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Staphylococcus aureus and methicillin-resistant staphylococci and mammaliicocci in the bulk tank milk of dairy cows from a livestock-dense area in northern Italy

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ABSTRACT

Staphylococcus aureus is the main etiologic agent of contagious dairy cow mastitis, while non-*aureus* staphylococci and mammaliicocci (NASM) are the bacteria most frequently isolated from milk. Beyond their impact on animal health, NASM can harbor antimicrobial resistance (AMR) genes with potential for bidirectional transfer with *S. aureus*, and methicillin-resistant (MR) staphylococci (MRS) can raise significant One Health concerns. In our study, we evaluated the prevalence and characteristics of MRS in the bulk tank milk (BTM) of 88 dairy farms in the livestock-dense province of Lodi, Lombardy, northern Italy. *S. aureus* was isolated from 32.95 % of BTM samples, with the Ribosomal Spacer PCR (RS-PCR) genotype B being the most prevalent, identified in 37.93 % of *S. aureus* positive farms. All isolates carried the *ica* genes (*ica*A, *ica*B, *ica*C, *ica*D) indicating the potential to produce biofilm. MRS were isolated in 56.81 % of farms. According to MALDI-TOF MS analysis, the most prevalent MR species included *S. epidermidis* (MRSE, 35.59 %) followed by *S. aureus* (MRSA, 18.64 %), *M. sciuri* (15.25 %), *S. saprophyticus* (11.86 %), *S. borealis* (6.78 %), *S. haemolyticus* (5.08 %), *M. fleurettii*, (3.39 %), *S. cohnii*, and *S. pettenkoferi* (1.70 % each). Most MR isolates carried the *mec*A gene, while none carried *mec*C. The staphylococcal cassette chromosome *mec* (SCC*mec*) was predominantly type V in MRSA (45.45 %) and type IV in MRSE (61.90 %). Given their relevance to One Health, monitoring AMR in all staphylococci and mammaliicocci isolated from milk is essential for understanding the prevalence, characteristics, and transmission dynamics of MR gene pools within dairy herds.

1. Introduction

Staphylococcus aureus is considered the most relevant major pathogen causing contagious mastitis in dairy cows. The highly variable cure rates and the consequent increase in public health risks are also linked to the growing phenomenon of antimicrobial resistance (AMR) (Abdi et al., 2018; Chandrasekaran et al., 2014). Methicillin-resistant *S. aureus* (MRSA) may show resistance to different beta-lactam antibiotics, limiting treatment options. This resistance is primarily due to the presence of staphylococcal cassette chromosome *mec* (SCC*mec*) elements that carry *mec* genes (*mecA/mecC*), conferring resistance to methicillin and nearly all other beta-lactam antibiotics (Abdelbary et al., 2017; Cheng and Han, 2020). In addition to being a significant problem for animal productions, these microorganisms belong to the ESKAPE group

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which is significant from a One Health perspective, due to their role in multi-drug resistant (MDR) infections in humans (De Oliveira et al., 2020; Mulani et al., 2019). Isolates of *S. aureus* from cow's milk have demonstrated resistance to multiple antimicrobials (Kumar et al., 2011). So far, the low prevalence of MRSA in dairy herds and the heat treatment of milk before human consumption has suggested a low risk of food-borne zoonotic infections with bovine MRSA strains (Schnitt and Tenhagen, 2020). In the last fifteen years, surveys using bulk tank milk (BTM) culture have revealed significant regional variations in MRSA prevalence, ranging from 0.3 % in the United Kingdom, based on 1500 BTM samples collected during 7 months (Paterson et al., 2012), to 4.4 % in Germany based on 768 BTM samples collected during 2 years (Kreausukon et al., 2012). Locatelli et al. (2016) evaluated the BTM from 224 *S. aureus*-positive dairy farms located in the farming district of northern Italy including the provinces of Brescia, Bergamo, and Mantova, detecting a prevalence of MRSA-positive herds of 4 %. In 2010, Benedetti and colleagues reported a non-negligible presence of MRSA among *S. aureus* isolated from bovine milk in the farms of a wider area (Po Valley) with similar characteristics. According to their data, 55 *S. aureus* strains from 20 different farms (13 % of the strains examined) carried the *mec*A gene. In the province of Lodi, the percentage of *mec*A positive *S. aureus* strains ranged from 17 % to 23 % between 1998 and 2008, suggesting an increase in prevalence and prompting additional studies.

Non-*aureus* staphylococci and mammaliicocci (NASM), formerly defined as coagulase-negative staphylococci (CNS or CoNS), are the bacterial group most frequently isolated from cow's milk (De Buck et al., 2021; Vanderhaeghen et al., 2014). These bacteria are often reported as physiological colonizers of the mammary gland and are a core component of the mammary gland microbiota (Addis et al., 2016; Oikonomou et al., 2020). While NASM can also cause intramammary infection (IMI), they are generally not assessed for antibiotic resistance because they are perceived as minor pathogens and antimicrobials are rarely used for their treatment. Nonetheless, NASM may serve as reservoirs for virulence and AMR genes. Similar mobile genetic elements have been detected in methicillin-resistant (MR) NASM and MRSA, suggesting that resistance genes could be bidirectionally transferred between MR NASM and *S. aureus* within a dairy farm (Schnitt et al., 2021). In Switzerland, 62 % of 211 BTM samples were reported to carry MR NASM (Huber et al., 2011). In Germany, MR NASM were found in 42.1 % of 19 BTM samples from preselected MRSA-positive herds (Schnitt et al., 2021). In Serbia, 24 % and 3.4 % of 283 BTM samples were positive for MR NASM and MRSA, respectively (Kos et al., 2023). In the US, the prevalence seems lower; a study found MR NASM in only 2.4 % BTM samples among 288 investigated conventional and organic farms (Cicconi-Hogan et al., 2014) and a more recent one (Goncalves et al., 2023) found a prevalence of 4.3 %, in absence of MRSA, over 300 dairy farms in Indiana, Ohio, and Michigan (Goncalves et al., 2023). In the UK, 4.1 % of BTM samples from 363 dairy farms carried MR NASM (Fisher and Paterson, 2020). Therefore, significant regional differences may exist in the prevalence of these microorganisms.

Data concerning MR NASM in dairy cows in Italy are limited. Most dairy farms are concentrated in the northern regions, with Lombardy accounting for approximately 46 % of the total national production from January to August 2023. With 4,082,355 tons delivered (https://www.clal.it/?section=quadro_lombardia), it is one of the European areas with the largest milk production volumes. In 2020, Lombardy had one of the highest stocking densities in Europe (> 4 units/ha, Eurostat), alongside being one of the most densely populated Italian and European regions (418/Km^2). Therefore, monitoring pathogens and AMR becomes even more relevant from a One Health perspective. Testing of BTM samples offers a practical and cost-effective approach easily implemented for disease surveillance on dairy farms (Nobrega et al., 2023).

With these premises, our study aimed to provide updated information on *S. aureus* and MRSA in the bulk tank milk from dairy cows in a densely populated, livestock-dense European region. In consideration of their role as minor pathogens, of their potential as resistance reservoirs, and of the limited information available in the literature, we also investigated and characterized MR *S. epidermidis* (MRSE) and other MR NASM.

2. Materials and methods

2.1. Milk samples and data collection

All the dairy cattle farms considered in this study ($n = 88$) were in the province of Lodi, an area of the Po valley of the Lombardy region, northern Italy (Fig. 1A). It is one of the major dairy production areas in Italy characterized by a high density of cattle farms, mainly of the Italian Friesian breed with an intensive production system. In Lombardy, the National Livestock Registry (https://www.vetinfo.it/, consulted in December 2022) records 5274 dairy farms housing 1,118,998 animals, accounting for 21.5 % and 42.4 %, respectively, of all farms and animals in Italy. As for the province of Lodi, 348 dairy cattle farms with a total of 107,845 animals are reported. Between October and November 2022, a convenience sample of 88 dairy herds was selected based on a list from the Regional Breeders Association of Lombardy and willingness to participate in this study, thus covering 26.83 % (88/328) of all dairy farms located in the selected province. One BTM sample was collected on each farm from the milk tank during routine mandatory quality controls, with the cooperation of the Regional Breeders Association of Lombardy, for a total of 88 BTM samples (one per farm). The samples were placed individually in plastic containers, labeled with the name and farm code, transported refrigerated (+4 °C) to the laboratory, and stored at −20 °C until microbiological analyses. Data on sampled herds were obtained from the managerial farm system Si@lleva (Italian Breeder Association, www.sialleva.it). For each dairy herd, data on geographical localization, herd consistency, parity, and medium age of the cows in the herd, were recorded. Data concerning the reproductive performances (i.e., number of inseminations for conception and period from calving to conception) and milk production (i.e., daily milk yield, 305-mature equivalent milk, % of fat and protein content in milk, and somatic cell count) were also recorded.

2.2. Milk bacteriology

For *S. aureus* isolation, 100 μL of BTM was seeded in duplicate on Baird Parker agar with rabbit plasma fibrinogen (BPA-RPF) (Microbiol, Cagliari, Italy). The identification of MRS was performed as described previously (Tenhagen et al., 2014) with minor modifications. For detection of methicillin-resistant (MR) staphylococci and mammaliicocci, 1 mL of BTM was incubated in 5 mL of Mueller Hinton broth (Oxoid, Basingstoke, UK) with 6.5 % NaCl for 18–20 h at 37 °C; after that, 100 μL and 10 μL of the enrichment were seeded on two selective Chromogenic MRSA (CMR) agar plates (Laboratorios Conda, Madrid, Spain). Isolated colonies were subjected to identification by MALDI-TOF mass spectrometry as described previously (Rosa et al., 2022). On colonies grown in BPA-RPF, a presumptive identification by MALDI-TOF MS was performed based on *S. aureus* typical morphology. Then, up to 5 colonies identified as *S. aureus* with high confidence (log score > 2.00) were reisolated to be frozen. All the colony forming units on both plates per sample were counted according to the *S. aureus* typical morphology. On CMR agar, Identification by MALDI-TOF MS was performed for colonies of the prevalent morphology and one colony per MRS species identified was re-isolated and archived. Most isolates were identified with high confidence (log score > 2.00) but two *S. fleurettii* and five *M. sciuri*, for which the identification log score was between 1.70 and 2.00. Isolates were stored at −80 °C with the Cryobank beads system for subsequent genotyping and molecular characterization.

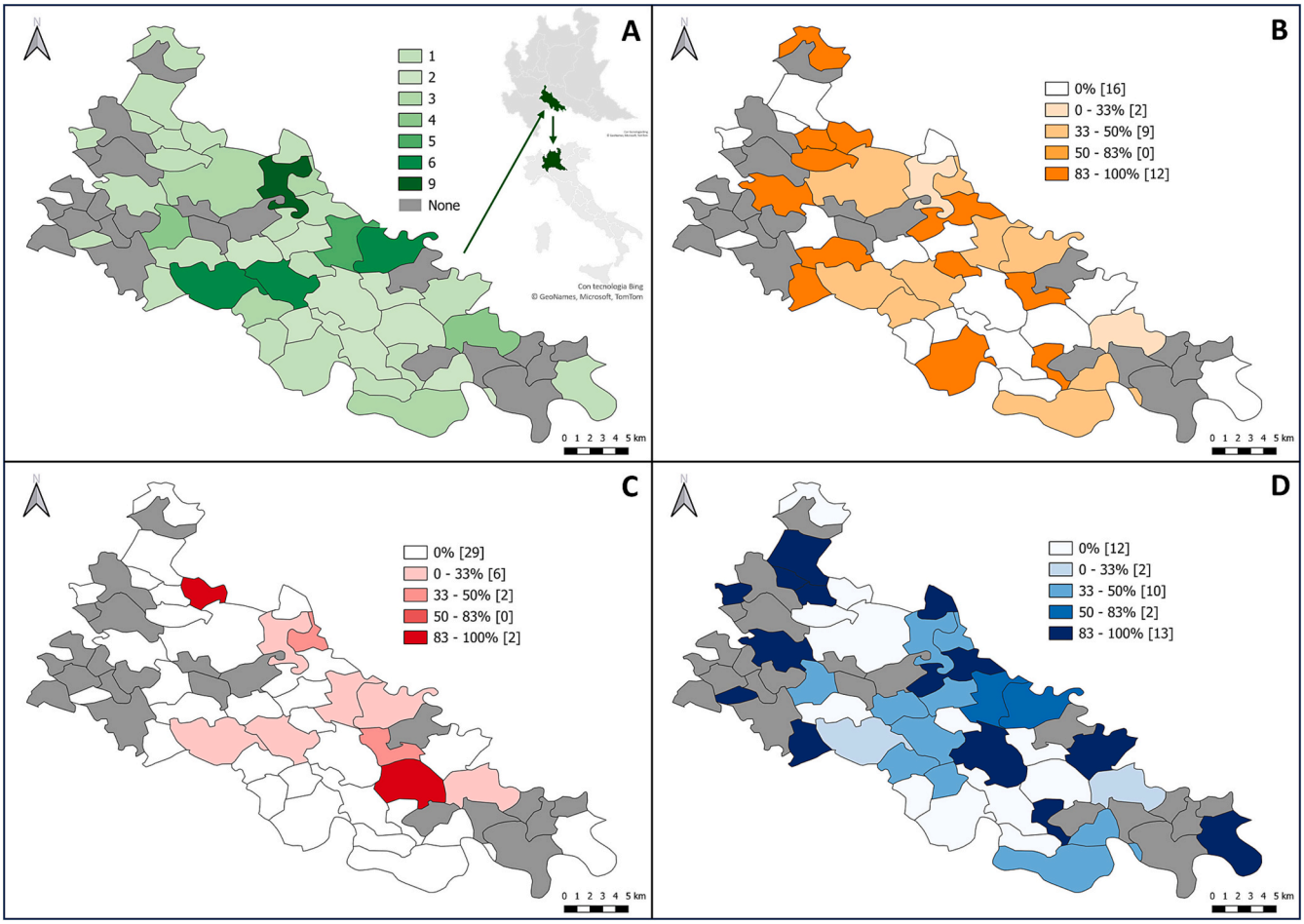

Fig. 1. Choropleth maps of the bacteriological results obtained for the 88 farms according to the municipalities of the Lodi province. A, Sampled farms. B, Farms positive for *Staphylococcus aureus*. C. Farms positive for phenotypically methicillin-resistant *S. aureus* (MRSA). D. Farms positive for phenotypically methicillin-resistant non-*aureus* staphylococci and mammaliicocci (MR NASM). The legends indicate the number of sampled farms (A) and the proportion of farms that were culture-positive for *S. aureus* (B), MRSA (C), and MR NASM (D), respectively. The location of the Lodi province within the Lombardy region and Italy is indicated in the top right of Fig. 1A.

2.3. Molecular characterization of S. aureus isolates

The DNA of *S. aureus* and MRSA isolates was extracted as previously described by Cremonesi et al. (2006). This method is based on the combination of a chaotropic agent, guanidium thiocyanate, with silica particles to obtain bacterial cell lysis and nuclease inactivation. After the measurement of its amount and quality by a NanoDrop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA), DNA was stored at −20 °C until use. The genotypes of *S. aureus* and MRSA isolates were determined by Ribosomal Spacer PCR (RS-PCR) (Cremonesi et al., 2015; Fournier et al., 2008; Graber et al., 2009). Each PCR reaction contained 12.5 μL of HotStarTaq Master Mix 2× (Qiagen, Hilden, Germany), 800 nM of each primer (G1 and L1), and 7 μL of DNA (originally extracted DNA diluted 1:100 in water) in a total reaction volume of 25 μL. Amplifications were carried out in a thermocycler (Bio-Rad, Segrate, Italy). A pre-PCR step was run at 95 °C for 15 min, followed by 27 cycles comprising denaturation at 94 °C for 1 min, a 2 min ramp, annealing at 55 °C for 7 min, a further 2 min ramp, and extension at 72 °C for 2 min; PCR was terminated by incubating at 72 °C for 10 min, followed by cooling down to 4 °C. One μL of each PCR product was then analysed by the miniaturized electrophoresis system (2100 Bioanalyzer system, Agilent, Santa Clara, CA, USA), as described by the manufacturer. Genotypes were identified by a freely available computer program developed in-house, calculating the corresponding Mahalanobis distance of informative peak sizes and comparing it to those of the reference strains using the "Mahalanobis Distances of *Staph.aureus* Genotypes" software (Syring et al., 2012). For the interpretation of the results, an electrophoretic pattern differing in only one band from a known genotype was considered as a genotypic variant, and genotypes with their variants were combined into Genotypic Clusters (CL) (Cosandey et al., 2016). The expression of genes involved in biofilm formation (*ica*A, *ica*D, *ica*B, *ica*C) was measured by qPCR as described previously (Federman et al., 2016) by selecting two colonies of each genotype detected in the farm. Reactions were carried out on a CFX Connect Real-Time PCR Detection System (Bio-Rad) in 15 μL total volume. Each reaction contained EvanGreen mix (Bio-Rad) and specific primers. GyrB was selected as the reference gene for normalization purposes. Each sample was tested in duplicate. Non-reverse transcribed controls and no template controls were included in the assays. The thermal profile was: 50 °C for 2 min, 95 °C for 5 min followed by 40 cycles at 95 °C for 10 s, 60 °C for 30 s. To assess melting curves, PCR products were incubated at 55 °C for 60 s, and the temperature was then increased to 95 °C at 0.5 °C increments for 10 s. All *S. aureus*, MRSA, MRSE, and other MR NASM isolates were tested for the presence of *mec*A and *mec*C genes by PCR using primers and amplification conditions as previously described (Monistero et al., 2020). All PCR reactions were carried out by using a thermocycler (Bio-Rad), in 0.2-mL tubes containing 12.5 μL of PCR Master Mix 2× (Thermo Scientific™), 0.2 μL of each primer (100 μM), 2 μL of genomic DNA (5 ng/μL) or sterile water for negative controls, and sterile water in a total reaction volume of 25

µL. As positive controls, *S. aureus* reference strain ATCC 700699 was used for *mecC* and IZSLER2 STAU26, previously isolated from the collection of IZSLER (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna) and analysed by molecular tests, was used for *mecA* in the PCR assays. All amplified PCR fragments were visualized on 2 % agarose gel electrophoresis (GellyPhor, Euroclone, Milan, Italy), stained with MIDORI Green Advance (Resnova, Rome, Italy), and visualized by iBright™ CL1500 Imaging System (Thermo Fisher Scientific, Life Technologies Corporation, Singapore). A 100 bp DNA ladder (Finnzymes, Espoo, Finland) was included in each gel.

MRSA, MRSE, and other MR NASM were typed and sub-typed for the SCC*mec* cassette by a PCR-based multiplex assay as described previously (Kondo et al., 2007; Longheu et al., 2021). The SCC*mec* nomenclature was as proposed by the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC), 2009). For brevity, the type is indicated by Roman numerals, the subtype, identified by a lower-case Latin letter. The *ccr* and *mec* gene complexes are indicated by an Arabic number and Latin letter, respectively, in parentheses.

2.4. Antimicrobial susceptibility testing

The phenotypic confirmation of methicillin resistance was carried out by the agar disk diffusion (ADD) method (John et al., 2009) on the isolates selected from CMR agar. The disk diffusion test was performed according to the procedure described in the Clinical and Laboratory Standards Institute (CLSI) guidelines Vet01S 5th edition (Clinical and Laboratory Standards Institute, 2020) with cefoxitin as marker of methicillin resistance (CLSI, 2013b). Oxacillin was also included for improved sensitivity and specificity (Khairullah et al., 2023; Wannet, 2002). Oxacillin disc diffusion can also be used for *S. epidermidis* (CLSI, 2020). *S. aureus* ATCC 25923 was used as a quality control strain according to the CLSI guidelines. The following antibiotic discs (Oxoid, Basingstoke, UK) were used to assess antimicrobial susceptibility: amoxicillin-clavulanic acid (AMC; 30 µg), cefoperazone (CFP; 75 µg), ciprofloxacin (CIP; 5 µg), ceftiofur (CTF; 30 µg), cefoxitin (FOX; 30 µg), gentamycin (GEN; 10 µg), kanamycin (KAN; 30 µg), oxacillin (OX; 5 µg), penicillin (PEN; 10 ui), trimethoprim-sulfamethoxazole (SXT; 25 µg), and tetracycline (TET; 30 µg). Isolates were classified as susceptible (S), increased exposure (I), or resistant (R) based on inhibition zone diameters using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) v.13.0 (PEN, GEN, KAN, CIP, SXT) or CLSI 2020 (AMC, CFP, CTF, FOX, OX, TET) breakpoint values. All MRSA and MRS were considered as multidrug resistant (MDR) (Magiorakos et al., 2012).

2.5. Data analysis and visualization

Data were entered and processed in Microsoft Excel 2016. Choropleth maps were elaborated and generated with QGIS Geographic Information System Version 3.34 Prizren, available at: http://qgis.org. Shapefiles were from Istituto Nazionale di Statistica and are available at: https://inspire-geoportal.ec.europa.eu/srv/api/records/istat:0000 21:20230411:123418?language=all. Farm data were analysed to determine which variables could be risk factors of the occurrence of *S. aureus* and MR staphylococci and mammaliicocci. Herd was considered as the statistical unit. Generalized linear models with binomial distribution were performed, and the binary outcome (presence/absence of *S. aureus* and presence/absence of MR staphylococci and mammaliicocci) based on milk bacteriology results were used as the dependent variables ($n = 88$). The following variables, i.e. herd consistency (determined as the total number of animals in the farm at the time of sampling), medium age, reproductive (number of inseminations for conception and the period from calving to conception), productive (daily milk yield, 305ME), and milk qualitative parameters (% of fat and protein content and milk somatic cell count - SCC) were separately considered and entered in each model as independent variables. Subsequently, all the variables with a P value ≤ 0.25 were inserted in a multivariate model developed through a backward selection procedure (significance level to remove variables from the model = 0.25) based on Akaike information criterion (AIC) values. Statistical analysis was performed using SPSS software (Statistical Package for Social Science, IBM SPSS Statistics for Windows, 25.0, Chicago, IL, USA).

3. Results

3.1. S. aureus and genotype distribution

Of 88 dairy farms distributed throughout the province of Lodi, 29 (32.95 %) tested positive for *S. aureus* upon screening of BTM on BPA-RPF. Sampled farms and *S. aureus*-positive farms are illustrated as choropleth maps in Fig. 1A and B, respectively. Of the positive 29 BTM samples, 17 had counts in the range of 10–100 colony-forming units (CFU)/mL, 7 between 101 and 1000 CFU/mL, and 5 greater than 1000 CFU/mL.

By RS-PCR genotyping, the 125 *S. aureus* colonies isolated from the 29 positive farms belonged to 15 different clusters including 23 different genotypes (Table 1). Cluster B (CLB) was the most represented with Genotype B (GTB) being the most frequent, accounting for 31 of the 125 *S. aureus* isolates (24.80 %) and present in seven out of 29 farms (24.13 %). In 23 farms, all the *S. aureus* colonies isolated and typed belonged to the same genotype, while two farms had two different subtypes of the same genotype (R and R^{I}; C and C^{II}), and four farms had two different genotypes (AF^{I} and BS^{I}; Z and CF; BIII and R; BS^{II} and R^{VII}). Out of 125 *S. aureus* isolates, eight (6.40 %) carried *mecA*. Five of them belonged to genotype CLQ and had been isolated from a single farm. The remaining three belonged to CLCF, CLS and AF I, each isolated from a single, different farm (Table 1). None of them carried *mec*C.

All the tested *S. aureus* isolates carried the *ica* genes (*ica*A, *ica*B, *ica*C, *ica*D), indicating the potential to produce biofilm.

3.2. Methicillin-resistant staphylococci and mammaliicocci

Following BTM enrichment and culture on CMR, presumptive MR colonies were observed in 50 out of 88 milk samples (56.81 %). Nine different species were identified by MALDI-TOF MS, for a total of 59

Table 1
RS-PCR genotypes of the 125 *S. aureus* isolated from the 29 positive bulk tank milk samples.

Cluster	Genotypes	*mecA*[a]	N. of isolates (%)[b]	N. of farms (%)[c]
CLB	B (24), B^{III} (7)	0 (0.00 %)	31 (24.80 %)	7 (24.13 %)
CLR	R (9), R^{I} (3), R^{IV} (5), R^{VII} (1)	0 (0.00 %)	18 (14.40 %)	6 (20.69 %)
CLC	C (1), C^{II} (1), C^{V} (11)	0 (0.00 %)	13 (10.40 %)	4 (13.79 %)
CLZ	Z (13)	0 (0.00 %)	13 (10.40 %)	5 (17.24 %)
CLBS	BS (4), BS^{I} (4), BS^{II} (1)	0 (0.00 %)	9 (7.20 %)	3 (10.34 %)
CLA	A^{I}	0 (0.00 %)	5 (4.00 %)	5 (17.24 %)
CLAU	AU	0 (0.00 %)	5 (4.00 %)	1 (3.45 %)
CLM	M^{I}	0 (0.00 %)	5 (4.00 %)	1 (3.45 %)
CLO	O	0 (0.00 %) 5[d] (17.24 %)	5 (4.00 %)	1 (3.45 %)
CLQ	Q	5 (4.00 %)	1 (3.45 %)	
CLS	S	1 (3.45 %)	5 (4.00 %)	1 (3.45 %)
CLBQ	BQ^{I}	0 (0.00 %)	4 (3.20 %)	1 (3.45 %)
CLCF	CF	1 (3.45 %)	4 (3.20 %)	1 (3.45 %)
CLY	Y	0 (0.00 %)	2 (1.60 %)	1 (3.45 %)
CLAF	AF^{I}	1 (3.45 %)	1 (0.80 %)	1 (3.45 %)

[a] Percent of isolates *mecA*-positive belonging to the cluster on the total number of isolates.
[b] Percent of isolates belonging to the cluster on the total number of isolates.
[c] Percent of farms harboring a *S. aureus* belonging to the cluster.
[d] All isolates were from the same farm.

isolates (Table 2). The farms positive for MRSA and MR NASM are illustrated as choropleth maps in Fig. 1C and Fig. 1D, respectively. The most frequently identified was MRSE (21, 35.59 %) followed by MRSA (11, 18.64 %). We also isolated MR *M. sciuri* (9, 15.25 %), *S. saprophyticus* (7, 11.86 %), *S. borealis* (4, 6.78 %), *S. haemolyticus* (3, 5.08 %), *M. fleurettii, (2, 3.39 %), S. cohnii*, and *S. pettenkoferi* (1, 1.70 % each). Nine farms had more than one MR species isolated (Table 2, highlighted in bold). Interestingly, four of the total 11 MRSA were isolated in CMR from farms where *S. aureus* had not been detected in the primary screening on BPA. There were no statistically significant associations between the occurrence of culturing *S. aureus*, MRSA, MRSE, and other MR staphylococci and mammaliicocci in the BTM samples and the tested herd level variables (Supplementary File 1).

3.3. Molecular characteristics of methicillin-resistant isolates

The PCR for *mec*A was negative in five out of 59 isolates, including three *S. aureus*, one *M. fleurettii*, and one *S. pettenkoferi*. Two *mec*A-negative MRSA carried the SCC recombinase C gene (*ccr*C), while one *mec*A-negative MRSA and the two *mec*A-negative MR NASM did not carry any of the genes assessed in the cassette typing panel. None of the isolates was positive for *mec*C. Of 11 MRSA, five (45.45 %) belonged to GTS, two (18.18 %) to GTBQ^{I}, and one each (0.91 %) to GTAF^{I}, GTA^{I}, GTQ, and GTZ. Three of the five GTS MRSA were isolated from BTM that was negative to the BPA-RPF screening. Table 2 lists the *mec* gene and SCC*mec* type detected in all MR staphylococci isolated from BTM upon culture on CMR. The main MRSA genotype was GTS (5 of 11, 45.45 %). The majority of MRSA carried SCC*mec* type V (5 out of 11, 45.45 %); four of them belonged to GTS. The majority of MRSE carried type IV SCC*mec* (13/21, 61.90 %), followed by type II and V (2/21 each, 9.52 %). Considering all MR staphylococci and mammaliicocci, the most prevalent SCC*mec* was type IV (14), followed by type V (9).

Table 2
Table listing the 59 methicillin-resistant staphylococci and mammaliicocci isolated and characterized in this study.

Species (MRSA genotype)	*mec*	SCC*mec*	N	Farm ID
S. epidermidis	A	IV	13	18, 48, 55, 58, 60, 66, **71**, 72, **94**, 99, 100, 105, **111**
S. epidermidis	A	V	2	9, 33
S. epidermidis	A	II	2	5, **85**
S. epidermidis	A	N. T.	4	63, 38, 46, 20
Total *S. epidermidis*			**21**	
S. aureus (S)	A	V	4	56, 67, 92, 106
S. aureus (BQ^{I})	A	V	1	19
S. aureus (A^{I})	A	IV	1	**94**
S. aureus (BQ^{I})	A	N.T.	1	104
S. aureus (AF^{I})	A	N.T.	1	**113**
S. aureus (Z)	-	*ccrC*	1	**71**
S. aureus(Q)	-	*ccrC*	1	**85**
S. aureus (S)	-	–	1	103
Total *S. aureus*			**11**	
M. sciuri	A	N.T.	5	57, **112**, **113**, 116, 51
M. sciuri	A	III	3	**53**, 77, 95
M. sciuri	A	II	1	47
Total *M. sciuri*			**9**	
S. saprophyticus	A	VII	2	69, 86
S. saprophyticus	A	N.T.	2	54, 115
S. saprophyticus	A	I	1	96
S. saprophyticus	A	II	1	**13**
S. saprophyticus	A	III	1	**47**
Total *S. saprophyticus*			**7**	
S. borealis	A	I	3	59, 110, 43
S. borealis	A	V	1	30
Total *S. borealis*			**4**	
S. haemolyticus	A	V	1	25
S. haemolyticus	A	N.T.	1	**13**
S. haemolyticus	A	N.T.	1	**53**
Total *S. haemolyticus*			**3**	
M. fleurettii	A	N.T.	1	**112**
M. fleurettii	-	–	1	**47**
Total *M. fleurettii*			**2**	
S. cohnii	A	VII	1	81
S. pettenkoferi	-	–	1	**111**

MRSA genotypes are reported in parentheses. Farms with more than one methicillin-resistant species are indicated in bold. N.T.: Non-typeable.

3.4. Antimicrobial susceptibility of MRSA and MRSE

The two most prevalent species of MR staphylococci, MRSE ($n = 21$) and MRSA ($n = 11$), were assessed for AMR (Table 3). Different patterns of resistance were observed in 13 isolates. Resistance to tetracycline was confirmed to be widespread both in MRSA (8/11, 72.73 %) and in MRSE (15/21, 71.43 %).

4. Discussion

This study investigated a livestock-dense and highly populated area of northern Italy to analyze the distribution and characteristics of *S. aureus* and MR staphylococci and mammaliicocci isolated from dairy cow BTM. The prevalence of *S. aureus* was found to be 32.95 %, which is lower than the 47.00 % reported by Gazzola et al. (2020). However, the latter study encompassed the entire Lombardy region, and local characteristics and farm management practices may contribute to the observed differences in the isolation rates observed by these authors. Consistent with previous findings, the *S. aureus* genotypes CLB and GTB predominated (Cremonesi et al., 2015). Notably, all the tested *S. aureus* isolates carried the *ica* gene locus. Although *S. aureus* biofilms can also occur in an *ica*-independent fashion (Archer et al., 2011), such a widespread presence of this gene locus indicates a significant potential for

Table 3
Antimicrobial resistance profiles of methicillin-resistant *S. aureus* (MRSA) and *S. epidermidis* (MRSE) isolated from bulk tank milk.

S. aureus (MRSA) – farm ID	Phenotypic resistance profile*
94[a]	CFP, OX, PEN
92, 106, 103[§]	AMC, FOX, OX, PEN, TET,
104	AMC, CFP, FOX, OX, PEN
85[c§]	AMC, CTF, FOX, OX, PEN
113, 71[b§]	AMC, CFP, FOX, OX, PEN, TET,
67	AMC, CTF, FOX, OX, PEN, TET,
19	AMC, CIP, CFP, CTF, FOX, OX, PEN, TET
56	AMC, CIP, CFP, CTF, FOX, OX, PEN, TET
***S. epidermidis* (MRSE) – farm ID**	**Phenotypic resistance profile***
63	FOX, OX
100	FOX, OX, PEN
105	CIP, FOX, OX, PEN
9, 55	FOX, OX, PEN, SXT
46	FOX, OX, PEN, TET
20	FOX, OX, PEN, TET
48	CFP, FOX, OX, PEN, TET
94[a]	CFP, KAN, FOX, OX, PEN
33	FOX, OX, PEN, SXT, TET
18	KAN, FOX, OX, PEN, TET
99	AMC, CFP, FOX, OX, PEN, TET
60, 66	CFP, KAN, FOX, OX, PEN, TET
58	CFP, KAN, FOX, OX, PEN, TET
72	CIP, KAN, FOX, OX, PEN, TET
85[c]	CFP, CTF, KAN, FOX, OX, PEN, TET
71[b]	CFP, CTF, KAN, FOX, OX, PEN, TET
11	CFP, KAN, FOX, OX, PEN, SXT, TET
5	AMC, CFP, CIP, KAN, FOX, OX, PEN, TET
38	AMC, CFP, CTF, KAN, FOX, OX, PEN, TET

***Abbreviations**: AMC, amoxicillin-clavulanic acid; CFP, cefoperazone; CIP, ciprofloxacin; CTF, ceftiofur; KAN, kanamycin; FOX, cefoxitin; GEN, gentamycin; OX, oxacillin; PEN, penicillin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline. Results classified as increased exposure are reported as sensitive. Superscript letters indicate the farms where both MRSA and MRSE were isolated. The symbol § indicates a *mec*A/*mec*C negative isolate.

biofilm formation. The possibility of forming biofilms on the surfaces of milking equipment represents a source of BTM contamination and a reservoir of *S. aureus* and NASM (Ibrahim et al., 2022; Osman et al., 2015).

Based on BTM surveys, the prevalence of MRSA in dairy farms in Europe has been reported to range from 0.3 % in the United Kingdom to 4.4 % in Germany, with a 4 % in northern Italy (Kreausukon et al., 2012; Locatelli et al., 2016; Paterson et al., 2012). We found a prevalence of 12.5 %, suggesting a higher diffusion of MRSA in the province of Lodi. A retrospective study carried out in the Po Valley and the province of Lodi in 2010 indicated that MRSA might have been underestimated (Benedetti et al., 2010). However, comparing MRSA prevalence across studies is challenging due to variations in sample types, inoculum volumes, pre-enrichment steps, and detection methods (Schnitt and Tenhagen, 2020). We enriched and cultured all BTM samples on CMR medium without preselecting for *S. aureus* positivity. Consequently, four out of 11 MRSA were isolated from farms that appeared negative for *S. aureus*, with tree belonging to the GTS genotype. Detection of *S. aureus* in BTM can be hindered by low microbial loads and intermittent shedding patterns in milk (Barkema et al., 2006; Keefe, 2012). Furthermore, genotypes more likely to carry MR, such as GTS, identified as the main MRSA in our study – often exhibit lower within-herd prevalence, making them more difficult to detect in BTM (Cremonesi et al., 2015; Fournier et al., 2008). Although sometimes required for practical reasons, preselecting for *S. aureus*-positive herds might therefore lead to an underestimation of MRSA prevalence.

Our study also revealed significant information regarding MR NASM, with a notable 56.80 % positivity rate among farms in the investigated area. Similar findings have been reported in BTM from Switzerland (62 % of 211 BTM samples) and Germany (42.1 % of 19 BTM samples from preselected MRSA-positive herds), while lower prevalence rates were reported in the US (2.4 % of 288 to 4.3 % of over 300) and UK (4.1 % of 363) (Cicconi-Hogan et al., 2014; Fisher and Paterson, 2020; Schnitt et al., 2021). Among MR NASM, MRSE is the most frequently detected one in the milk of cows with mastitis (Nobrega et al., 2018; Osman et al., 2016; Schnitt et al., 2021; Seixas et al., 2014), followed by MR *M. sciuri* (Cicconi-Hogan et al., 2014; Fisher and Paterson, 2020; Mahato et al., 2017). In clinical settings, MRSE is considered a major pathogen associated with immunocompromised patients and foreign body infections (Becker et al., 2020). In this study, we found MRSE as the most prevalent MR *Staphylococcus* sp., with 23.86 %. A recent retrospective study by our group indicated that the most frequently isolated NASM from herd survey milk in northern Italy was by far *S. chromogenes* (44.49 %), while *S. epidermidis* constituted only 17.84 % (Addis et al., 2023). Notably, we did not detect any MR *S. chromogenes* in the present study, despite its predominant presence in dairy herds. The detection of methicillin resistance (MR) in staphylococcal species that are more commonly associated with human skin rather than the cow udder raises questions about the role of antimicrobial use in humans in contact with animals in the development and transfer of MR species. However, it is also worth considering that the closer phylogenetic relationship between *S. aureus* and *S. epidermidis*, compared to *S. chromogenes*, might play a role in this observation (Lamers et al., 2012).

The isolation of MRSA, MRSE and other MR NASM from BTM samples has significant One Health implications, particularly for individuals who frequently interact with animals during milking and with unpasteurized milk (Gajewska et al., 2023). Data from various European countries indicate a risk for bidirectional transmission between humans and livestock; however, the zoonotic risk remains to be fully elucidated (Anjum et al., 2019). Additionally, the potential spread to other livestock should be considered since 45.45 % of all identified MRSA strains were GTS (Addis et al., 2022; Capra et al., 2017; Cremonesi et al., 2015). Implementing BTM sampling for detecting MRSA, MRSE and other MR NASM at herd level can enhance surveillance efforts and aid in understanding reservoirs and transmission routes within a One Health framework.

In the study by Schnitt et al. (2021) nine NASM species were identified by MALDI-TOF MS, and *M. sciuri* was the most frequently detected MR NASM. The second most common MR NASM was *M. fleurettii*, followed by *S. haemolyticus*, *S. cohnii*, and *S. epidermidis*. A considerable difference with our study, however, is that these authors assessed prescreened MRSA-positive farms. Adding to possible underestimation, prescreening might lead to biases in NASM species detection in consideration of the possible mutually exclusive mammary gland colonization of *S. aureus* versus certain NASM (Piepers et al., 2013; Piepers et al., 2010; Vanderhaeghen et al., 2014). The choropleth maps illustrate the heterogeneous distribution of *S. aureus* and MR staphylococci and mammaliicocci, and the presence of MR NASM also in the BTM of MRSA-negative farms. On the other hand, as already mentioned above, it should be noted that other protocol variables including pre-enrichment steps and selective media used for MR NASM isolation do also have an impact on the observed results.

Recent investigations have explored the relationships between farm characteristics and BTM positivity rates. Kos et al. (2023) found significant associations with milking equipment conditions and Total Bacterial Count in BTM samples. Goncalves et al. (2023) observed a significant association of herd size and MR in BTM, but only for herds with fewer than 200 lactating cows when swine were also present on the same farm. In our study, no variable, herd characteristic or parameter demonstrated a significant odds-ratio on the presence of *S. aureus*, MRSA or other MR staphylococci or mammaliicocci. This observation could be due to the similarities in herd management within the Lodi Province. Within-herd variables were out of the purposes of this work, so we did not consider data about *S. aureus* herd prevalence, clinical mastitis rate, antibiotics use, treatment policy or cure rate, as the focus was on the herd as statistical unit.

In our study, most MRSA isolates belonged to GTS (5 out of 11), in line with previous observations (Cremonesi et al., 2015). *S. aureus* GTS have a broad host range and affect various livestock species, especially swine, as well as humans (McCarthy et al., 2012). Conversely, GTB strains are better adapted to the mammary gland environment and typically exhibit a higher within-herd prevalence, but are less likely to carry MR traits (Addis et al., 2022; Capra et al., 2017). Despite the widespread diffusion of GTB among dairy herds, none of our MRSA belonged to this genotype.

Most MRSA, MRSE and MR NASM isolated in this study carried the *mec*A gene while none carried the *mec*C gene, consistent with previous studies reporting the low prevalence of this gene among cattle populations (Duijkeren et al., 2014; Monistero et al., 2020). On the other hand, we found three phenotypically resistant *mec*A/*mec*C-negative *S. aureus* isolates. Although this may result from PCR sensitivity issues, it should be mentioned that *mec*-independent mechanisms can also play a role in broad-spectrum beta-lactam resistance in *S. aureus*, including, among others, beta-lactamase hyperproduction, production of methicillinases, acquisition of structurally modified normal penicillin-binding proteins, the appearance of small colony variants, alterations in the peptidoglycan biosynthesis pathway or changes affecting autolytic enzymes (Abebe and Birhanu, 2023; Brakstad and Maeland, 1997; Massidda et al., 1996). As we did not perform disc diffusion tests on all the 125 *S. aureus* isolates from the BPA-RPF screening, phenotypically resistant, *mec*A/*mec*C-negative strains may have been underestimated in our study.

The classification of SCC*mec* is based on variations in the *mec* gene complex and other associated genes. Different cassette types are related to differences in the level and spectrum of antibiotic resistance. At the time of this writing, SCC*mec* types I to XIV have been identified (Uehara, 2022). Each type represents a unique combination of resistance genes and genetic elements. Some are more commonly found in healthcare-associated MRSA (HA-MRSA), others are linked to community-acquired MRSA (CA-MRSA), and others are more common in livestock-associated MRSA (LA-MRSA). HA-MRSA generally contain SCC*mec* type I, II, III, V, and VIII; most of them are large and complex

and generally cause resistance to multiple antibiotics (Uehara, 2022). In LA-MRSA, SCC*mec* are mainly SCC*mec* IV and SCC*mec* V, although non-typeable cassettes and type XI containing *mec*C have also been found. In this study, MRSA carried mainly SCC*mec* V (5 of 11 isolates, of which four were GTS), followed by one type IV. The type V cassette in MRSA isolated from dairy cow milk is described in the Lodi area since 2010 (Benedetti et al., 2010), when it was found in 4 out of 14 isolates and 4 out of 9 farms. The same authors highlighted the widespread diffusion of SCC*mec* type IV, detected in 10 of 14 isolates and 5 out of 9 farms. In a recent study, of the 14 LA-MRSA isolates from blood cultures of patients admitted to Policlinico San Matteo in Pavia, eight had a type V, and five had a type IV SCC*mec* cassette (Merla et al., 2022).

We observed a higher prevalence of MRSE compared to MRSA. We found type IV and type V SCC*mec* cassettes almost exclusively in MRSE, except for one *S. borealis* and one *S. haemolyticus*. Notably, these are species that colonize human skin and nares and share resistance characteristics, opening the way to considerations about the human-to-livestock directionality of MRS transmission as mentioned above and highlighted by several authors (Coates-Brown et al., 2018; Haran et al., 2012; Zadoks et al., 2011).

That MR NASM might exchange resistance genes with *S. aureus* has been suggested by numerous studies. The transfer of SCC*mec* elements between MR NASM and *S. aureus* was demonstrated in vitro (Chlebowicz et al., 2014; Morikawa et al., 2012; Ray et al., 2016), although the underlying in vivo mechanisms are still unknown (Fisher and Paterson, 2020; Haaber et al., 2017; Miragaia, 2018). The review by Hanssen and Ericson Sollid (2006) summarizes the large body of evidence about the relevance of MRSE as reservoirs of resistance for *S. aureus*, including their higher prevalence and larger SCC*mec* reservoirs, their role as colonizers, and the long stretches of homology of other SCCs found in MRSA isolates. On the other hand, they also reported the opinion of other authors suggesting that the main source of SCC*mec* could be MRSA itself and that the acquisition from NASM would be an infrequent event (Aires de Sousa and de Lencastre, 2004).

In conclusion, adding to MRSA, also MRSE and other MR NASM isolated from milk deserve future investigations for clarifying prevalence, resistance characteristics, relevance as IMI agents, and role as potential AMR carriers and reservoirs, in view of their relevance for animal health but also of their potential impact on human health.

Ethical statement

The study did not involve invasive procedures on animals as it was carried out on milk samples collected from the farm bulk milk tank.

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CRediT authorship contribution statement

Sara Fusar Poli: Writing – original draft, Methodology, Formal analysis, Data curation. **Clara Locatelli:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Valentina Monistero:** Writing – review & editing, Methodology, Formal analysis. **Gustavo Freu:** Writing – review & editing, Methodology, Formal analysis. **Paola Cremonesi:** Writing – review & editing, Methodology, Formal analysis. **Bianca Castiglioni:** Writing – review & editing, Methodology. **Cristina Lecchi:** Writing – review & editing, Methodology, Funding acquisition, Formal analysis. **Carla Maria Longheu:** Methodology, Formal analysis. **Sebastiana Tola:** Writing – review & editing, Methodology, Data curation. **Alessandro Guaraglia:** Writing – review & editing, Visualization, Methodology, Formal analysis, Data curation. **Carolina Allievi:** Writing – review & editing, Methodology, Formal analysis. **Luca Villa:** Writing – review & editing, Methodology, Formal analysis. **Maria Teresa Manfredi:** Writing – review & editing, Project administration, Methodology, Funding acquisition. **Maria Filippa Addis:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization.

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.

Data availability

The data collected and used to support this article can be offered by the corresponding author upon request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2024.105482.

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