindamycin resistance was assessed by the D-test, placing erythromycin and clindamycin discs 16 ± 4 mm apart (Steward et al., 2005).

Minimum inhibitory concentrations (MICs) were determined by the broth microdilution method using 96-well microtitre panels (Sensititre™, Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. *E. coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as quality-control strains. The antimicrobials and concentration ranges tested by broth microdilution were amoxicillin/clavulanic acid (AUG; 0.25/0.12–16/8 µg/mL), cefazolin (KZ; 0.25–8 µg/mL), erythromycin (E; 0.03–8 µg/mL), tetracycline (TE; 0.25–16 µg/mL), sulfamethoxazole/trimethoprim (SXT; 0.12/2.38–8/152 µg/mL), rifampicin (RD; 0.06–2 µg/mL), ampicillin (AMP; 0.03–16 µg/mL), penicillin (PEN; 0.03–16 µg/mL), ceftiofur (XNL; 0.25–8 µg/mL), clindamycin (DA; 0.5–2 µg/mL), oxacillin plus 2% NaCl (OXA+; 0.25–4 µg/mL), florfenicol (FFC; 2–8 µg/mL), kanamycin (KAN; 8–32 µg/mL), and sulfisoxazole (FIS; 128–512 µg/mL). For each isolate, a bacterial suspension of approximately 1.5×10^8 CFU/mL (0.5 McFarland) was prepared in sterile saline solution and diluted to 5×10^5 CFU/mL in cation-adjusted MH broth. Each well was inoculated with 100 µL of the final suspension, and plates were incubated at 37 °C ± 1 °C for 18 h. The MIC was recorded as the lowest antimicrobial concentration showing no visible growth.

For cefoxitin (FOX), ceftaroline (CPT), levofloxacin (LEV), norfloxacin (NOR), linezolid (LNZ), and gentamicin (CN), MICs were determined using MIC Test Strips (Liofilchem srl, Roseto degli Abruzzi, Italy), following the manufacturer's instructions and using *S. aureus* ATCC 25923 as quality control. MIC values were interpreted according to CLSI M100 clinical breakpoints (35th edition, 2025), where available. Specifically, SXT was interpreted using CLSI MIC breakpoints, whereas FIS was interpreted using EUCAST ECOFF/TECOFF criteria. For antimicrobials lacking applicable CLSI clinical breakpoints or requiring epidemiological interpretation, MICs were interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) (European Committee on Antimicrobial Susceptibility Testing, 2025) epidemiological cut-off values (ECOFFs) or tentative epidemiological cut-off values (TECOFFs). Based on these cut-offs, isolates were classified as wild-type or non-wild-type, indicating the likely absence or possible presence of acquired resistance mechanisms, respectively.

2.6. In vitro evaluation of surface adhesion

Based on phenotypic AMR profiles detected by disc diffusion and MIC testing and considering the isolate obtained from the sample positive for SEs, 39 isolates were selected for assessment of surface-associated adherent biomass. The assay was performed using a crystal violet microtitre-plate method adapted from Stepanović et al. (2000, 2007). Briefly, one colony from a TSA culture incubated for 24 h at 37 °C was inoculated into 5 mL tryptic soy broth (TSB) and incubated for 24 h at 37 °C. After incubation, the optical density at 600 nm (OD600) was measured using an Eppendorf BioPhotometer D30 (Eppendorf, Hamburg, Germany). Cultures were adjusted to approximately 10^6 cells/mL in TSB supplemented with 1% glucose (TSB + G), and 200 µL aliquots were inoculated into flat-bottom 96-well polystyrene microplates (CytoOne, Starlab International GmbH, Germany). For each isolate, six wells were inoculated per plate and each assay was performed in triplicate. Negative-control wells contained 200 µL of sterile TSB + G only.

After 24 h of incubation at 37 °C, wells were emptied by inversion and washed three times with 200 µL sterile phosphate-buffered saline (PBS, pH 7.4) to remove non-adherent cells. Adherent cells were fixed with 150 µL of 99% methanol for 20 min at room temperature. Plates were then emptied, dried, stained with 200 µL of 2% (*v*/v) crystal violet for 15 min at room temperature, washed three times with PBS to remove excess stain and dried under a chemical hood. The bound dye was solubilised with 150 µL of 33% (*v*/v) acetic acid for 30 min, and absorbance was measured at 540 nm (OD540) using a Tecan Sunrise microplate reader (Tecan Group Ltd., Männedorf, Switzerland) with Magellan software version 7.2.

Surface adhesion was classified according to the optical density cut-off (ODc), calculated as the mean OD of negative control wells plus three times their standard deviation across the three replicates. Isolates were classified as non-adherent (OD ≤ ODc), weakly adherent (ODc < OD ≤ 2 x ODc), moderately adherent (2 x ODc < OD ≤ 4 x ODc) or strongly

adherent (OD > 4 x ODc).

2.7. Whole-genome sequencing

Selected isolates were subjected to WGS. Total bacterial genomic DNA was extracted from cultures grown on SBA for 18–24 h at 37 °C using the Extractme Genomic DNA isolation kit (Blirt S.A., Gdańsk, Poland), according to the manufacturer's instructions. DNA concentration was measured using the Qubit 2.0 fluorometer and the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). Approximately 100 ng of DNA in a final volume of 30 μL was used for library preparation with the Illumina DNA Library Prep Kit (Illumina, San Diego, CA, USA), consisting mainly of tagmentation, amplification and library purification steps. Library fragment distribution was checked by analysing 1 μL of each library with an Agilent 2100 Bioanalyzer using the High Sensitivity DNA chip (Agilent Technologies, Santa Clara, CA, USA), verifying the expected fragment-size profile of approximately 250–1000 bp. Libraries were further quantified using the Qubit dsDNA HS Assay Kit. Based on mean fragment size and fluorometric concentration, libraries were diluted in resuspension buffer to 4 nM. A 2% (*v*/v) PhiX internal sequencing control was added before paired-end sequencing on an Illumina MiSeq platform using the MiSeq Reagent Kit v3 (600-cycle), setting 151 cycles for each read.

Raw sequencing data were analysed mainly through the web-based Galaxy platforms (https://usegalaxy.org and https://galaxy.sciensano.be). Read quality was assessed using FastQC, and adapters and low-quality bases were removed using Trimmomatic (Bolger et al., 2014). Nextera and other Illumina-specific adapter sequences were removed using the paired-end Nextera adapter file (ILLUMINACLIP), low-quality bases were trimmed from the beginning and end of reads (LEADING:10; TRAILING:10), and reads were trimmed when the average Phred quality score fell below 20 within a four-base sliding window (SLIDINGWINDOW:4:20). Reads shorter than 40 bp after trimming were discarded (MINLEN:40). Trimmed reads were assembled *de novo* using Unicycler (Wick et al., 2017) with the bridging mode set to “normal”, and contigs shorter than 200 bp were excluded from the final FASTA files. Assembly quality was assessed using QUAST (Mikheenko et al., 2018), and only genome assemblies with a mean sequencing depth of at least 30× were retained for downstream genomic analyses.

Specific genes were searched in assembled genomes using NCBI BLAST+ blastn (Cock et al., 2015). Isolates were further characterized using the *Staphylococcus* pipeline v.1.2 available on the Galaxy platform (Timmermans et al., 2022), including detection of AMR determinants, SE genes, selected genes associated with adhesion and biofilm development, and multilocus sequence typing (MLST). The localization of AMR genes on plasmid or chromosomal contigs was assessed using PlasmidFinder, provided by the Center for Genomic Epidemiology, Technical University of Denmark (Carattoli et al., 2014), and MOB-recon, a module of MOB-suite developed for plasmid clustering, reconstruction, and typing from draft genome assemblies (Robertson & Nash, 2018). PlasmidFinder was used for *in silico* detection of plasmid replicons in assembled genomes, whereas MOB-recon was used to reconstruct plasmid sequences and infer plasmid mobility. These analyses supported the classification of resistance genes as chromosomal, plasmid-associated, or located on putatively mobilizable plasmids.

2.8. Phylogenetic analysis

Pairwise single-nucleotide polymorphism (SNP) distances were calculated from the high-quality SNP alignment generated by CSI Phylogeny 1.4 (Call SNPs and Infer Phylogeny), available through the Center for Genomic Epidemiology (CGE), Technical University of Denmark (DTU) (https://www.genomicepidemiology.org/) (Kaas et al., 2014). Because no validated SNP threshold is available for distinguishing environmental persistence from recurrent introduction of *S. aureus* in small-ruminant dairy farms, SNP distances were interpreted as measures of genomic relatedness rather than as definitive evidence of either epidemiological mechanism. The thresholds of 15 core-genome SNPs and 25 whole-genome SNPs proposed by Coll et al. (2020) for recent MRSA transmission were used only as literature-based benchmarks. Isolates below these benchmarks were considered compatible with recent common ancestry, but epidemiological linkage was required for interpretation.

2.9. Statistical analyses

Differences in *S. aureus* positivity among sample groups, sample types, farms, and seasons were assessed using Pearson's χ^2 test. When appropriate, exploratory post-hoc comparisons were performed using adjusted pairwise comparisons or two-sided Fisher's exact tests, with Holm correction applied within each predefined family of comparisons. Farm-specific seasonal differences were assessed by comparing positive and negative samples between the warm and cold seasons using two-sided Fisher's exact tests, with Holm correction across farms. Viable counts were analysed only for *S. aureus*-positive samples. Counts were log10-transformed before analysis and compared among sample categories using one-way ANOVA followed by Tukey's HSD post-hoc test. For selected isolates, exploratory multivariate association analyses were performed separately for phenotypic AMR profiles, AMR gene profiles, and SE/SE-like gene profiles. Each isolate-level data block was analysed as a binary matrix, with resistance/non-wild-type phenotypes or detected genes coded as present and susceptible/wild-type phenotypes or undetected genes coded as absent. Associations between each data block and grouping variables, including farm, source type, sampling season, sequence type, and MRSA status, were assessed using permutation-based multivariate testing. Global χ^2 tests and one-way ANOVA were not adjusted for multiple comparisons. Holm correction was applied only to categorical follow-up comparisons and separately within each isolate-level multivariate data block, whereas Tukey's HSD was used for pairwise comparisons of viable counts. Only results remaining significant after correction were reported. Statistical significance was set at $p < 0.05$. Analyses were performed in Python 3.11 using SciPy, statsmodels, scikit-learn, NumPy, and pandas.

3. Results

3.1. Occurrence and contamination levels of S. aureus

Among the 425 samples collected from 15 ovine-caprine dairy farms with on-site processing facilities, including four farms in Umbria region and eleven farms in Marche region, *S. aureus* was detected in 208 samples (48.9%), comprising isolates recovered from food, environmental, and operators' hand swab samples.

As reported in Table 1, food samples showed the highest occurrence of *S. aureus* (62.1%), followed by environmental samples (44.9%) and operator hand swabs (43.3%). Positivity differed significantly among the three main sample groups ($\chi^2 = 9.50$, df = 2, $p = 0.0087$), with food samples showing significantly higher prevalence than environmental samples. Among food matrices, curd showed the highest positivity rate (75.0%), followed by finished products (67.6%), although differences among food sample types were not statistically significant ($p = 0.118$). In contrast, positivity differed significantly among environmental sample types ($\chi^2 = 19.14$, df = 8, $p = 0.014$), with cheese molds/baskets showing the highest prevalence (66.7%) and multipurpose equipment control panels the lowest (1/14; 7.1%).

Quantitative analysis of positive samples showed that finished products had the highest mean viable *S. aureus* counts overall (4.45 ± 1.34 log CFU/g). Significantly lower mean contamination levels were observed in bulk tank milk, curd, sinks, and handles, ranging from 2.76 to 3.32 log CFU/unit (Table 2).

At farm level (Table 3), *S. aureus* positivity varied markedly, ranging from 0.0% in farm 3 to 94.1% in farm 17, with significant differences

Table 1
Occurrence of *Staphylococcus aureus* in food, environmental, and human samples collected from ovine-caprine dairy farms in Central Italy.

Sample group	Sample type	Positive/Total samples (%)
Food	Bulk tank milk	15/29 (51.7%)
	Curd	21/28 (75.0%)
	Cheeses during ripening	5/12 (41.7%)
	Finished products	23/34 (67.6%)
	Total	64/103 (62.1%)[a]
Environmental	Milking liners	15/37 (40.5%)
	Multipurpose equipment/containers for curd production	16/27 (59.3%)
	Cheese molds/baskets	22/33 (66.7%)*
	Processing tables/work surfaces	20/43 (46.5%)
	Ripening/drying surfaces, cold rooms, refrigerators	15/36 (41.7%)
	Aprons	7/16 (43.8%)
	Sinks	25/55 (45.5%)
	Handles	10/31 (32.3%)
	Multipurpose equipment control panels	1/14 (7.1%)*
	Total	131/292 (44.9%)[b]
Human	Operator/dairy worker hand swabs	13/30 (43.3%)
	Total	13/30 (43.3%)[ab]
Overall		208/425 (48.9%)

Different superscript letters indicate significant differences among main sample groups (Pearson's χ^2 test with adjusted pairwise comparisons, $p < 0.05$); * environmental sample types with markedly different positivity rates in exploratory post-hoc one-*vs*-rest comparisons ($p < 0.05$).

Table 2
Staphylococcus aureus contamination levels in positive samples by sample category.

Sample category	log CFU/unit (mean ± SD)
Bulk tank milk	2.76 ± 0.70[b]
Curd	3.32 ± 1.28[b]
Cheeses during ripening	3.21 ± 0.95[ab]
Finished products	4.45 ± 1.34[a]
Milking liners	3.45 ± 1.34[ab]
Multipurpose equipment and containers used for milk and curd	3.54 ± 1.00[ab]
Molds	3.44 ± 1.22[ab]
Processing tables and work surfaces	3.38 ± 0.91[ab]
Ripening and drying surfaces, cold rooms and refrigerators	3.43 ± 1.36[ab]
Aprons	2.91 ± 0.63[ab]
Sinks	2.77 ± 0.63[b]
Handles	2.94 ± 0.96[b]
Multipurpose equipment control panels	2.30 ± n.d[ab]
Operator hands	3.33 ± 1.06[ab]

Different superscript letters indicate significant differences among sample categories, as determined by one-way ANOVA followed by Tukey's HSD post-hoc test ($p < 0.05$).

among farms overall. Farms 17 and 25 showed significantly higher positivity than the remaining farms, whereas farms 2 and 3 showed significantly lower positivity. To assess whether this heterogeneity was related to sampling season, farm-level positivity was stratified by warm season (March–August) and cold season (September–February). Overall, *S. aureus* positivity was 112/212 (52.8%) in the warm season and 96/213 (45.1%) in the cold season, with no statistically significant association between positivity and seasonality at the overall level. Most farms showed no significant seasonal difference. The only exception was farm 19, where positivity was significantly higher in the warm season than in the cold season. These findings indicate that the marked farm-level variability was not primarily explained by seasonality but was mainly associated with farm-level differences.

Table 3
Seasonal and farm-level occurrence of *Staphylococcus aureus*-positive samples in ovine–caprine dairy farms located in the Marche and Umbria regions of Central Italy.

Region	Farm ID	Positive/Total samples (%)		
		Warm season (March–August)	Cold season (September–February)	Total
Marche	2	0/12 (0.0%)	2/16 (12.5%)	2/28 (7.1%)[L]
	3	0/12 (0.0%)	0/13 (0.0%)	0/25 (0.0%)[L]
	4	4/11 (36.4%)	1/11 (9.1%)	5/22 (22.7%)
	6	6/14 (42.9%)	11/16 (68.8%)	17/30 (56.7%)
	13	11/16 (68.8%)	8/16 (50.0%)	19/32 (59.4%)
	17	15/17 (88.2%)	17/17 (100.0%)	32/34 (94.1%)[H]
	19	9/15 (60.0%)[a]	1/16 (6.3%)[b]	10/31 (32.3%)
	22	13/17 (76.5%)	9/16 (56.3%)	22/33 (66.7%)
	25	11/13 (84.6%)	10/13 (76.9%)	21/26 (80.8%)[H]
	26	2/16 (12.5%)	7/13 (53.8%)	9/29 (31.0%)
	28	8/17 (47.1%)	6/17 (35.3%)	14/34 (41.2%)
Umbria	36	9/13 (69.2%)	8/14 (57.1%)	17/27 (63.0%)
	40	5/13 (38.5%)	3/12 (25.0%)	8/25 (32.0%)
	45	11/16 (68.8%)	6/13(46.2%)	17/29 (58.6%)
	53	8/10 (80.0%)	7/10 (70.0%)	15/20 (75.0%)
Overall		112/212 (52.8%)	96/213 (45.1%)	208/425 (48.9%)

Different lowercase letters within the same row indicate significant seasonal differences. Superscripts H and L indicate farms with significantly higher or lower total positivity than the pooled remaining farms, respectively. Fisher's exact tests with separate Holm corrections for seasonal and farm-level comparisons; Holm-adjusted $p < 0.05$. Overall farm effect: Pearson's $\chi^2 = 113.67$, df $= 14$, $p < 0.001$.

3.2. Staphylococcal enterotoxin detection and molecular screening of the isolates

Thirty-four finished products were tested for the presence of SEs A–E. Among these, only one sheep cheese sample from farm 13 tested positive. Since the assay does not differentiate among enterotoxin types, the specific toxin detected could not be determined. The positive sample showed a test value of 0.40, above the negative cut-off value of 0.13, and had a coagulase-positive staphylococci count, confirmed as *S. aureus*, of 6.63 log CFU/g.

A total of 1662 colonies recovered from the 208 *S. aureus*-positive samples were confirmed molecularly as *S. aureus* by detection of the 16S rRNA and *nuc* targets and screened for *mecA* and *mupA*. Nine isolates were *mecA*-positive and were therefore classified as MRSA, while no isolate carried *mupA*. All MRSA isolates were obtained from the farms in Marche region. Seven MRSA isolates were obtained from farm 26, specifically from a cold-room handle and from the hand of an operator, while two MRSA isolates were obtained from a ready-to-eat sheep cheese with ripening time below 30 days collected in farm 28. Further characterization showed that the seven isolates from farm 26 were indistinguishable from each other, as were the two isolates from farm 28. Therefore, only two distinct MRSA strains were identified, one from farm 26 and one from farm 28.

3.3. Phenotypic antimicrobial susceptibility profiles

From the 1662 confirmed *S. aureus* isolates, 51 were selected for extended characterization. Given the low prevalence of MRSA detected in the sampled farms, isolate selection was expanded to include

approximately two isolates per sampling session, when available, one from a food matrix and one from an environmental sample, prioritizing matrices with the highest *S. aureus* counts and samples positive for MRSA and/or SEs. The isolation source and sampling season for each selected isolate are reported in Supplementary Table 1. Two MRSA isolates from farm 26, recovered from the operator's hand and the cold-room handle, showed identical profiles and were therefore considered as a single strain (SA39/44). Consequently, 50 isolates were included in the comparative phenotypic and genotypic analyses.

Phenotypic antimicrobial susceptibility testing showed heterogeneous resistance and non-wild-type profiles among the 50 selected *S. aureus* isolates (Fig. 2, Supplementary Table 2). Tetracycline

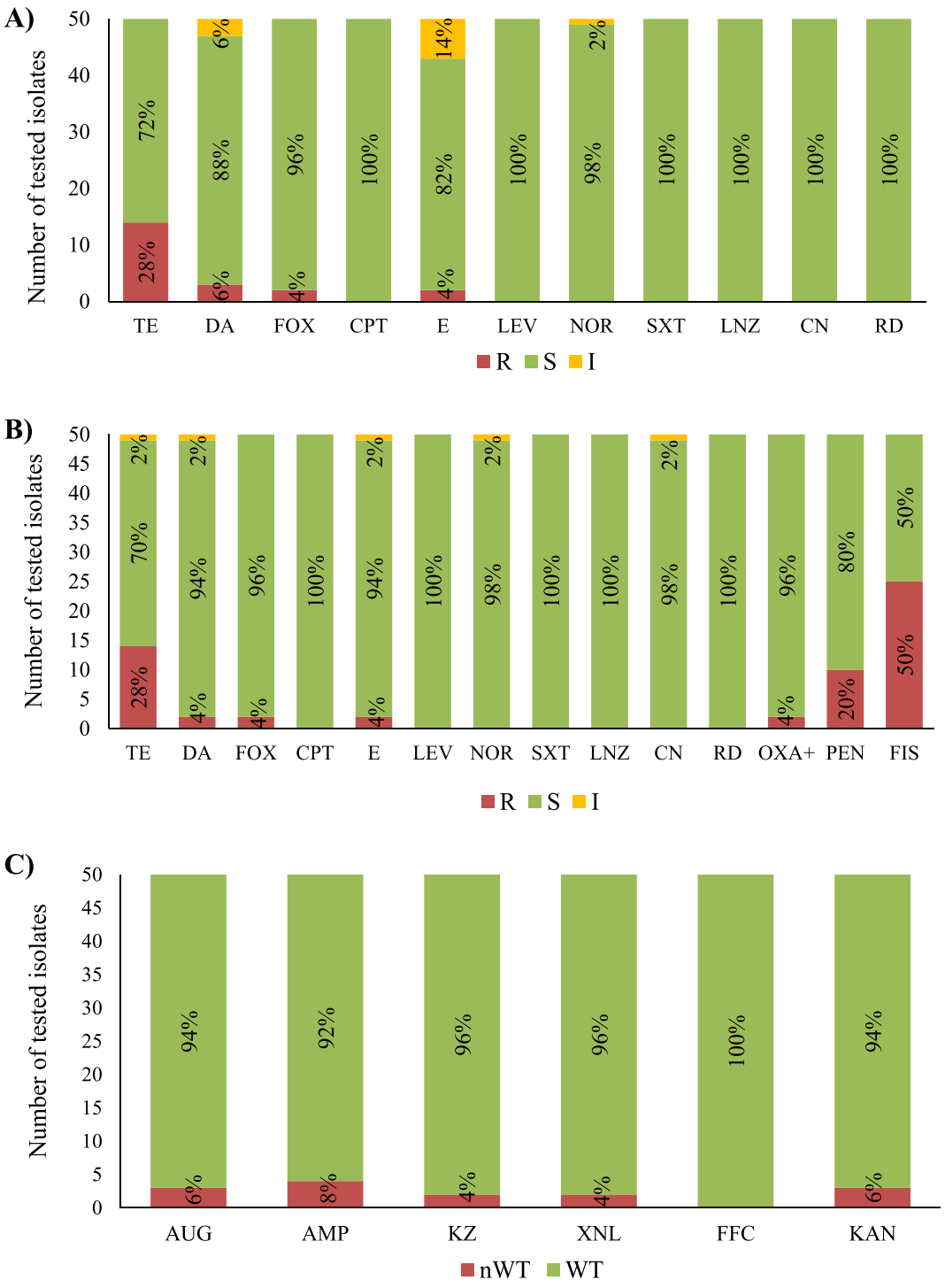


Fig. 2. Phenotypic antimicrobial susceptibility profiles of selected *Staphylococcus aureus* isolates assessed by disc diffusion and minimum inhibitory concentration (MIC) testing. (A) Disc diffusion test results classified according to Clinical and Laboratory Standards Institute (CLSI) clinical breakpoints. (B) MIC results classified according to CLSI clinical breakpoints. (C) MIC results classified as wild-type (WT) or non-wild-type (nWT) according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off values. R, resistant; I, intermediate; S, susceptible; TE, tetracycline; DA, clindamycin; FOX, cefoxitin; CPT, ceftaroline; E, erythromycin; LEV, levofloxacin; NOR, norfloxacin; SXT, sulfamethoxazole/trimethoprim; LNZ, linezolid; CN, gentamicin; RD, rifampicin; OXA+, oxacillin +2% NaCl; PEN, penicillin; FIS, sulfisoxazole; AUG, amoxicillin/clavulanic acid; AMP, ampicillin; KZ, cefazolin; XNL, ceftiofur; FFC, florfenicol; KAN, kanamycin.

resistance was detected in 14/50 isolates (28.0%) by both disc diffusion and MIC testing. MIC testing also identified resistance to sulfisoxazole in 25/50 isolates (50.0%) and to penicillin in 10/50 isolates (20.0%). Lower levels of resistance or non-susceptibility were observed for clindamycin, erythromycin, cefoxitin/oxacillin, norfloxacin, and gentamicin, whereas all isolates were susceptible to levofloxacin, sulfamethoxazole/trimethoprim, linezolid, rifampicin, and ceftaroline. Inducible clindamycin resistance was detected in two isolates. Using EUCAST ECOFFs or TECOFFs, isolates were classified as wild-type or non-wild-type, indicating the likely absence or possible presence of acquired resistance mechanisms, respectively. Non-wild-type profiles were detected for amoxicillin/clavulanic acid, ampicillin, cefazolin, ceftiofur, and kanamycin, while all isolates were wild-type for florfenicol.

At farm level, resistance and non-wild-type profiles were unevenly distributed. Isolates from farms 22, 36, and 45 showed consistent tetracycline resistance, whereas isolates from farm 26 and farm 28 included the most complex resistance profiles, corresponding to the two MRSA strains SA39/44 and SA46. In particular, SA39/44 from farm 26 showed resistance to tetracycline, clindamycin, cefoxitin, erythromycin, oxacillin, and penicillin, together with non-wild-type profiles for amoxicillin/clavulanic acid, ampicillin, cefazolin, ceftiofur, and kanamycin. Similarly, SA46 from farm 28 showed resistance to tetracycline, clindamycin, cefoxitin, oxacillin, penicillin, and sulfisoxazole, with non-wild-type profiles for the same antimicrobials interpreted using ECOFFs. In contrast, several farms included isolates that were susceptible or wild-type for all tested antimicrobials, indicating substantial variability in antimicrobial susceptibility profiles among farms (Supplementary Table 2).

3.4. Surface adhesion under in vitro conditions

Based on AMR profiles and the recovery of one isolate from the SE-positive product, 39 isolates were selected for assessment of surface-associated adherent biomass using the crystal-violet microtiter plate assay. As shown in Table 4, all tested isolates showed adhesive capacity. Specifically, eight isolates (20.5%) were classified as weakly adherent, 22 (56.4%) as moderately adherent, and nine (23.1%) as strongly adherent. Both MRSA strains and the isolate associated with the SE-positive cheese sample (SA7), were classified as moderately adherent.

3.5. Whole-genome sequencing-based genomic characterization

WGS produced data of sufficient quality for 40/50 selected isolates. Genome coverage ranged from 94.57% to 99.82%, allowing downstream characterization and molecular typing. The genome sequences were deposited in the Galaxy/S.I.R.I.O. platform (*S. aureus* Intensive Server for Research in Omics data), managed by the National Reference Laboratory for Coagulase-Positive Staphylococci, including *S. aureus* (Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Turin, Italy), and are available from the authors upon request.

As reported in Table 5, MLST analysis identified 11 sequence types (STs) (1, 45, 72, 133, 398, 522, 700, 1290, 2011, 3044, and 3224) among the successfully sequenced isolates. STs were assigned by analysing assembled contigs according to the MLST scheme available in Public databases for molecular typing and microbial genome diversity database (https://pubmlst.org/organisms/staphylococcus-aureus) (Jolley et al., 2018). ST133, ST700, and ST522 were the most frequently detected sequence types. Six isolates were assigned to three clonal complexes (CCs), namely CC1, CC8, and CC45. The two MRSA strains differed genetically: SA39/44 belonged to ST1/CC1, whereas SA46 belonged to ST398.

WGS confirmed the presence of several AMR genes among the selected *S. aureus* isolates (Table 5). Overall, AMR genes were detected in 17/40 successfully sequenced isolates. The most frequently detected gene was *blaZ* (9/40), followed by *tet(M)* (7/40) and *tet(K)* (6/40). The *mecA* gene was detected in two isolates, corresponding to the two MRSA strains SA39/44 and SA46. Other resistance genes were detected sporadically, including *erm(T)*, *erm(C)*, *aac(6′)-aph(2″)*, *aph(3′)-III*, *lsa(E)*, *lnu(B)*, *dfrG*, and *ant(9)-Ia*, each identified in a single isolate. Gene-location analysis showed that several resistance determinants were chromosomally located, including *mecA*, *tet(M)*, *erm(T)*, *blaZ* in some isolates, and the resistance genes detected in SA46. Plasmid-associated resistance genes were also identified. In particular, *aac(6′)-aph(2″)* and *aph(3′)-III* were located on mobilizable plasmids, while *tet(K)* and *erm(C)* were detected on non-mobilizable plasmids. The *blaZ* gene was detected in both chromosomal and plasmid-associated contexts. The highest number of resistance genes was observed in SA46 from farm 28, which carried eight AMR genes, followed by SA39/44 from farm 26, which carried five AMR genes.

WGS analysis also revealed a widespread distribution of SE and SE-like genes among the sequenced isolates (Table 5). Classical SE genes included *sea*, *seb*, sec*2*, sec-*bovine*, *sed*, and *see*, whereas the remaining detected genes were considered SE-like genes. All successfully sequenced isolates carried at least one SE or SE-like gene. Genes encoding classical enterotoxins were detected in 8/40 isolates (20.0%). Specifically, sec-*bovine* was identified in five isolates, sec*2* in two isolates, and *sed* in one isolate, while *sea*, *seb*, and *see* were not detected. The *seg* gene was detected in four isolates, including the two sec*2*-positive isolates. Among SE-like genes, *selw* and *selx* were the most

Table 4
In vitro surface adhesion and whole-genome sequencing-based profiles of biofilm-associated genes in selected *Staphylococcus aureus* isolates.

Farm ID	Isolate ID	Surface-adhesion category	Biofilm-associated genes				
			icaA	*icaB*	*icaC*	*icaD*	*icaR*
2	SA2	WA	+	+	+	+	+
6	SA5	WA	+	+	+	+	+
	SA6	MA	+	+	+	+	+
	SA30	WA	+	+	+	+	+
13	SA7	MA	+	+	+	+	+
	SA8	MA	+	+	+	+	+
	SA54	MA	na	na	na	na	na
	SA55	SA	na	na	na	na	na
17	SA9	MA	+	+	+	+	+
	SA10	MA	+	+	+	+	+
19	SA11	MA	+	+	+	+	+
	SA12	SA	+	+	+	+	+
22	SA13	MA	+	+	+	+	+
	SA14	MA	+	+	+	+	+
	SA34	SA	+	+	+	+	+
	SA35	MA	+	+	+	+	+
25	SA15	MA	+	+	+	+	+
	SA16	MA	+	+	+	+	+
	SA36	MA	+	+	+	+	+
	SA37	SA	na	na	na	na	na
26	SA17	MA	na	na	na	na	na
	SA18	MA	+	+	+	+	+
	SA38	MA	+	+	+	+	+
	SA39/44	MA	+	+	+	+	+
28	SA19	SA	+	+	+	+	+
	SA20	SA	+	+	+	+	+
	SA46	MA	+	+	+	+	+
	SA48	MA	+	+	+	+	+
36	SA21	WA	+	+	+	+	+
	SA22	WA	+	+	+	+	+
	SA56	MA	na	na	na	na	na
	SA57	MA	+	+	+	+	+
40	SA23	SA	+	+	+	+	+
	SA24	SA	+	+	+	+	+
45	SA25	WA	+	+	+	+	+
	SA26	SA	+	+	+	+	+
	SA50	WA	na	na	na	na	na
	SA51	WA	na	na	na	na	na
53	SA53	MA	na	na	na	na	na

WA, weakly adherent; MA, moderately adherent; SA, strongly adherent; +, biofilm-associated gene detected; na, whole-genome sequence data not available.

Table 5
Sequence types, clonal complexes, antimicrobial resistance genes, and staphylococcal enterotoxin (SE)/SE-like gene profiles of selected *Staphylococcus aureus* isolates subjected to whole-genome sequencing.

Farm ID	Isolate ID	Sequence type	Clonal complex	Antimicrobial resistance genes												Staphylococcal enterotoxin (SE) and SE-like genes																				
				mecA	*tet (M)*	*tet (K)*	*erm (T)*	*erm (C)*	*aac(6′)-aph(2″)*	*blaZ*	*aph (3′)-III*	*lsa (E)*	*lnu (B)*	*dfrG*	*ant (9)-Ia*	*sea*	*seb*	*sec2*	*sec-bovine*	*sed*	*see*	*seg*	*seh*	*sei*	*sel*	*selc4*	*selc-ovine*	*selj*	*selu2*	*selw*	*selx*	*sem*	*sen*	*seo*	*ser*	*sey*
2	SA1	133	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	–	+ c	–	–	+ c	+ c	–	–	–	–	–
	SA2	398	n/a	–	–	–	+ c	–	+ m	+ c	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	–	–	–	–	–	–
4	SA3	133	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	+ c	–	–	–	–	–
	SA4	133	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	+ c	–	–	–	–	–
	SA29	133	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	+ c	–	–	–	–	–
6	SA5	522	n/a	–	+ c	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	+ c	–	–	–	–	–
	SA6	1290	CC1	–	–	–	–	–	–	+ c	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	+ c	–	–	–	–	–
	SA30	2011	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	+ c	–	–	–	–	–
	SA31	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
13	SA7	700	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	–	–	–	–	–	+ c	–	–	–	–	+ c	+ c	–	–	–	–	–
	SA8	2011	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	+ c	–	–	–	–	–
	SA54	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
	SA55	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
17	SA9	700	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	+ c	+ c	–	–	–	–	–
	SA10	700	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	+ c	+ c	–	–	–	–	–
	SA32	133	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	–	+ c	–	+ c	+ c	+ c	–	–	–	–	–
	SA33	133	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	+ c	+ c	–	–	–	–	–
19	SA11	700	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	–	–	–	–	+ c	–	–	–	+ c	+ c	+ c	–	–	–	–	–
	SA12	700	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	–	–	–	–	+ c	–	–	–	+ c	+ c	+ c	–	–	–	–	–
22	SA13	522	n/a	–	+ c	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	+ c	+ c	–	–	–	–	–
	SA14	522	n/a	–	+ c	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	+ c	+ c	–	–	–	–	–
	SA34	522	n/a	–	+ c	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	+ c	+ c	–	–	–	–	–
	SA35	522	n/a	–	+ c	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	+ c	+ c	–	–	–	–	–
25	SA15	72	CC8	–	–	–	–	–	–	+ c	–	–	–	–	–	–	–	–	–	–	–	+ c	–	+ c	–	–	–	+ c	+ c	+ c	+ c	+ c	+ c	–	–
	SA16	45	CC45	–	–	+ n	–	–	–	+ n	–	–	–	–	–	–	–	+ c	–	–	–	+ c	–	+ c	+ c	+ c	–	–	+ c	+ c	+ c	+ c	+ c	+ c	–	–
	SA36	72	CC8	–	–	–	–	–	–	+ c	–	–	–	–	–	–	–	–	–	–	–	+ c	–	+ c	–	–	–	–	+ c	+ c	+ c	+ c	+ c	+ c	–	–
	SA37	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
26	SA17	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
	SA18	3224	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	–	–	–	–	+ c	–	–	–	–	–	–	–	+ c
	SA38	3044	n/a	–	–	–	–	–	–	+ n	–	–	–	–	–	–	–	–	–	+ m	–	–	–	–	–	–	–	+ m	–	+ c	+ c	–	–	–	+ m	–
	SA39/44	1	CC1	+ c	–	+ n	–	+ n	–	+ n	+ m	–	–	–	–	–	–	–	–	–	–	–	+ c	–	–	–	–	–	+ c	+ c	–	–	–	–	–
28	SA19	133	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	–	+ c	–	–	+ c	+ c	–	–	–	–	–
	SA20	133	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	–	+ c	–	–	+ c	+ c	–	–	–	–	–
	SA46	398	n/a	+ c	+ c	+ n	–	–	–	+ c	–	+ c	+ c	+ c	+ c	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	–	–	–	–	–	–
	SA48	133	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	–	+ c	–	–	+ c	+ c	–	–	–	–	–
36	SA21	3044	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ c	+ c	–	–	–	–	–
	SA22	45	CC45	–	–	–	–	–	–	+ n	–	–	–	–	–	–	–	+ c	–	–	–	+ c	–	+ c	+ c	+ c	–	–	+ c	+ c	+ c	+ c	+ c	+ c	–	–

(*continued on next page*)

Table 5 (*continued*)

Farm ID	Isolate ID	Sequence type	Clonal complex	Antimicrobial resistance genes												Staphylococcal enterotoxin (SE) and SE-like genes																				
				mecA	*tet (M)*	*tet (K)*	*erm (T)*	*erm (C)*	*aac(6′)-aph(2″)*	*blaZ*	*aph (3′)-III*	*lsa (E)*	*lnu (B)*	*dfrG*	*ant (9)-Ia*	*sea*	*seb*	sec2	sec-*bovine*	*sed*	*see*	*seg*	*seh*	*sei*	*sel*	*selc4*	*selc-ovine*	*selj*	*selu2*	*selw*	*selx*	*sem*	*sen*	*seo*	*ser*	*sey*
	SA56	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
	SA57	522	n/a	–	+[c]	+[n]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+[c]	+[c]	–	–	–	–	–
40	SA23	133	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+[c]	+[c]	–	–	–	–	–
	SA24	133	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+[c]	+[c]	–	–	–	–	–
	SA49	133	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+[c]	–	–	–	–	+[c]	–	–	–	–	–	–
45	SA25	700	n/a	–	–	+[n]	–	–	–	–	–	–	–	–	–	–	–	–	+[c]	–	–	–	–	–	+[c]	–	–	–	–	+[c]	+[c]	–	–	–	–	–
	SA26	700	n/a	–	–	+[n]	–	–	–	–	–	–	–	–	–	–	–	–	+[c]	–	–	–	–	–	+[c]	–	–	–	–	+[c]	+[c]	–	–	–	–	–
	SA50	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
	SA51	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
53	SA27	133	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+[c]	–	+[c]	–	–	+[c]	+[c]	–	–	–	–	–
	SA28	133	n/a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+[c]	–	+[c]	–	–	+[c]	+[c]	–	–	–	–	–
	SA52	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
	SA53	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
Total isolates				2	7	6	1	1	1	9	1	1	1	1	1	0	0	2	5	1	0	4	2	4	15	2	7	2	3	39	36	4	4	4	1	1

na, sequence data not available; n/a, clonal complex not assigned; +, gene detected; −, gene not detected. Superscript letters following + indicate gene location: [c], chromosome; [m], mobilizable plasmid; [n], non-mobilizable plasmid.

frequently detected, being present in 39/40 (97.5%) and 36/40 (90.0%) isolates, respectively. Other SE-like genes were less frequent, including *sel* in 15 isolates, *selc-ovine* in seven isolates, *sei*, *sem*, *sen*, and *seo* in four isolates each, *selu2* in three isolates, *seh*, *selc4*, and *selj* in two isolates each, and *ser* and *sey* in one isolate each. The detected SE and SE-like genes were chromosomal, with the exception of *sed*, *selj*, and *ser*, which were detected in isolate SA38 and identified on mobilizable plasmid-associated contigs. The isolate SA7, recovered from the cheese sample positive for SEs, carried sec-*bovine* together with *sel*, *selw*, and *selx*.

Finally, all adhesion-tested isolates that were successfully sequenced carried the *ica* operon genes *icaA*, *icaB*, *icaC*, *icaD*, and *icaR* (Table 4).

3.6. Phylogenetic relatedness and isolate-level associations

SNP-based phylogenetic analysis identified several groups of closely related or near-identical *S. aureus* isolates within individual farms (Fig. 3). A cluster comprising SA13, SA14, SA34, and SA35 from farm 22 grouped ST522 isolates recovered from both food and environmental sources and showed very limited pairwise variation, differing by 0–3 SNPs. Similarly, SA15 and SA36 from farm 25, both belonging to ST72 and recovered from food matrices during different sampling seasons, differed by 3 SNPs. Isolates SA3, SA4, and SA29 from farm 4, all belonging to ST133, also clustered together, with pairwise differences of 0–4 SNPs, suggesting the circulation of a farm-associated lineage. The two MRSA isolates from farm 26, SA39 and SA44, clustered together, belonged to ST1, and showed 0 SNP differences, supporting their classification as the same strain profile. Paired or grouped isolates from farms 17, 19, 28, 40, and 53 also showed close phylogenetic relatedness within the same farm. The complete pairwise SNP distance matrix is provided in Supplementary Table 3. Overall, these low SNP distances indicate recent common ancestry and are compatible with within-farm circulation over time. However, they do not distinguish continuous environmental persistence from repeated introduction from a stable animal, human, or environmental reservoir.

In agreement with these observations, exploratory isolate-level analyses showed significant associations between STs and phenotypic AMR profiles, AMR gene profiles, and SE/SE-like gene profiles, while farm ID was significantly associated with SE/SE-like gene profiles (Fig. 4).

4. Discussion

This study provides an integrated microbiological, phenotypic, and genomic assessment of *S. aureus* circulating in small-scale ovine-caprine dairy farms with on-site raw milk processing in Central Italy. Overall, *S. aureus* was detected in almost half of the analysed samples, confirming that this pathogen is widely distributed across food matrices, processing environments, and operator-associated samples in this production setting. The highest occurrence was observed in food samples, particularly curd and finished products, whereas cheese molds/baskets represented the most frequently contaminated environmental surfaces. These findings identify curd handling, molding, and ripening-related steps as critical points for amplification and transfer of *S. aureus* along the production chain, and are consistent with previous observations that direct manual handling, repeated contact with reusable equipment, and non-standardized artisanal procedures can facilitate contamination in small-scale dairy systems (Latorre et al., 2020; Mezher et al., 2023; Papadopoulos et al., 2019).

The significant heterogeneity in observed sample positivity among farms indicates that contamination was unevenly distributed across the investigated production systems. This variability may reflect differences in animal reservoirs, hygiene practices, equipment design, product handling, sanitation procedures, and the composition of the sampled matrices (Kalayu et al., 2020; Papadopoulos et al., 2019). However, because the analysis pooled different matrix types and sampling seasons, these findings should be interpreted as exploratory, unadjusted

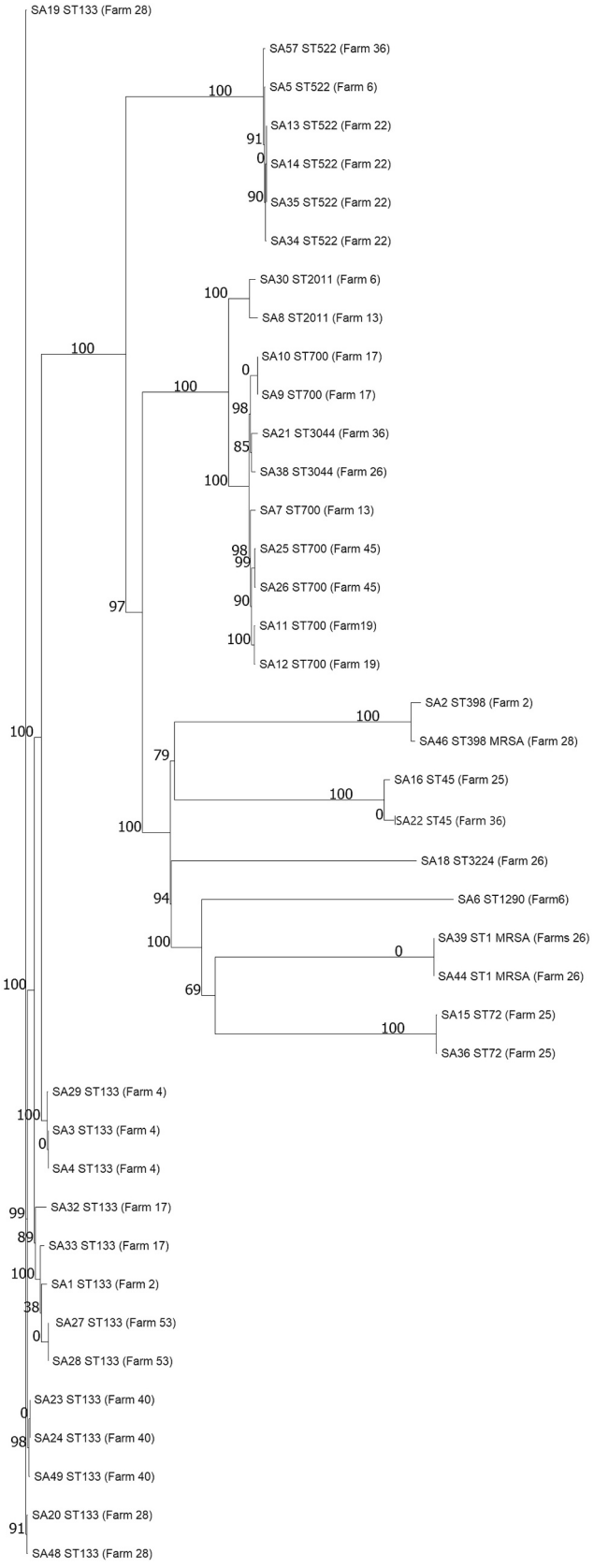


Fig. 3. Phylogenetic relationship of selected *Staphylococcus aureus* isolates based on single nucleotide polymorphism (SNP) analysis.

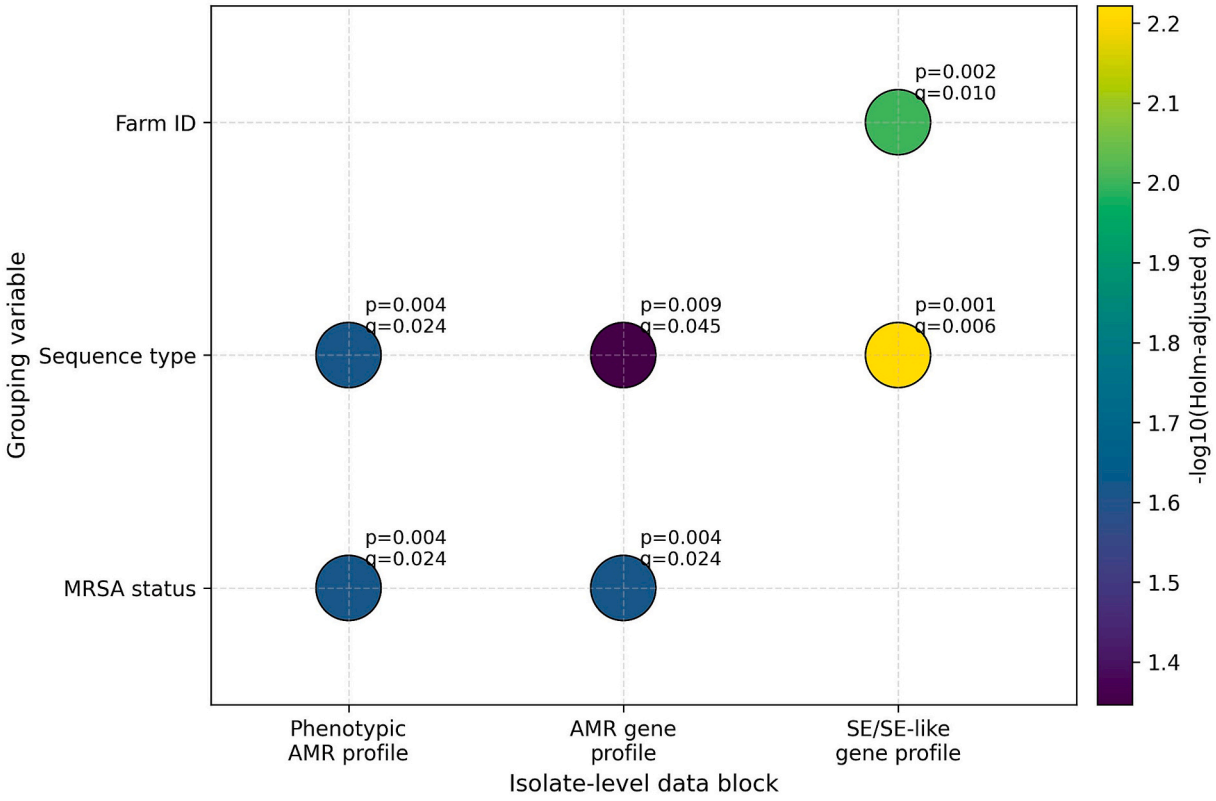


Fig. 4. Significant isolate-level associations detected by exploratory multivariate analyses. Data blocks were analysed as binary matrices and corrected separately using Holm correction. Only significant associations are shown ($q < 0.05$); labels indicate unadjusted p and Holm-adjusted q values. Dot colour reflects the adjusted significance level. AMR, antimicrobial resistance; MRSA, methicillin-resistant *Staphylococcus aureus*; SE, staphylococcal enterotoxin.

comparisons and do not establish causal farm effects. Nevertheless, the magnitude of the observed heterogeneity supports the identification of farm-specific critical contamination points and the implementation of tailored control measures. The seasonal stratification further supported this interpretation, as overall positivity did not differ significantly between warm and cold seasons, and the most extreme farms maintained consistent patterns across both sampling periods. Thus, the observed heterogeneity is more likely to reflect farm-specific management, hygiene, processing practices, and sampled matrices than a general seasonal effect. Similar farm-to-farm variability has been reported in dairy systems and is generally attributed to differences in udder health, animal reservoirs, operator carriage, cleaning procedures, equipment design, and separation between clean and contaminated processing areas (Kalayu et al., 2020; Papadopoulos et al., 2019). Importantly, high *S. aureus* occurrence did not necessarily correspond to complex AMR profiles, as isolates from farm 17 showed limited resistance. Nevertheless, high contamination levels remain relevant from a food safety perspective because they increase the probability of transfer to ready-to-eat products and may create favourable conditions for SE production.

The detection of SE in only one finished product may appear limited, but the finding is relevant from a food-safety perspective because the positive cheese also showed a high *S. aureus* load (6.63 log CFU/g). However, as this represented a single positive sample from one farm, it should not be interpreted as evidence of widespread SE contamination across the production system. Rather, it demonstrates that, under specific farm and product conditions, *S. aureus* can reach high concentrations associated with conditions permissive for enterotoxin production. Staphylococcal food poisoning is typically associated with the ingestion of preformed, heat-stable SEs produced during bacterial growth in food, and high bacterial concentrations in ready-to-eat matrices represent a recognized risk factor (Cieza et al., 2024; Fisher et al., 2018; Hennekinne et al., 2012). The isolate SA7, recovered from the SE-positive cheese, carried sec-*bovine* together with *sel*, *selw*, and *selx*. The sec-*bovine* variant belongs to the staphylococcal enterotoxin C group and has been described as a host-associated type C enterotoxin variant, with relevance in ruminant-associated *S. aureus* and dairy contexts (Marr et al., 1993). Its detection in an isolate recovered from an SE-positive cheese sample is therefore consistent with the presence of a classical SE-associated genetic background. The additional detection of *sel*, *selw*, and *selx* indicates a broader SE/SE-like gene repertoire. Although SE-like proteins have not always been conclusively implicated in emetic intoxication, they may contribute to the pathogenic potential of *S. aureus* because several display superantigenic or immunomodulatory activities and are frequently found alongside classical SE genes in enterotoxigenic strains (Cieza et al., 2024; Fisher et al., 2018). SE and SE-like genes have also been reported in staphylococci from raw milk and raw milk cheeses, confirming that dairy environments can harbour strains with enterotoxigenic potential (Moawad et al., 2025; Wiśniewski et al., 2024). In the present study, most detected SE and SE-like genes showed chromosomal localization, including sec-*bovine*, *sel*, *selw*, and *selx* in SA7. This finding is consistent with the diverse genomic organization of the enterotoxin family, whose genes may occur on chromosomes or on mobile genetic elements depending on the toxin type and strain background (Cieza et al., 2024; Fisher et al., 2018). Notably, isolate SA38 carried *sed*, *selj*, and *ser* on mobilizable plasmid-associated contigs. This is biologically relevant because several SE genes occur on plasmids, prophages, pathogenicity islands, genomic islands, or enterotoxin gene clusters, contributing to variability in enterotoxin gene repertoires among *S. aureus* isolates (Cieza et al., 2024; Fisher et al., 2018). In particular, the co-occurrence of *sed*, *selj*, and *ser* is compatible with previously described plasmid-borne enterotoxin gene combinations, although plasmid-associated localization alone does not demonstrate horizontal transfer under dairy-production conditions (Fisher et al., 2018). Importantly, immunoenzymatic assays used for food testing

detect SEs but do not necessarily identify the specific toxin variant involved, and the presence of an SE or SE-like gene alone does not demonstrate gene expression or toxin production under the conditions of cheese manufacture and storage (Fetsch et al., 2014; Schelin et al., 2011; Wiśniewski et al., 2024). Future studies should therefore evaluate representative isolates carrying classical SE genes under simulated cheese-ripening conditions, combining genomic screening with reverse transcription quantitative polymerase chain reaction (RT-qPCR) to assess enterotoxin gene expression and enzyme-linked immunosorbent assay (ELISA) or liquid chromatography–tandem mass spectrometry (LC-MS/MS) to quantify actual toxin production. Thus, the concordance between SE detection in the cheese and the presence of sec-*bovine* in SA7 supports, but does not prove, the involvement of this isolate in enterotoxin production. Overall, the combination of raw milk processing, high *S. aureus* loads in some matrices, classical SE genes in selected isolates, and plasmid-associated SE/SE-like genes in one isolate supports the need for strict control of bacterial growth during curd handling, early ripening, and storage of short-ripened products.

The low prevalence of MRSA is reassuring but remains important from a One Health perspective. *S. aureus* is a major opportunistic pathogen in humans and animals, and MRSA continues to be one of the most relevant AMR threats globally (World Health Organization, 2024a). In the present study, MRSA was confined to farms in the Marche region and was represented by two distinct genomic backgrounds. SA39/44, recovered from the operator's hand and a cold-room handle in the same farm, belonged to ST1/CC1. The occurrence of the same strain profile on a human-associated sample and an environmental surface is compatible with cross-contamination within the processing environment and supports the role of operators as possible reservoirs or vectors. ST1/CC1 MRSA with human-associated markers has been previously reported in livestock-related contexts, including dairy sheep farms (Alba et al., 2015; Carfora et al., 2016). In contrast, SA46, recovered from a cheese during ripening, belonged to ST398 which is widely recognized as a livestock-associated lineage with the capacity to colonize animals and humans and to circulate across animal, food, environmental, and occupational interfaces (Tian et al., 2025). Its detection is therefore relevant even in the context of low MRSA prevalence, because raw milk dairy products can act as a vehicle for strains carrying clinically important resistance determinants. The distinct genetic backgrounds of SA39/44 and SA46 are consistent with different epidemiological origins rather than a single common MRSA dissemination event. The low MRSA occurrence observed here contrasts with the higher prevalence reported in some Northern and Central European dairy surveys. German studies detected MRSA in 9.7% and 8.5% of conventional dairy-herd bulk-tank milk samples in 2014 and 2019, respectively, with prevalence increasing with herd size and livestock density (Tenhagen et al., 2018; Tenhagen et al., 2023). These differences may partly reflect variation in production intensity, herd structure, antimicrobial exposure, and the regional ecology of livestock-associated MRSA, particularly CC398. In contrast, MRSA carriage was detected in only 1.1% of sheep farmers in an Italian survey (Mascaro et al., 2019), suggesting a more limited MRSA reservoir in comparable small-ruminant systems. This is consistent with the lower occurrence observed in our small-scale ovine–caprine farms and may reflect the combined influence of lower herd density, different production structures, and the local distribution of MRSA lineages.

Phenotypic antimicrobial susceptibility testing revealed a heterogeneous but biologically coherent resistance pattern. Sulfisoxazole resistance was the most frequent MIC-based finding, followed by tetracycline and penicillin resistance. Tetracycline resistance was supported by the detection of *tet(M)* and *tet(K)*, whereas penicillin resistance was mainly associated with *blaZ*. This finding is relevant from a public-health perspective because several of the antimicrobial classes involved, including beta-lactams, tetracyclines, macrolides, aminoglycosides, and sulfonamides, include compounds used in human medicine and are listed among medically important antimicrobials (World Health Organization, 2024b). In particular, *blaZ*-mediated beta-lactamase production may compromise penicillins and aminopenicillins such as ampicillin and amoxicillin, while *mecA* is associated with resistance to most beta-lactams used for staphylococcal infections. Thus, contaminated raw-milk dairy matrices may act not only as a source of resistant *S. aureus*, but also as a potential vehicle for resistance determinants affecting antimicrobial classes of human clinical relevance, although actual transfer or infection risk depends on bacterial viability, exposure, host susceptibility, and processing/storage conditions (European Food Safety Authority and European Centre for Disease Prevention and Control, 2026; World Health Organization, 2024b). The agreement between phenotypic and genomic data was especially evident in multideterminant isolates such as SA39/44 and SA46, which also carried *mecA*, the key determinant of methicillin resistance in *S. aureus*. The usefulness of WGS for predicting AMR phenotypes in *S. aureus* has been previously demonstrated (Gordon et al., 2014; Nurjadi et al., 2021). The absence of *mupA* in all screened isolates is reassuring with regard to high-level mupirocin resistance, as *mupA* is the main determinant associated with high-level resistance. However, continued monitoring is justified in a One Health framework because mupirocin resistance can emerge under selective pressure and has been reported globally (Deepak et al., 2024). The localization of AMR determinants on plasmid-associated sequences increases the relevance of these findings. In particular, *aac(6′)-aph(2″)* and *aph(3′)-III* were located on predicted mobilizable plasmids, *tet(K)* and *erm(C)* were detected on non-mobilizable plasmids; while *blaZ* was found in both chromosomal and plasmid-associated contexts. These genomic findings indicate potential mobility of some resistance determinants. However, horizontal transfer was not experimentally demonstrated and would require functional validation through conjugation or transformation assays. Mobile genetic elements are central drivers of AMR dissemination and may enable resistance determinants to move within complex microbial communities present in raw milk, on processing surfaces, and during cheese manufacture (Partridge et al., 2018). This is particularly important in artisanal raw milk systems, where complex microbial communities can remain viable throughout production and ripening and may provide opportunities for the persistence and potential exchange of AMR genes. Accordingly, the detection of resistance determinants to antibiotics with human therapeutic relevance, even when sporadic, supports the interpretation of raw-milk dairy chains as potential reservoirs and transmission interfaces within a One Health AMR framework, rather than as isolated food-safety findings alone (European Food Safety Authority and European Centre for Disease Prevention and Control, 2026; World Health Organization, 2024b). Therefore, even sporadic detection of mobilizable resistance genes in *S. aureus* should be considered relevant for surveillance in raw milk dairy chains (Alexa et al., 2024; Partridge et al., 2018).

Surface adhesion and subsequent biofilm development may contribute to environmental persistence and recurrent contamination in dairy settings. In the present study, all tested isolates produced detectable adherent biomass on polystyrene, and most were classified as moderately or strongly adherent under the tested static conditions. Both MRSA isolates and the SE-associated isolate SA7 were moderately adherent, indicating that isolates carrying public health-relevant traits also exhibited appreciable surface-attachment potential. However, the crystal-violet microtiter plate assay measures total surface-associated biomass and does not directly demonstrate cell viability or mature biofilm architecture (Azeredo et al., 2017; Stepanović et al., 2007). Furthermore, adhesion to polystyrene cannot be directly extrapolated to stainless steel, wooden molds, rubber milk liners, or other dairy-processing materials, which have different physicochemical properties and surface structures. Surface composition, temperature, milk residues, and sanitation conditions may substantially influence attachment and biofilm development (Di Ciccio et al., 2015; Medina et al., 2025). Therefore, the observed adhesion phenotype should not be considered direct evidence of biofilm formation or persistence on commercial dairy-farm surfaces. Nevertheless, biofilms containing *S. aureus* have been

documented on milk-contact equipment and may act as reservoirs for recurrent milk contamination, supporting careful sanitation of molds and baskets, cold rooms, ripening surfaces, sinks, and handles (Latorre et al., 2020). All adhesion-tested isolates that were successfully sequenced carried the *icaA, icaB, icaC, icaD,* and *icaR* genes, which are associated with polysaccharide intercellular adhesin/poly-*N*-acetylglucosamine-mediated biofilm development. Although these determinants have been associated with stronger biofilm phenotypes in MRSA, their presence alone does not demonstrate gene expression or predict biofilm formation under dairy-processing conditions (Yu et al., 2024). The variation in adherent biomass despite the widespread presence of the *ica* operon therefore suggests an additional influence of gene regulation, strain background, and environmental conditions.

Phylogenetic and exploratory isolate-level analyses supported a predominantly farm-specific structure of *S. aureus* diversity. MLST analysis identified a diverse ST distribution, with ST133, ST700, and ST522 as the most frequent sequence types. This pattern is partly consistent with recent WGS data from small-ruminant mastitis in Sardinia, where ST133 and ST700 were dominant, and with earlier Sardinian ovine-mastitis data identifying CC130/ST700/t1773 as a prevalent lineage (Azara et al., 2017; Moawad et al., 2025). The detection of ST133 is also in line with previous reports from Mediterranean small-ruminant flocks, where this lineage has been described among the main *S. aureus* genetic backgrounds associated with sheep and goat milk or mastitis (Dore et al., 2016; Gelasakis et al., 2015). Overall, the presence of ST133 and ST700 supports the circulation of small-ruminant-associated *S. aureus* lineages in Mediterranean dairy systems, whereas the relatively prominent ST522 component in our dataset may reflect local farm-level or regional variation. Among MRSA isolates, the detection of ST1/CC1 and ST398 is consistent with previous reports of zoonotic MRSA lineages in Italian small-ruminant dairy chains and Mediterranean dairy-farm settings, including bulk-tank milk, farm-related humans, and dairy-farm environments (Caruso et al., 2016; Macori et al., 2017; Papadopoulos et al., 2019). However, ST398 was detected in only two isolates in the present study, contrasting with other geographical areas, particularly Northern Europe, where ST398 has more frequently been reported as a livestock-associated *S. aureus* lineage (Li et al., 2022; Moawad et al., 2023). Overall, our data support a mixed population structure dominated by small-ruminant-associated STs, with sporadic MRSA lineages relevant to the human–animal interface. Closely related isolates were identified within the same farms, sometimes across different matrices and sampling seasons, supporting farm-associated circulation compatible with either local persistence or repeated contamination, rather than isolated contamination events. This pattern may reflect introduction from stable reservoirs, such as colonized animals or operators, survival in inadequately sanitized niches, surface attachment followed by biofilm development under suitable conditions, or a combination of these factors. Consistently, sequence type was significantly associated with phenotypic AMR profiles, AMR gene profiles, and SE/SE-like gene profiles, while farm ID was associated with SE/SE-like gene profiles. Overall, isolate clustering appeared to be driven mainly by genetic background and farm-level factors, supporting the need for farm-tailored control measures.

Previous studies have investigated *S. aureus* at different levels of dairy production systems. In Italy, enterotoxigenic isolates have been reported in raw ovine milk, while methicillin-resistant and methicillin-susceptible strains have been characterized in dairy sheep and in-contact humans, providing evidence of possible intra-farm circulation between animal and human hosts (Carfora et al., 2016; Giacinti et al., 2011). Production-chain studies conducted in Italian artisanal dairies, Greece, Norway, Austria, and Poland have further documented *S. aureus* in raw milk, intermediate and finished products, personnel, equipment, and processing environments using chain-wide microbiological sampling, molecular typing, antimicrobial-susceptibility testing, virulence and adhesion-related characterization, or WGS (Gajewska et al., 2022, 2023; Johler et al., 2018; Jørgensen et al., 2005; Papadopoulos et al., 2018; Walcher et al., 2014). The specific contribution of the present study therefore lies not in the combination of these analytical methods *per se*, but in the insights obtained by integrating them within a multi-farm and two-season investigation of small-scale ovine–caprine raw-milk production chains. This integration showed that contamination was strongly farm-dependent and concentrated at specific processing steps, whereas a high occurrence of *S. aureus* did not necessarily correspond to a greater AMR burden. It also identified distinct MRSA lineages in different epidemiological contexts and genetically related isolates recovered from different matrices and, in some farms, across sampling seasons, findings compatible with farm-associated circulation, recurrence, or persistence. Taken together, these results indicate that surveillance based exclusively on product contamination or AMR prevalence would provide an incomplete assessment and support control measures tailored to the contamination patterns and strain characteristics of individual farms. For small artisanal dairies, such measures may include targeted mastitis management, strict hand and milking hygiene, systematic sanitation of critical surfaces, and simple farm-specific cleaning checklists focused on recurrent contamination points.

5. Conclusions

This study demonstrates that *S. aureus* is widely distributed along ovine–caprine raw-milk dairy chains in Central Italy, with marked heterogeneity in sample positivity among farms. Curd handling, cheese molding, ripening environments, sinks, handles, and manual operations emerged as key points for bacterial transfer or persistence. Although MRSA showed low sample prevalence, the detection of distinct MRSA strain profiles, plasmid-associated AMR determinants, classical SE genes, and surface-adhesion potential under the tested conditions highlights the One Health relevance of *S. aureus* in this production setting. Importantly, the occurrence of resistance determinants affecting antimicrobial classes used in human medicine, including beta-lactams, suggests that raw milk dairy chains may act as potential reservoirs and vehicles of AMR traits with clinical relevance. WGS and pairwise SNP analysis identified genetically related, near-identical *S. aureus* isolates within individual farms across matrices and, in some cases, sampling seasons, supporting farm-associated circulation compatible with either local persistence or repeated contamination from a stable reservoir. Control measures should prioritize production stages and product categories in which *S. aureus* growth may occur during production, particularly curd and other intermediate matrices and short-ripened products. A risk-based, farm-specific approach is therefore recommended, combining control of contamination and bacterial growth at these stages, improved sanitation of recurrently contaminated equipment and environmental surfaces, strengthened milking and handling hygiene, and targeted surveillance for MRSA, enterotoxigenic strains, and clinically relevant AMR determinants. These measures should be adapted to the contamination patterns and critical points identified at each farm.

CRediT authorship contribution statement

David Savelli: Writing – review & editing, Investigation, Formal analysis, Data curation, Conceptualization. **Claudia Gabucci:** Writing – review & editing, Investigation, Formal analysis, Data curation, Conceptualization. **Vesna Milanović:** Writing – original draft, Formal analysis, Data curation. **Di Lullo Stefania:** Writing – review & editing, Investigation, Formal analysis. **Angelo Romano:** Investigation, Formal analysis. **Andrea Osimani:** Visualization, Resources. **Federica Cardinali:** Data curation. **Francesca Romana Massacci:** Writing – review & editing. **Sara Primavilla:** Investigation, Formal analysis. **Lucilla Cucco:** Investigation, Formal analysis. **Marta Paniccià:** Writing – review & editing. **Cinzia Lorenzetti:** Investigation, Formal analysis. **Eleonora Scoccia:** Formal analysis. **Giuliana Blasi:** Writing – review & editing. **Piermario Mangili:** Investigation, Formal analysis. **Fabio Zuccon:**

Investigation, Formal analysis. **Annalisa Petruzzelli:** Writing – review & editing, Resources, Funding acquisition.

Fundings

This work was supported by the Italian Ministry of Health within the framework of the research project "Persistenza di *Staphylococcus aureus* antibiotico-resistenti e potenzialmente tossigeni nella filiera ovi-caprina del territorio umbro-marchigiano: approccio integrato in un'ottica "One Health" (grant number IZSUM 08/2022).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2026.120793.

Data availability

Data will be made available on request.

References

Alba, P., Feltrin, F., Cordaro, G., Porrero, M. C., Kraushaar, B., Argudín, M. A., … Battisti, A. (2015). Livestock-associated methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* sequence type (CC)1 in European farmed animals: High genetic relatedness of isolates from Italian cattle herds and humans. *PLoS One, 10*(8), Article e0137143. https://doi.org/10.1371/journal.pone.0137143

Alexa, E. A., Cobo-Díaz, J. F., Renes, E., O'Callaghan, T. F., Kilcawley, K., Mannion, D., … Alvarez-Ordóñez, A. (2024). The detailed analysis of the microbiome and resistome of artisanal blue-veined cheeses provides evidence on sources and patterns of succession linked with quality and safety traits. *Microbiome, 12*, 78. https://doi.org/10.1186/s40168-024-01790-4

Azara, E., Piras, M. G., Parisi, A., & Tola, S. (2017). Antimicrobial susceptibility and genotyping of *Staphylococcus aureus* isolates collected between 1986 and 2015 from ovine mastitis. *Veterinary Microbiology, 205*, 53–56. https://doi.org/10.1016/j.vetmic.2017.05.006

Azeredo, J., Azevedo, N. F., Briandet, R., Cerca, N., Coenye, T., Costa, A. R., … Sternberg, C. (2017). Critical review on biofilm methods. *Critical Reviews in Microbiology, 43*(3), 313–351. https://doi.org/10.1080/1040841X.2016.1208146

Barritt, M. M. (1936). The intensification of the Voges-Proskauer reaction by the addition of α-naphthol. *Journal of Pathology and Bacteriology, 42*(2), 441–454. https://doi.org/10.1002/path.1700420212

Beshiru, A., Igbinosa, I. H., Akinnibosun, O., Ogofure, A. G., Dunkwu-Okafor, A., Uwhuba, K. E., & Igbinosa, E. O. (2024). Characterization of resistance and virulence factors in livestock-associated methicillin-resistant *Staphylococcus aureus*. *Scientific Reports, 14*, 13235. https://doi.org/10.1038/s41598-024-63963-3

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics, 30*(15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Carattoli, A., Zankari, E., García-Fernández, A., Voldby Larsen, M., Lund, O., Villa, L., … Hasman, H. (2014). In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrobial Agents and Chemotherapy, 58*(7), 3895–3903. https://doi.org/10.1128/AAC.02412-14

Carfora, V., Giacinti, G., Sagrafoli, D., Marri, N., Giangolini, G., Alba, P., Feltrin, F., Sorbara, L., Amoruso, R., Caprioli, A., Amatiste, S., & Battisti, A. (2016). Methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* in dairy sheep and in-contact humans: An intra-farm study. *Journal of Dairy Science, 99*(6), 4251–4258. https://doi.org/10.3168/jds.2016-10912

Caruso, M., Latorre, L., Santagada, G., Fraccalvieri, R., Miccolupo, A., Sottili, R., … Parisi, A. (2016). Methicillin-resistant *Staphylococcus aureus* (MRSA) in sheep and goat bulk tank milk from southern Italy. *Small Ruminant Research, 135*, 26–31. https://doi.org/10.1016/j.smallrumres.2015.12.023

Cieza, M. Y. R., Bonsaglia, E. C. R., Rall, V. L. M., dos Santos, M. V., & Silva, N. C. C. (2024). Staphylococcal enterotoxins: Description and importance in food. *Pathogens, 13*(8), 676. https://doi.org/10.3390/pathogens13080676

Clinical and Laboratory Standards Institute. (2025). *Performance standards for antimicrobial susceptibility testing (CLSI M100, 35th ed.)*.

Cock, P. J. A., Chilton, J. M., Grüning, B., Johnson, J. E., & Soranzo, N. (2015). NCBI BLAST+ integrated into galaxy. *GigaScience, 4*(1), 39. https://doi.org/10.1186/s13742-015-0080-7

Coll, F., Raven, K. E., Knight, G. M., Blane, B., Harrison, E. M., Leek, D., … Peacock, S. J. (2020). Definition of a genetic relatedness cutoff to exclude recent transmission of meticillin-resistant *Staphylococcus aureus*: A genomic epidemiology analysis. *The Lancet Microbe, 1*(8), e328–e335. https://doi.org/10.1016/S2666-5247(20)30149-X

Cortimiglia, C., Bianchini, V., Franco, A., Caprioli, A., Battisti, A., Colombo, L., … Luini, M. (2015). Short communication: Prevalence of *Staphylococcus aureus* and methicillin-resistant *S. Aureus* in bulk tank milk from dairy goat farms in northern Italy. *Journal of Dairy Science, 98*(4), 2307–2311. https://doi.org/10.3168/jds.2014-8923

Dawan, J., Pomseethong, P., Hwang, J. S., Kim, J. K., Lee, S.-S., & Kim, J.-C. (2025). Enhanced eradication of Staphylococcus aureus biofilms by combination disinfectants: Comparative analysis of antimicrobial and anti-biofilm efficacy. *Biotechnology and Bioprocess Engineering, 30*, 862–873. https://doi.org/10.1007/s12257-025-00220-y

Deepak, S. J., Kannan, P., Savariraj, W. R., Ayyasamy, E., Alagesan, S. K. T. M., Ravindran, N. B., … Amachawadi, R. G. (2024). Characterization of *Staphylococcus aureus* isolated from milk samples for their virulence, biofilm, and antimicrobial resistance. *Scientific Reports, 14*, 25635. https://doi.org/10.1038/s41598-024-75076-y

Di Ciccio, P., Vergara, A., Festino, A. R., Paludi, D., Zanardi, E., Ghidini, S., & Ianieri, A. (2015). Biofilm formation by *Staphylococcus aureus* on food contact surfaces: Relationship with temperature and cell surface hydrophobicity. *Food Control, 50*, 930–936. https://doi.org/10.1016/j.foodcont.2014.10.048

Dore, S., Liciardi, M., Amatiste, S., Bergagna, S., Bolzoni, G., Caligiuri, V., … Cannas, E. A. (2016). Survey on small ruminant bacterial mastitis in Italy, 2013–2014. *Small Ruminant Research, 141*, 91–93. https://doi.org/10.1016/j.smallrumres.2016.07.010

European Committee on Antimicrobial Susceptibility Testing. (2025). *MIC and zone diameter distributions and ECOFFs*. Retrieved from https://mic.eucast.org.

European Food Safety Authority, & European Centre for Disease Prevention and Control. (2026). The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2023–2024. *EFSA Journal, 24*(2), Article e9887. https://doi.org/10.2903/j.efsa.2026.9887

European Parliament and Council of the European Union. (2004). Regulation (EC) no. 853/2004 of 29 April 2004 laying down specific hygiene rules for food of animal origin. *Official Journal of the European Union, L139*, 55–205.

Feßler, A. T., Olde Riekerink, R. G. M., Rothkamp, A., Kadlec, K., Sampimon, O. C., Lam, T. J. G. M., & Schwarz, S. (2012). Characterization of methicillin-resistant *Staphylococcus aureus* CC398 obtained from humans and animals on dairy farms. *Veterinary Microbiology, 160*(1–2), 77–84. https://doi.org/10.1016/j.vetmic.2012.05.005

Fetsch, A., Contzen, M., Hartelt, K., Kleiser, A., Maassen, S., Rau, J., Kraushaar, B., Layer, F., & Strommenger, B. (2014). *Staphylococcus aureus* food-poisoning outbreak associated with the consumption of ice-cream. *International Journal of Food Microbiology, 187*, 1–6. https://doi.org/10.1016/j.ijfoodmicro.2014.06.017

Fisher, E. L., Otto, M., & Cheung, G. Y. C. (2018). Basis of virulence in enterotoxin-mediated staphylococcal food poisoning. *Frontiers in Microbiology, 9*, Article 436. https://doi.org/10.3389/fmicb.2018.00436

Gajewska, J., Chajęcka-Wierzchowska, W., & Zadernowska, A. (2022). Occurrence and characteristics of *Staphylococcus aureus* strains along the production chain of raw milk cheeses in Poland. *Molecules, 27*(19), 6569. https://doi.org/10.3390/molecules27196569

Gajewska, J., Chajęcka-Wierzchowska, W., & Zadernowska, A. (2023). Genomic characterisation of the pathogenicity of *Staphylococcus aureus* isolates recovered from the cheese chain production. *International Dairy Journal, 147*, Article 105774. https://doi.org/10.1016/j.idairyj.2023.105774

Gelasakis, A. I., Angelidis, A. S., Giannakou, R., Filioussis, G., Kalamaki, M. S., & Arsenos, G. (2015). Bacterial subclinical mastitis and its effect on milk yield in low-input dairy goat herds. *Journal of Dairy Science, 98*(6), 3698–3708. https://doi.org/10.3168/jds.2014-8747

Giacinti, G., Amatiste, S., Tammaro, A., Sagrafoli, D., Giangolini, G., & Rosati, R. (2011). *Staphylococcus aureus* enterotossinogeni isolati da latte crudo ovino: Enterotoxigenic *Staphylococcus aureus* in sheep raw milk. *Italian Journal of Food Safety, 1*(1zero), 281–282. https://doi.org/10.4081/ijfs.2011.889

Gordon, N. C., Price, J. R., Cole, K., Everitt, R., Morgan, M., Finney, J., … Walker, A. S. (2014). Prediction of *Staphylococcus aureus* antimicrobial resistance by whole-genome sequencing. *Journal of Clinical Microbiology, 52*(4), 1182–1191. https://doi.org/10.1128/JCM.03117-13

Hennekinne, J. A., De Buyser, M. L., & Dragacci, S. (2012). *Staphylococcus aureus* and its food poisoning toxins: Characterization and outbreak investigation. *FEMS Microbiology Reviews, 36*(4), 815–836. https://doi.org/10.1111/j.1574-6976.2011.00311.x

International Organization for Standardization. (2017a). *ISO/IEC 17025:2017. General requirements for the competence of testing and calibration laboratories*. ISO.

International Organization for Standardization. (2017b). In International Organization for (Standardization) (Ed.), *Microbiology of the food chain — Horizontal method for the immunoenzymatic detection of staphylococcal enterotoxins in foodstuffs (ISO 19020: 2017)*.

International Organization for Standardization. (2024). *ISO 7218:2024. Microbiology of the food chain — General requirements and guidance for microbiological examinations*. ISO.

Johler, S., Macori, G., Bellio, A., Acutis, P. L., Gallina, S., & Decastelli, L. (2018). Short communication: Characterization of *Staphylococcus aureus* isolated along the raw milk cheese production process in artisan dairies in Italy. *Journal of Dairy Science, 101*(4), 2915–2920. https://doi.org/10.3168/jds.2017-13815

Jolley, K. A., Bray, J. E., & Maiden, M. C. J. (2018). Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. *Wellcome Open Research, 3*, 124. https://doi.org/10.12688/wellcomeopenres.14826.1

Jørgensen, H. J., Mørk, T., & Rørvik, L. M. (2005). The occurrence of *Staphylococcus aureus* on a farm with small-scale production of raw milk cheese. *Journal of Dairy Science, 88*(11), 3810–3817. https://doi.org/10.3168/jds.S0022-0302(05)73066-6

Kaas, R. S., Leekitcharoenphon, P., Aarestrup, F. M., & Lund, O. (2014). Solving the problem of comparing whole bacterial genomes across different sequencing platforms. *PLoS One, 9*(8), Article e104984. https://doi.org/10.1371/journal.pone.0104984

Kalayu, A. A., Woldetsadik, D. A., Woldeamanuel, Y., Wang, S. H., Gebreyes, W. A., & Teferi, T. (2020). Burden and antimicrobial resistance of *S. Aureus* in dairy farms in Mekelle, northern Ethiopia. *BMC Veterinary Research, 16*(1), 20. https://doi.org/10.1186/s12917-020-2235-8

Latorre, A. A., Pachá, P. A., González-Rocha, G., San Martín, I., Quezada-Aguiluz, M., Aguayo-Reyes, A., … Muñoz, M. A. (2020). On-farm surfaces in contact with milk: The role of *Staphylococcus aureus*-containing biofilms for udder health and milk quality. *Foodborne Pathogens and Disease, 17*(1), 44–51. https://doi.org/10.1089/fpd.2019.2704

Li, T., Lu, H., Wang, X., Gao, Q., Dai, Y., Shang, J., & Li, M. (2022). Molecular characteristics of Staphylococcus aureus causing bovine mastitis between 2014 and 2020 in China. *Frontiers in Veterinary Science, 9*, Article 1003792. https://doi.org/10.3389/fvets.2022.1003792

Lienen, T., Schnitt, A., Cuny, C., Maurischat, S., & Tenhagen, B.-A. (2021). Phylogenetic tracking of LA-MRSA ST398 intra-farm transmission among animals, humans and the environment on German dairy farms. *Microorganisms, 9*(6), 1119. https://doi.org/10.3390/microorganisms9061119

Lim, S.-K., Nam, H.-M., Jang, G.-C., Lee, H.-S., Jung, S.-C., & Kim, T.-S. (2013). Transmission and persistence of methicillin-resistant *Staphylococcus aureus* in milk, environment, and workers in dairy cattle farms. *Foodborne Pathogens and Disease, 10*(8), 731–736. https://doi.org/10.1089/fpd.2012.1436

MacFaddin, J. F. (2000). *Biochemical tests for identification of medical bacteria* (3rd ed.). Lippincott Williams & Wilkins.

Macori, G., Giacinti, G., Bellio, A., Gallina, S., Bianchi, D. M., Sagrafoli, D., … Decastelli, L. (2017). Molecular epidemiology of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* in the ovine dairy chain and in farm-related humans. *Toxins, 9*(5), 161. https://doi.org/10.3390/toxins9050161

Magro, G., Rebolini, M., Beretta, D., & Piccinini, R. (2018). Methicillin-resistant *Staphylococcus aureus* CC22-MRSA-IV as an agent of dairy cow intramammary infections. *Veterinary Microbiology, 227*, 29–33. https://doi.org/10.1016/j.vetmic.2018.10.021

Marr, J. C., Lyon, J. D., Roberson, J. R., Lupher, M., Davis, W. C., & Bohach, G. A. (1993). Characterization of novel type C staphylococcal enterotoxins: Biological and evolutionary implications. *Infection and Immunity, 61*(10), 4254–4262. https://doi.org/10.1128/iai.61.10.4254-4262.1993

Mascaro, V., Squillace, L., Nobile, C. G. A., Papadopoli, R., Bosch, T., Schouls, L. M., … Pavia, M. (2019). Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) carriage and pattern of antibiotic resistance among sheep farmers from southern Italy. *Infection and Drug Resistance, 12*, 2561–2571. https://doi.org/10.2147/IDR.S211629

Medina, C., Manriquez, D., Gonzalez-Córdova, B. A., Pacha, P. A., Vidal, J. M., Oliva, R., & Latorre, A. A. (2025). Biofilm-forming ability of *Staphylococcus aureus* on materials commonly found in milking equipment surfaces. *Journal of Dairy Science, 108*(4), 3382–3389. https://doi.org/10.3168/jds.2024-25416

Mezher, Z., Titarenko, E., Morena, V., Giangolini, G., & Condoleo, R. (2023). Survey on husbandry and cheese manufacturing practices in small ruminants' farmhouse dairies in Central Italy. *Veterinaria Italiana, 59*(3). https://doi.org/10.12834/VetIt.2687.19358.2

Mikheenko, A., Prjibelski, A., Saveliev, V., Antipov, D., & Gurevich, A. (2018). Versatile genome assembly evaluation with QUAST-LG. *Bioinformatics, 34*(13), i142–i150. https://doi.org/10.1093/bioinformatics/bty266

Moawad, A. A., Dore, S., Lollai, S., El-Adawy, H., Brangsch, H., Monecke, S., … Tomaso, H. (2025). Genomic characterization of *Staphylococcus aureus* isolated from mastitis in small ruminants in Sardinia, Italy. *Frontiers in Microbiology, 16*, Article 1661122. https://doi.org/10.3389/fmicb.2025.1661122

Moawad, A. A., El-Adawy, H., Linde, J., Jost, I., Tanja, G., Katja, H., … Tomaso, H. (2023). Whole genome sequence-based analysis of *Staphylococcus aureus* isolated from bovine mastitis in Thuringia. *Germany. Frontiers in Microbiology, 14*, Article 1216850. https://doi.org/10.3389/fmicb.2023.1216850

Nurjadi, D., Zizmann, E., Chanthalangsy, Q., Heeg, K., & Boutin, S. (2021). Integrative analysis of whole genome sequencing and phenotypic resistance toward prediction of trimethoprim-sulfamethoxazole resistance in *Staphylococcus aureus*. *Frontiers in Microbiology, 11*, Article 607842. https://doi.org/10.3389/fmicb.2020.607842

Pal, M., Shuramo, M. Y., Tewari, A., Srivastava, J. P., & Steinmetz, C. H. D. (2023). *Staphylococcus aureus* from a commensal to zoonotic pathogen: A critical appraisal. *International Journal of Clinical and Experimental Medicine Research, 7*(2), 220–228. https://doi.org/10.26855/ijcemr.2023.04.023

Papadopoulos, P., Angelidis, A. S., Papadopoulos, T., Kotzamanidis, C., Zdragas, A., Papa, A., … Sergelidis, D. (2019). *Staphylococcus aureus and methicillin-resistant S. aureus (MRSA) in bulk tank milk, livestock and dairy-farm personnel in north-central and north-eastern Greece: Prevalence, characterization and genetic relatedness*. *Food Microbiology, 84*, Article 103249. https://doi.org/10.1016/j.fm.2019.103249

Papadopoulos, P., Papadopoulos, T., Angelidis, A. S., Boukouvala, E., Zdragas, A., Papa, A., … Sergelidis, D. (2018). Prevalence of *Staphylococcus aureus* and of methicillin-resistant *S. Aureus* (MRSA) along the production chain of dairy products in North-Western Greece. *Food Microbiology, 69*, 43–50. https://doi.org/10.1016/j.fm.2017.07.016

Partridge, S. R., Kwong, S. M., Firth, N., & Jensen, S. O. (2018). Mobile genetic elements associated with antimicrobial resistance. *Clinical Microbiology Reviews, 31*(4), Article e00088-17. https://doi.org/10.1128/CMR.00088-17

Robertson, J., & Nash, J. H. E. (2018). MOB-suite: Software tools for clustering, reconstruction and typing of plasmids from draft assemblies. *Microbial Genomics, 4*(8), Article e000206. https://doi.org/10.1099/mgen.0.000206

Romanò, A., Gazzola, A., Bianchini, V., Cortimiglia, C., Maisano, A. M., Cremonesi, P., … Luini, M. (2020). *Staphylococcus aureus* from goats are genetically heterogeneous and distinct to bovine ones. *Frontiers in Veterinary Science, 7*, 628. https://doi.org/10.3389/fvets.2020.00628

Schelin, J., Wallin-Carlquist, N., Cohn, M. T., Lindqvist, R., Barker, G. C., & Rådström, P. (2011). The formation of *Staphylococcus aureus* enterotoxin in food environments and advances in risk assessment. *Virulence, 2*(6), 580–592. https://doi.org/10.4161/viru.2.6.18122

Stepanović, S., Vuković, D., Dakić, I., Savić, B., & Švabić-Vlahović, M. (2000). A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods, 40*(2), 175–179. https://doi.org/10.1016/S0167-7012(00)00122-6

Stepanović, S., Vuković, D., Hola, V., Di Bonaventura, G., Djukić, S., Ćirković, I., & Ruzicka, F. (2007). Quantification of biofilm in microtiter plates: Overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS, 115*(8), 891–899. https://doi.org/10.1111/j.1600-0463.2007.apm_630.x

Steward, C. D., Raney, P. M., Morrell, A. K., Williams, P. P., McDougal, L. K., Jevitt, L., … Tenover, F. C. (2005). Testing for induction of clindamycin resistance in erythromycin-resistant isolates of *Staphylococcus aureus*. *Journal of Clinical Microbiology, 43*(4), 1716–1721. https://doi.org/10.1128/JCM.43.4.1716-1721.2005

Tenhagen, B.-A., Alt, K., Grobbel, M., & Maurischat, S. (2023). MRSA in bulk tank milk of dairy herds in Germany – Changes over time. *Tierärztliche Praxis. Ausgabe G, Grosstiere/Nutztiere, 51*(2), 63–69. https://doi.org/10.1055/a-2004-1474

Tenhagen, B.-A., Alt, K., Pfefferkorn, B., Wiehle, L., Käsbohrer, A., & Fetsch, A. (2018). Short communication: Methicillin-resistant *Staphylococcus aureus* in conventional and organic dairy herds in Germany. *Journal of Dairy Science, 101*(4), 3380–3386. https://doi.org/10.3168/jds.2017-12939

Tian, X., Zhang, Z., Chang, J., Hou, W., Li, J., & Shi, C. (2025). Driver identification for resistome transmission of food-associated methicillin-resistant *Staphylococcus aureus* ST398. *Food Bioscience, 68*, Article 106517. https://doi.org/10.1016/j.fbio.2025.106517

Timmermans, M., Bogaerts, B., Vanneste, K., De Keersmaecker, S. C. J., Roosens, N. H. C., Kowalewicz, C., … Boland, C. (2022). Large diversity of linezolid-resistant isolates discovered in food-producing animals through linezolid selective monitoring in Belgium in 2019. *Journal of Antimicrobial Chemotherapy, 77*(1), 49–57. https://doi.org/10.1093/jac/dkab376

Walcher, G., Gonano, M., Kümmel, J., Barker, G. C., Lebl, K., Bereuter, O., … Stessl, B. (2014). *Staphylococcus aureus* reservoirs during traditional Austrian raw milk cheese production. *Journal of Dairy Research, 81*(4), 462–470. https://doi.org/10.1017/S0022029914000417

Wick, R. R., Judd, L. M., Gorrie, C. L., & Holt, K. E. (2017). Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Computational Biology, 13*(6), Article e1005595. https://doi.org/10.1371/journal.pcbi.1005595

Wiśniewski, P., Gajewska, J., Zadernowska, A., & Chajęcka-Wierzchowska, W. (2024). Identification of the enterotoxigenic potential of *Staphylococcus* spp. from raw milk and raw milk cheeses. *Toxins, 16*(1), Article 17. https://doi.org/10.3390/toxins16010017

World Health Organization. (2024a). *WHO bacterial priority pathogens list, 2024: Bacterial pathogens of public health importance to guide research, development and strategies to prevent and control antimicrobial resistance*. World Health (Organization).

World Health Organization. (2024b). *WHO list of medically important antimicrobials: A risk management tool for mitigating antimicrobial resistance due to non-human use*. World Health Organization.

Yu, J., Han, W., Xu, Y., Shen, L., Zhao, H., Zhang, J., … Yu, F. (2024). Biofilm-producing ability of methicillin-resistant *Staphylococcus aureus* clinically isolated in China. *BMC Microbiology, 24*, 241. https://doi.org/10.1186/s12866-024-03380-8

Zhang, K., Sparling, J., Chow, B. L., Elsayed, S., Hussain, Z., Church, D. L., … Conly, J. M. (2004). New quadriplex PCR assay for detection of methicillin and mupirocin resistance and simultaneous discrimination of *Staphylococcus aureus* from coagulase-negative staphylococci. *Journal of Clinical Microbiology, 42*(11), 4947–4955. https://doi.org/10.1128/JCM.42.11.4947-4955.2004