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Analysis of bovine intramammary bacteriome, resistome and of the bacterial transmission within dairy herds

PhD Thesis submitted by

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Abstract

Mastitis is the most important and costly disease in dairy cows worldwide. Bovine intramammary infection (IMI) caused by pathogenic bacteria is common and well understood but very little is known about the bacteria and their resistome present in the mammary gland (= intramammary resistome, IR) of healthy and untreated cows whose milk is regularly delivered for human consumption. The aim that our research project wanted to: i) describe the bacteriome of healthy cows means all the bacteria that we can isolate from the milk of healthy cow, ii) describe the resistome means the antibiotic resistance correlated with the bacteriome isolated with the use of phenotypic and genomic methods iii) comparison of the bacteriome and resistome with the environmental isolates.

Healthy, untreated cows of nine dairy herds from the Swiss Canton Tessin were analyzed three times within one year to identify the most abundant species of the intramammary bacteriome of healthy animals. Aseptically collected milk samples were cultured and bacteria identified using MALDI-TOF mass spectrometry. To describe the intramammary resistome, 350 strains of the predominant species were selected and subjected to short-read based whole genome sequencing (WGS) combined with phenotypic analyses and antibiotic resistance gene profiling. Both chromosomes and mobile genetic elements were examined for antibiotic resistance genes (ARGs) using in-house and online software tools. ARGs were then associated with phenotypic antibiotic resistance profiling data from minimum inhibitory concentration assays. Furthermore, the phylogeny of the two main species isolated *Staphylococcus xylosus* (*S. xylosus*) and *Mammaliicoccus sciuri* (*M. sciuri*) was assessed using 70 housekeeping genes and the maximum likelihood approach (PhyML). Additionally statistical analyses were carried out. Of 256 cows (1024 single quarters) analyzed, 96% were bacteriologically positive and 80% of the quarters were positive for at least one bacterial species. 84.5% of the quarters were healthy with somatic cell counts (SCC) < 200'000 cells/ml, whereas 15.5% of the quarters showed a subclinical mastitis (SCC ≥ 200'000 cells/ml). The 1288 isolates were assigned to 104 different bacterial species including 24 predominant species. *Staphylococcaceae* were most prevalent (14 different species; 73.5% bacteria-positive quarters) with *S. xylosus* and *M. sciuri* accounting for 41.5% of the strains. Our study demonstrates that *Staphylococcaceae* could be consider part of the healthy milk bacteriome. Furthermore, the different farm-specific patterns of the bacteriome are associated with the use of different bedding in the herd. The non-*aureus* staphylococci and mammalicocci (NASM) have been regarded as minor mastitis pathogens being the most abundantly observed bacteria in mastitis milk samples. Based on our study, however, their function needs to be reconsidered. We could assume that different subtypes of NASM may colonize the mammary gland whereas other subtypes may cause minor mastitis.

Phenotypic and genomic antimicrobial resistance (AMR) was bacterium-specific whereby resistance to clindamycin and oxacillin was most frequently observed (40% and 30%). In contrast, AMR to penicillin, although massively used for mastitis treatment during last decades, was rarely observed. The phenotypic findings, not in all the cases, could be linked to chromosomal or plasmid-borne ARGs, demonstrating a lack in understanding the mechanisms that lead to the observed phenotypic AMRs in the isolated bacteria. For some species/AMR, the observed phenotypic AMR could be explained. This is true for *M. sciuri* and its resistance to clindamycin (*salA* gene) as well as *S. xylosus*, *M. sciuri* and a few other bacteria in the case of tetracycline. For all of them, a small plasmid was found carrying the *tetK* or another tetracycline ARG. The presence of tetracycline AMR was herd dependent and was observed in various isolates of the same farm indicating a possible horizontal gene transfer among the different bacteria on the same farm, particularly among *S. xylosus* and *M. sciuri*.

The phylogenetic studies involved *S. xylosus* and *M. sciuri;* these bacteria were the most abundant isolated and were collected from both milk and environmental samples. *M. sciuri* was found predominantly in environmental samples (particularly straw bedding and teat liners before milking). Differently, the *S. xylosus* strains were mostly isolated from milk samples. Based on these results we could conclude that *S. xylosus* is mainly udder adapted whereas the habitat of *M. sciuri* is more environmentally related. Detailed phylogenetic analyses for *M. sciuri* revealed two main clades whereby the smaller one included the *M. sciuri* type strain. Typically, the strains forming this clade were almost exclusively isolated from bedding, milk, and teats, whereas those of the other clade were isolated from milk, teats, and liners, but hardly from bedding. These results suggest that *M. sciuri* circulates clade dependently more among environment and mammary gland (type strain clade) whereas *M. sciuri* of the other clade circulates more among mammary gland and milking equipment. These findings are confirmed by genotype analysis, inferring MLST, demonstrating that the distribution of the sequence types (STs) between milk and environment revealed only a few common STs. Interestingly, *M. sciuri* showed a remarkable variability at the STs level with the consequence that a number of different STs were observed on the same farm. In the case of *S. xylosus*, three main clades were observed. As in *M. sciuri*, the phylogenetic distances within each clade were very small showing that the taxa/strains evolved minimally and that they are genetically very similar. For all three clades, the taxa were largely milk associated whereas environmental taxa were hardly observed. These findings indicate that *S. xylosus* circulates primarily among mammary glands and the taxa found in the environment are more the result of a contamination by milk.

As a conclusion, the research project demonstrates that bacteria, particularly NASM, are very common in the mammary gland of healthy cows, a fact that needs to be considered when interpreting bacteriological results obtained from clinical milk samples. Furthermore, AMR in NASM is uncommon, also against penicillin, although it has been massively used for mastitis treatment during last decades. Unfortunately, AMR in these bacteria remains largely unexplained by genomic analyses. Furthermore, AMR against antibiotics used in human medicine is rare. Finally, phylogenetic studies demonstrate that *M. sciuri* and *S. xylosus* formed only a few major clades and within each clade the strains were genetically very similar; for *M. sciuri* the habitat was clade dependent.

It is essential to get more knowledge about the bacteriome of the mammary glands of healthy and diseased cows to understand the microbiota, hinder pathogens to gain a foothold and, in the long term, prevent the development and spread of resistances. WGS represents an important tool for detecting ARGs but still needs to be associated with phenotypic analysis and with gene and/or protein expression analyses. Screening for new genes associated with AMR and an increase of the ARG databases will be essential, especially for the One Health concept.

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1. Introduction

1.1 General overview about bovine mastitis

Bovine mastitis, also referred as bovine intramammary infection (IMI) caused by pathogens, is the most important and costly disease of dairy cows worldwide (Seegers et al., 2003). Severe economic losses in dairy cattle herds involve different factors such as the reducing milk yield, unsuitability of the milk for consumption, the costs for antibiotic therapy, and, in the worse scenario, the culling of animals caused by treatment failure (Ruegg, 2017). In Switzerland, the estimated cost for the engage to mastitis is approximately of 131 million $ each year (Heiniger et al., 2014). Another important factor is the effect of mastitis on the welfare of the dairy cows. The consequences on cow's behavior showed a general depression, loss of the weight, decreased in the interplay with the other animals, reduced feed consumption, and a decreased in the time of lying (Petersson-Wolfe et al., 2018). Different factors need to be taking into account for controlling the mastitis as the nature of the causing of mastitis (normally bacteria), the host, meaning the cow, and the general environment of the herd (NMC, 2017).

Depending on the severity of the mastitis, a classification in subclinical and clinical mastitis can be made (Adkins and Middleton, 2018). In clinical mastitis, the milk showed alteration with the presence of clots, flakes and, in some cases, a watery consistency (Cobirka et al., 2020). Additionally, clinical mastitis showed visible signs of swelling at the udder level with general inflammation in the mammary gland expressed in redness, heat and pain (Adkins and Middleton, 2018). In subclinical mastitis, the milk and the udder do not showed alternation with a lack of visible sign (Ruegg, 2017). Unfortunately, even if no visual signs could be identified, a decrease in milk production is possible. As demonstrated in recent years, subclinical mastitis is more common than clinical mastitis, with the duration of the disease generally longer than clinical mastitis and the positive cow more difficult to identify (Seegers et al., 2003). The recognition of subclinical mastitis, differently from the clinical cases, requires the use of diagnostic methods.

Over the last few years, different diagnostic methods were used for the identification of mastitis. In general, the diagnostic of mastitis can be divided into the measuring of the inflammation inputs and the identification of the bacterial that caused the illness (Adkins and Middleton, 2018). To measure inflammation inputs different analyses could be done, including direct analysis of the milk (lactose, lactate dehydrogenase, NAGase, acute phase proteins, and conductivity), but the main widespread diagnostic method is the counting of the somatic cell

count (SCC), due to its limited cost and the easy availability of data collection (Adkins and Middleton 2018). The SCC is defined as an indicator of milk quality that describes the total amount of immune cells in the milk (Pyörälä 2003). The different concentration of the different immune type cells involved in the infection as differential somatic cell count (DSCC) was suggested as the best parameter to take into account (Halasa and Kirkeby, 2020). This differentiation is important because the amount of polymorphonuclear neutrophils (PMNs) has been found to predominate in the early stage of infection compared to lymphocytes and macrophages (Damm et al., 2017).

In the laboratory, the SCC could be measured with two different types of methods: the direct microscopy SCC method and the flow cytometry (Schwarz et al., 2011a, Schwarz et al., 2011b). Different studies correlate the high SCC with signs of mastitis (Schukken et al., 2003). On the other hand, the identification of mastitis only based on SCC could be unprecise due to a combination of factors involved in the illness, including: the individually response of the immune system of each single cow, the characteristics of the cow, the nature of the pathogen, the herd environment, the season of the sampling, the lactation stage, the milking procedure, and, lastly, other external stressors factors (Williamson et al., 2022).

Notwithstanding the above, to determine the origin of the mastitis and for selecting the best antibiotic treatment to use, bacterial culturing identification is essential and remain the gold standard procedure worldwide (IDF, 2022). The bacterial identification could be performed on the bulk tank milk, composite milk or on single quarters milk samples. The culturing methods included the use of general agar media as, for examples, the blood agar or specific media for the identification of the pathogens called selective agar that include chromogenic agar media which differentiate the bacterial species based on the color of the colony speeding up the process of identification (Perry 2017, Garcia et al., 2021). The main issue correlated with the culturing method is that the different bacteria need specific growth media and conditions to be isolated. In some cases, even with indications of mastitis, the bacterial culture could be negative due to low concentration of the bacteria, sub-optimal condition for growth and the presence of inhibitory substances in the milk, resulting in a high number of undetected mastitis cases (Taponen et al., 2009). The growth of the bacteria alone is, however, not enough for the species identification; additional tests should be performed. These tests include: biochemical-based phenotypic tests and, only recently, the direct identification with the Matrix-Assisted Laser Desorption/Ionization – Time of Flight mass spectrometry (MALDI-TOF MS) (Singhal et al.,

2015). The MALDI-TOF technique is based on specific spectrum generated by the instrument called peptide mass fingerprinting and the comparison of the fingerprint in a curated databased. Based on this, the species identification could be assigned comparing the ribosomal proteins from an unknown sample (Murray et al., 2012). The MALDI-TOF is a fast high throughput technique but the cost for the instrument and the need of culturing of the bacteria are important limitation in the use of this powerful tool (Haider et al., 2023). Furthermore, for some bacterial species, the implementation of the library is necessary for the correct identification of uncommon or less common species, e.g. for non-aureus staphylococci and mammalicocci (NASM) (Cameron et al., 2018).

Another method used for the identification of pathogen bacteria based on molecular biology is the use of real-time multiplex polymerase chain reaction (RT-PCR) (Taponen et al., 2009). The advantages of RT-PCR are rapid results and a non-culture-based method that detects even bacteria that require more time and specific conditions to grow. However, the disadvantage of this technique is that even DNA from dead bacteria could be detected with a false-positive sample result (Wolffs et al., 2005).

Over the past year, research works that has identified bacterial species using sequencing technology has increased (Woo et al., 2008). For the bacterial identification, the 16s rRNA was chosen as a gene with hypervariable regions that can be compared between bacteria species (Clarridge, 2004). The sequencing technology, as the other molecular technology, do not request the culturing of the bacterial but high cost and long timing to performing are necessary (Besser et al., 2018). To identify new bacterial species and characterize them by identifying antimicrobial resistant genes and understanding the genome, whole genome sequencing (WGS) is required (Cremonesi et al., 2022). Epidemiological analysis to identify clones among different strains isolated from mastitis samples is a powerful application of WGS (Cremonesi et al., 2022). Furthermore, WGS data, which include all genes belonging to the bacteria, could be used directly as a high-powered discriminatory method, especially when comparing different strains involved in an infection (Slott Jensen et al., 2020).

1.2 **Mastitis pathogens**

Despite all the factors involved in bovine mastitis, the intrinsic properties of the bacterial pathogen involved in the disease remain one of the most important determining factors. Based on the work of Radostits published in 2007, 140 bacterial species caused bovine mastitis have

been described (Radostits, 2007). Although this high number of bacterial species caused mastitis, the main pathogens can be clustered into few main species including *Staphyloccus aureus* (*S. aureus*), *Streptococcus agalactiae* (*Strep. agalactiae*) and *Streptococcus uberis* (*Strep. uberis*), *Escherichia coli* (*E. coli*), and non-*aureus* staphylococci and mammaliicocci (NASM). A classification of the mastitis pathogens is based on minor or main pathogens or based on the origin of them (contagious or environmental) (Cobirka et al., 2020). The bacterial species *S. aureus* and *E. coli* are of particular concern because they are zoonotic agents that might possibly be transmitted to humans through close contact with animals infected (Johnson et al., 2008, Fergestad et al., 2021). Despite their potential, mastitis strains are not actually of public health concern (Barlow 2021, Enger and Middleton 2021).

For example, in Switzerland, the low number of methicillin resistant *Staphylococcus aureus* (MRSA) isolated from mastitis milk and the higher percent detected in humans, point out a really low risk of transmission (Nemati et al., 2023).

The next subchapters included a short overview of the main mastitis pathogens. A deeper look regarding the species *S. aureus* and the NASM was done due to the extensive involvement of these bacteria species in our research's projects.

Staphylococcus aureus

S. aureus still remains one of the main bacterial sources causing mastitis. *S. aureus* is mainly associated with subclinical and chronic mastitis, is normally more common in late lactation, but can cause clinical mastitis in some cases (Barkema et al., 2006, Tenhagen et al., 2009). As *S. aureus* is a contagious bacterium, the spread of the bacterium is mainly associated with the milking process through the contaminated milking machine from cow to cow, the farmer's hands and the washcloths used to clean the udders (Zadoks et al., 2002). The transmission of the bacterium by flies has also been detected (Gioia et al., 2022). The variable success rate of antibiotic treatment in the lactation period empathized the importance of this pathogen at herd level (Barkema et al., 2006).

Intrinsic properties of the bacterium seem to be involved in the low cure rate of treatments; *S. aureus* is able to hide itself by forming micro-abscesses that cannot be recognized by neutrophils and causing damage in the secretory tissue of the mammary gland (Mullarky et al., 2001, Erskine et al., 2003). In recent years, several genotyping methods have been developed, whether sequence-based or not. The characteristics of a good genotype method are the facility in the execution, short time for performing, reliability, reproducibility, and high discrimination between different strains (van der Zee et al., 2019). The 16S-23S ribosomal

spacer PCR (RS-PCR) is a method developed for the genotyping during the recent years (Fournier et al., 2008, Graber et al., 2009). This PCR has a high discriminating power; by examining the bulk tank milk, an initial screening between contagious and non-contagious genotypes can be carried out (Gazzola et al., 2020).

In Switzerland, mainly two different genotypes were identified genotype B (GTB) and genotype C (GTC); the other genotypes (GTOG) accounted for only 20% of the isolates (Fournier et al., 2008). The GTB is a contagious genotype characterized by a prevalence of up to 87% at the herd level (Fournier et al., 2008, Graber et al., 2009). GTB was not only isolated in Switzerland but was also the main contagious genotype in other European countries (Cosandey et al., 2016). Several genotypes have been isolated from non-European countries, North and South America, South and North Africa (Tunisia) with the genotype R (GTR) predominating (Monistero et al., 2018). Consequently, it is possible to identify different genotypes based on the different geographical origin of the strains. Additionally, after performing the genotyping Monistero and co-workers exanimated a pattern of virulence genes. The final conclusion of the article is that there are differences between the strains isolated from different countries and that further studies on possible markers of virulence and contagious are needed (Monistero et al., 2018).

Recently, Sartori and colleagues, developed a new quantitative PCR (qPCR) for the detection of a gene called adhesion-like bovine protein (*adlb*) related to genotypes (in particular the GTB) in which high contagiousness was found at the herd level (Sartori et al., 2017). Screening the herds for the presence of the gene *adlb* has been one of the main successful tools used to sanitize the *S. aureus* GTB in Tessin, one region of Switzerland (Sartori et al., 2018, GTB sanitation paper, in preparation). Last year, Maisano et al., carried out a cross-sectional study in Italy in which, a statistical association was found between the high prevalence of intrammary infection caused by *S. aureus* and the presence of the *adlb* gene (Maisano et al., 2023).

The recent development of sequenced-based genotyping methods, such as *spa* typing and multi locus sequence typing (MLST), has been a great step forward due to their reproducibility and ease of inter-laboratory exchange of results (Enright et al., 2000, Harmsen et al., 2003). Since 2014, in addition to these sequencing-based methods, the core genome MLST has been introduced (Leopold et al., 2014).

Escherichia coli

E. coli is classified as an opportunistic environmental mastitis pathogen (Zadoks et al., 2011). *E. coli* mastitis are normally characterized by causing acute clinical mastitis (Shpigel et al., 2008, Blum et al., 2017). Despite this, this bacterium can also cause subclinical mastitis (Zaatout 2022). The genome of *E. coli* is characterized by so-called genome plasticity. Consequently, specific subtypes of *E. coli* have been identified with acquire adapted virulence factors necessary to live in the mammary gland of the cows (Blum et al., 2015, Goldstone et al., 2016). When we mention these types of bacteria, we can also call them mastitis pathogen *E. coli* (MPEC) (Goulart and Mellata, 2022). Despite the characteristics of the pathogen, the immune status of the cow, and in particular a deficit of the leucocyte immune system in the peripartum period, are also important factors in the development of the disease (LeBlanc, 2020). Although many works studied the virulence factors involved in mastitis caused by *E. coli*, it has not been possible to identify a common pattern of virulence factors related to the severity of the illness (Zadoks et al., 2011). In contrast, a different genetic pattern of virulence factors was identified between *E. coli* strains from the mastitis and *E. coli* strains from the herd environment (Blum et al., 2008). The factors that seem to be involved in the development of the illness are the survival of the bacteria in an anaerobic environment and the ability to utilize the lactose substrate (Cobirka et al., 2020).

As for *S. aureus*, several techniques have been used to genotype *E. coli* strains. In particular molecular typing methods as ribotyping, amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE) typing, multiple-locus variable-number tandem repeat analysis (MLVA), and different sequencing methods such as MLST and cgMLST have been developed over the last decade (Goulart and Mellata, 2022).

As mentioned in the first paragraph, *E. coli* could be a public health problem for human due to the zoonotic characteristics of the bacteria. *E. coli* may be a carrier of antimicrobial resistant genes (ARGs) that could be transmitted by horizontal gene transfer (HGT) to other pathogens (Zhang et al., 2020, Goulart and Mellata, 2022). In particular, strains of *E. coli* carrying the extended spectrum β-lactamase enzymes are involve in *E. coli*'s mastitis infection (Klaas and Zadoks, 2018, Yang et al., 2018). In 2017, the WHO listed a list of antibiotic-resistant "priority pathogens" including ESBL (WHO, 2017). The first ESBL *E. coli* was detected in cattle feces in China already in 2004 (Shiraki et al., 2004). Different countries, widespread around the world, detected the presence of ESBL strains in cases of mastitis,

including the European countries Germany and Greece (Goulart and Mellata, 2022). In particular, the risk of transmission of ESBL could be due to the consuming of raw milk and raw milk products derivates (Freitag et al., 2017). Different concentration of ESBL were detected in the milk in a range between 0.4 to 25.4% (Freitag et al., 2017, Dahmen et al., 2013, Goulart and Mellata, 2022).

Streptococcus agalactiae

Strep. agalactiae is mainly classified as a contagious mastitis bacterium, however, cases of this pathogen have also been detected at the environmental level (Cheng and Han, 2020, Kabelitz et al., 2021). Despite the reduction of the mastitis caused by *Strep. agalactiae* due to an increasing in the hygienic procedure at the herd level, mastitis caused by this pathogen has been found and varies between the countries involved in the studies, and in some cases, it appears that the bacterium may be a reemerging pathogen (Lyhs et al., 2016, Ruegg 2017, Kabelitz et al., 2021). The pathogenicity of the illness caused by *Strep. agalactiae* depends on the formation of biofilm at the level of the mammary gland level and, consequently, its persistence in the udder (Cheng and Han, 2020). As a consequence, *Strep. agalactiae* may also be involved in chronic mastitis (Keefe, 1997). The main source of transmission of *Strep. agalactiae* is during the milking process. To better understand the mechanism of propagation within the herds, Jensen conduct an experiment involving strains isolated from human and bovine infection (Jensen, 1982). The experiment demonstrated a different immune response of the bovine udder to infection by *Strep. agalactiae* from human and bovine origin (Jensen, 1982). This study emphasized how the intrinsic genetic characteristics of the strains can influence the course of the infection. Another important factor to take in consideration is the serotype of the *Strep. agalactiae*. Previous studies have shown that *Strep. agalactiae* serotype IV has been detected in strains isolated from bovine mastitis but also from human strains (Lyhs et al., 2016).

In recent years, several molecular methods have been developed to study the clonality of the strains of *Strep. agalactiae* such as randomly amplified polymorphic DNA (RAPD) analysis, multilocus enzyme electrophoresis, pulsed-field gel electrophoresis (PFGE) of restriction enzyme digest products of genomic DNA (Bohnsack et al., 2004). In 2003, thanks to the development of the MLST for *Strep. agalactiae*, epidemiologic correlation between the human and bovine strains were identify and the study of the population biology was deepened (Jones et al., 2003, Bohnsack et al., 2004). Based on studies on MLST, the strains colonize human and bovine are genetically different excluding the bovine's genotypes ST23 and ST61 that are genetically related with the human's genotypes ST23 and ST17 (Pereira et al., 2010,

Lyhs et al., 2016, Kabelitz et al., 2021). In the diagnosis of the infection by *Strep. agalactiae* that cause bovine mastitis, SCC remain one of the best parameters using for the identification of the infection; mastitis caused by *Strep. agalactiae* are characterized by high SCC, in some cases extremely elevated (Keefe, 2012).

Although, *Strep. agalactiae* still poses a problem at the farm level, antibiotic therapy, mainly based on β-lactam antibiotics, remain the best and most successful strategy for the treatment (Ruegg 2017, Gomes and Henriques, 2016). The use of penicillin as antibiotic treatment remains the first line and most successful treatment (Erskine et al., 2003, Krömker et al., 2014). However, the high use of antibiotics for the therapy may select antimicrobial resistant strains (Gomes and Henriques, 2016). In particular, when comparing the resistance of the different *Streptococcus* spp. isolated from cattle, a large number of antibiotic resistance strains of *Strep. agalactiae* were isolated, in particular to the antibiotics sulfametroprim, tetracycline, and erythromycin (Erskine et al., 2003). However, misinterpretation due to the lack of breaking point for strains isolated from bovine mastitis must be taken into account (El Garch et al., 2020).

Streptococcus uberis
Strep. uberis differently from *Strep. agalactiae* is classified as mastitis environmental pathogen. Despite this classification, cases of contagious *Strep. uberis* were found (Wente et al., 2019, Leelahapongsathon et al., 2020, Cremonesi et al., 2022). *Strep. uberis* first reservoir in the herd environment is the bedding; a high amount of the bacteria was isolated from straw bedding comparing to sand and sawdust (Hillerton et al., 2003). Manure is essential for the survival of the bacterium but even without it, an experimental study suggested that the bacterium can survive in straw for 35 days, highlight the higher risk of finding *Strep. uberis* in organic material comparing to the inorganic (Sherwin et al., 2021). Although the differences between the bedding types used, the survival rate in the bedding depends mainly on the genetic characteristics between the strains (Davies et al., 2016).

The first case of *Strep. uberis* in bovine mastitis was reported in 1932 (Oliver et al., 2011). Since the first detection, *Strep. uberis* has been isolated at different stage of lactation, heifers, dry cows, and also in multiparous cows (Oliver et al., 2011, Kabelitz et al., 2021). During the last years, an increasing of mastitis caused by *Strep. uberis* and environmental mastitis pathogens in general, has been observed (Hillerton et al., 2003, Bradley et al., 2007). The different level of pathogenicity of infection related to *Strep. uberis* depends on several factors involved such as the animal's immune system and the specific characteristics of the pathogen (Tassi et al., 2013). The intramammary infection by *Strep. uberis* are normally

acquired during the dry cow period as clinical form; if not treated, acute cases occur during lactation (Cobirka et al., 2020). Differently from other bacteria, the infection caused by *Strep. uberis* is often express in a long duration and high bacterial count (Schukken et al., 2011). The infection may be visible with elevated SCC and subclinical chronic mastitis (Wyder et al., 2011). If the infection persists, after six days *Strep. uberis* may invade the alveolar tissue from the mammary gland and, in some cases, cause a state of fibrosis similar to the infection caused by *S. aureus* (Cobirka et al., 2020). In persistence infection, antibiotics treatment may not be effective (Milne et al., 2005).

Despite the persistence infection cases, conventional antibiotic treatment showed high cure rate against the *Strep. uberis* infection (Erskine et al., 2003, Cobirka et al., 2020). A recent paper by El Garch and collaborators analyzed the resistance of *Strep. uberis* strains isolated from eight European countries in 2015-2016 (El Garch et al., 2020).

Previous research has been conducted by the same group and a comparison of the antimicrobial resistance between strains isolated from 2002-06, 2009-12 was performed. A general low resistance to penicillin, normally used against the *Streptococcus* spp. infection, was detected, in agreement with other researches (Overesch et al., 2013, Käppeli et al., 2019). On the other hand, a slight increase in the resistance for erythromycin and tetracycline was observed (El Garch et la., 2020). Continuous monitoring of antimicrobial resistance is essential to understand whether the commercial antimicrobials in use are still effective and to monitor the risk of emergence resistant bacteria.

Non-*aureus* staphylococci and mammaliicocci (NASM)

Nowadays, the term NASM included all the *Staphylococcus* spp. and *Mammaliicoccus* spp. different from the major pathogen *S. aureus* (Rosa et al., 2022). The NASM group currently include 63 different species of *Staphylococcus* and five species of *Mammaliicoccus* (LPSN, 2023). Only recently, some *Staphylococcus* species as *Staph. fleuretti, Staph. lentus, Staph. sciuri, Staph. stefanovicii,* and *Staph. vitulinus* were reassigned to the *Mammaliicoccus* genus with *Mammaliicoccus sciuri* (*M. sciuri*) as the type species (Madhaiyan et al., 2020). The reassignment was based on an accurate bacterial reclassification established by a phylogenetic study of the 16S rRNA gene and core genome complemented by genome-based indices (Madhaiyan et al., 2020).

Quoting the title of Hamel and co-authors, the role of NASM as agent of infection correlated to bovine mastitis or source from contamination is still unclear (Hamel et al., 2020). In 2021, a review by De Buck and co-workers, showed the current knowledge gaps on this large

group of bacteria (De Buck et al., 2021). Due to the different characteristics of the various bacterial species, it is a good practice to no longer consider these bacteria as a group, but rather, individual species (De Visscher et al., 2015, Hamel et al., 2020).

The distribution and the prevalence of the NASM species varies between countries, regions, and environments (De Buck et al., 2021). Worldwide, the prevalent NASM species identified in different countries by different research groups was *Staphylococcus chromogenes* (*S. chromogenes*) (De Buck et al., 2021). In a Canadian study analyzing mastitis strains, 50% were recognized as *S. chromogenes* (Condas et al., 2017). However, based on a recent paper using meta-analysis and mapping review, the same species was identified in 6.7% milk quarters from apparently healthy cattle (Kurban et al., 2022). According to the study by Hamel et al., increased SCC cannot always be associated with IMI caused by NASM infection (Hamel et al., 2020). According to this study, differently, a higher concentration of the bacteria could be a factor to take in consideration in cows in which NASM caused IMI was detected (infection by *S. chromogens*, *Staphylococcus epidermidis*, and *Staphylococcus simulans*) (Hamel et al., 2020). Another study, from De Vliegher and co-workers, consider NASM as commensal bacteria that may have a slight or negative effect on SCC and other factors such as the milk production and milk quality (De Vliegher et al., 2012). Furthermore, another study by Braem et al., includes NASM as part of the cow's udder microbiome, proposing a possible mechanism of competition against the mastitis pathogens (Braem et al., 2014). Several studies have found strains of *S. chromogenes* isolated from healthy animals inhibit the growth of mastitis pathogens and biofilm formation (Isaac et al., 2017, Brouillette et al., 2021, Toledo-Silva et al., 2022, Vander Elst et al., 2023). The work of Srithanasuwan and co-workers, studied the interaction between main bovine mastitis such as *S. aureus*, *Strep. uberis* and *E. coli* and NASM (Srithanasuwan et al., 2023). In this study, NASM were co-infected and survived to the main pathogens. In some samples, *S. chromogenes* was able to degraded *Strep. agalactiae*, empathized the role of this bacteria as a promising alternative to the wide use of antibiotics (Srithanasuwan et al., 2023). These studies highlight the possible role of some species of NASM as an alternative to the antimicrobial therapy. However, further research involving genotyping and, consequently, a better understanding of their genomic characteristics and properties are needed.

In previous works, antimicrobial-resistant NASM have been isolated (Moniri et al., 2007, Gizaw et al., 2020). The prevalence of NASM antibiotic resistance (AMR) varies from

country to country and depends on the different species (De Buck et al., 2021). An investigation of phenotypic antimicrobial resistance, as for other mastitis pathogens, was conducted by El Garch and co-authors by monitoring antibiotic resistance in NASM strains isolated in 2009-12 and 2015-16 (El Garch et al., 2020). During this time period, an increase in tetracycline resistance was observed (from 7.3% to 18%). In contrast, the resistance to penicillin remained steady with a percentage of 29.1%. The antibiotic resistance to oxacillin was 43% with the detection of the gene *mecA* in 15.75% of the strains that were phenotypically resistant. A general low presence of strains methicillin resistance NASM was found, in line also with the MRSA detection (Frey et al., 2013, De Buck et al., 2021, Fergestad et al., 2021). Another issue related to antibiotic resistance and NASM is the possibility of the spread of ARGs to the pathogen *S. aureus*, although the AMR for this group is low (Persoon Waller et al., 2011). Another important aspect is the formation of the biofilm that can facilitate the colonization and attachment of bacteria to the udder epithelium, minimizing the effect of an antibiotic treatment (Zigo et al., 2021, Zigo et al., 2022). In addition, fewer virulence factors have been identified in NASM, with the exception of biofilm as described (Zigo et al., 2022). Studies have shown that positivity to biofilm was related with clinical strains more than with strains derived from the bulk tank milk and is mainly associated with the species *Staphylococcus haemolyticus* and *S. chromogenes* (Tremblay et al., 2013, Srednik et al., 2017, Zigo et al., 2022). Several studies have shown the correlation between biofilm and the presence of multidrug resistance in other bacterial species (e.g. *Klebsiella oxytoca*), but the correlation between multi drug resistance and the biofilm positivity of NASM is still unclear and should be further investigated (Phophi et al., 2019, Karimi et al., 2021). In this regard, the differences between the potential for biofilm formation in strains of different species found in various studies highlight the importance of characterizing the genotype of the strain. On the other hand, some NASM strains could might have a positive effect against biofilm formation by mastitis pathogens biofilm such as *Staph. aureus* (Goetz et al., 2017).

Continuous monitoring of antimicrobial-resistance bacteria, even for this minor mastitis pathogen, is essential to prevent the increase of antimicrobial resistance worldwide. The antimicrobial therapy to NASM infections depends on the different countries. For example, in the USA the infection caused by NASM may not be treated or may be treated as a group of bacteria without taking into account the characteristics of the individual bacterial species (Cameron et al., 2014, Jenkins et al., 2019). In Switzerland, the antibiotic therapy suggested

against NASM as mastitis pathogens is based on a combination of benzylpenicillin and aminoglycosides (BLV, 2019). Despite the different strategies used for the antibiotic treatment, a study of Taponen and co-workers showed high cure rate (86%) in case of clinical and subclinical mastitis involved NASM. In contrast, cows not treated for NAMS infection had only the 46% of cure rate (Taponen et al., 2006). Additionally to their role in bovine mastitis, NASM strains positive to enterotoxins isolated in food matrixes were detected highlighted their possible enterotoxigenic potential (Carneiro Aguiar et al., 2022, Freitas et al., 2023). However, enterotoxins required appropriate condition of pH, temperature and water activity to be produced and remain mainly related to the presence of the pathogenic bacterium *S. aureus* (Freitas et al., 2023).

In summary, as suggested by Ruiz-Romero and Vargas-Bello-Pérez in their review on NASM published in 2023 and the previous review by De Buck and co-authors (De Buck et al., 2021, Ruiz-Romero and Vargas-Bello-Pérez, 2023) different aspects needs to be further explore regarding the NASM. Nowadays, it is clear that they should not be considered as a group. The characteristics of each species as reservoir of virulent factors or antimicrobial resistance genes need to be studied more thoroughly. Despite these aspects, some studies have shown that NASM could be a possible alternative to antibiotics, but further experiments need to be conducted, ideally using cellular or animal model (Ruiz-Romero and Vargas-Bello-Pérez, 2023).

1.3 Actual knowledge about the bovine bacteriome

There are several factors involved in the severity of mastitis, one of them is the nature of the pathogenic mastitis bacteria. Given the crucial implication of this factor, it is important to define the role of the bacteria colonizing the mammary gland to understand their implication as pathogenic, healthy or commensal bacteria.

According to an old dogma of veterinary medicine, milk was considered a sterile matrix due to the high quantity of milk quarters in which no bacterial growth was possible to identify (Metzger et al., 2018). In the last years, this old dogma has been questioning by the introduction of culture-independent approach based on metagenomic sequencing (Quigley et al., 2013, Metzger et al., 2018, Oikonomou et al., 2020). Several studies implied the use of this technologies and conclude with a common thought that the healthy udder microbiome could be a composition of a complex microbial community of different bacterial species (Addis et al., 2016).

The following sub-chapters provide a brief overview of the cultured- and sequence-based studies carried out in last year's analyzing healthy and clinical mastitis cases.

Culture identification is a simple and essential tool for the identification of mastitis used by the diagnostic laboratory. The correct identification and implication of bacteria species in mastitis infection remains the best strategy to reduce the use of antibiotics and, consequently, the possible increase of antibiotic resistant bacteria (Viora et al., 2014).

In a previous Swiss study investigating the presence of NASM with cultured-based methods, Frey and co-workers included, in addition to clinical mastitis samples, quarters of milk previously treated for mastitis and considered healthy at the time of the study (Frey et al., 2013). The results of the study showed the presence of NASM (n= 47, 11%) also in healthy quarters highlighting the possible role of the bacteria in both healthy and clinical quarters.

In another Swiss study performed by our group, a cultured-based approach was used to identify the bacteria present in herds that were involved in a study for the eradication of the *S. aureus* GTB (Sartori et al., 2018). The bacteria isolated and identified with the MALDI-TOF were mainly NASM and *Streptococcus* spp. In particular, a high percentage of *S. chromogenes* and *Staphylococcus xylosus* were isolated.

In a study prior to those mentioned above, milk samples from cows with clinical symptoms and from healthy cows with negative cultures were analyzed with a sequencing approach (Kuehn et al., 2013). The results of the study showed differences between the bacterial species isolated from the clinical and healthy quarters of the cows, with the main group isolated as *Staphylococcus* spp. in both clinical and healthy animals.

The review by Kurban et al., analyzed quarter milk samples of apparently healthy cows and cows with clinical mastitis (Kurban et al., 2022). The article was based on data collected from previous studies or voluntary data; the important feature of the samples was the common identification of strains with MALDI-TOF spectrometry. This study showed that some bacterial species were isolated from both healthy cows and clinical cases and that a knowledge gap was visible for some not common mastitis species.

As mentioned in the first chapter, in recent years, thanks to improved technology, more studies have been conducted involving the use of sequencing to study the bacteriome.

The milk microbiome, especially the bulk tank milk, could be influence by different external factors, for instance, the environment and practices of the herd, the countries and different

seasons in which the milk was collected, the milking machine and the bacteria colonized the teat apex (Skeie et al., 2019, Parente et al., 2020, Porcellato et al., 2021). Several aspects influencing the udder microbiome were considered in the review by Derakhshani et al. (Derakhshani et al., 2018). The review considered all the factors that might influence the udder microbiome, including housing condition, type of bedding, antibiotic use at the herd level and the influence of "other microbiome" such as the teat apex and the teat canal microbiota (Derakhshani et al., 2018). In addition to the herd and other geographical/management and environmental factors, different methods are usually used during the sampling procedure and different methods to perform metagenomic analysis such as DNA extraction, purification kit, choice of target gene, number of PCR cycles and primers, and different platform used for the sequencing (Metzger et al., 2018, Steinberg et al., 2022). A necessary standardization of the methods used for metagenomics is essential for further comparison between different studies, as differences in bacteria genus distribution have been observed studies (Parente et al., 2020). Possible changes in the microbiota were underlined by a longitudinal study performed by Winther and co-authors (Winther et al., 2022). In this study, using metagenomics for analyze milk from five cows with low and five cows with high SCC differences in the microbiota of these two categories were visible. When the samples had low SCC, a large variability of bacteria was found. Differently, samples with high SCC showed a low prevalence of bacteria, especially as agent of bacterial infection. This suggests the possible presence of a healthy microbioma in the udder. Despite the factors that can influence the studies, in several metagenomics studies the common bacteria isolated from milk belonged mainly to the phyla of Firmicules, Actinobacteria, Bacteroides, and Proteobacteria (Bonsaglia et al. 2017, Taponen et al. 2019, Steinberg et al., 2022). In particular, as found in many studies the genus *Staphylococcus* is the most prevalent in the milk microbiota (Oikonomou et al., 2014, Steinberg et al., 2022). This genus was found to be abundant in studies involving both healthy and subclinical milk samples (Bonsaglia et al. 2017, Steinberg et al., 2022).

Recent studies well established that a possible healthy bacteriome could exist (Porcellato et al., 2020, Park et al., 2022). These studies however, are based only on metagenomics studies missing the important fact regarding the growing of the microorganism. The metagenomics data includes all the bacteria that will be found in the milk based on a species identification with the 16S rRNA region. Due to this fact, the possible DNA also of dead bacteria might be possibly identify. The study by Oikonomou et al., compared the microbiome

of healthy cows and clinical mastitis using culture and pyrosequencing methods (Oikonomou et al., 2012). The results of this study showed the presence of *Staphylococcus* spp. and *Streptococcus* spp. isolated from healthy cows that might be "normal" constituent of the microbiome (Oikonomou et al., 2012, Oikonomou et al., 2014). The research also showed differences between milk from healthy and diseased cows, emphasized the possible role of anaerobic bacteria as part of the microbiome. A study by Guo et al., analyzing and comparing the bacteriome of raw milk collected from healthy cows in different China regions showed a greater influence of seasonality than the geographical origin of the samples (Guo et al., 2021). In another research performed by Park and co-authors, *S. xylosus*, *Staphylococcus epidermidis* and *Aerococcus urinaeequi* belonged to the healthy microbiome of the cow's (Park et al., 2022). In this study, *S. aureus* infected and uninfected quarters from the same cows were sequenced by 16S rRNA amplicon sequencing. Based on the results, quarters showing low levels of inflammation belonged to samples positive for *S. xylosus, S. epidermidis,* and *Aerococcus urinaeequi.* This fact, highlight a possible use of these bacteria as anti-*Staph. aureus* for clinical use as found in other studies (Carson et al., 2017).

Despite the articles regarding the microbiome of healthy cows, a recent article from Rainard put some doubt about the existence of a living healthy bacteriome (Rainard, 2017, Taponen et al., 2019). The existence of a healthy bacteriome is still a complex, controversial and actual topic of discussion.

1.4 Use of antibiotics for the bovine mastitis treatment

Antibiotic treatment remains the best strategies against the mastitis disease. In the agricultural field, at least in Switzerland, the main antibiotics used at the livestock level are penicillins, sulfonamides, and tetracyclines (Federal Office of Public Health and Federal Food Safety and Veterinary Office, 2022). However, a continuous decrease in antibiotic sales has been reported since 2012, reaching a total decrease of 51% (Federal Office of Public Health and Federal Food Safety and Veterinary Office, 2022). This was mainly caused by the decrease in the use of "critical antibiotics" as macrolides, fluoroquinolones, and, third and fourth generation cephalosporins, due to the Ordinance on Veterinary Medicinal Products that did not allow their stock feeding on farms livestock since April 2016 (Federal Office of Public Health and Federal Food Safety and Veterinary Office, 2022).

The reduction in the use of the antibiotics may also be due to the Swiss Antibiotic Resistant Strategies (StAR) program which promote a more conscious use of drugs by the

veterinarians and the farmers (Swiss Confederation, 2023). Additionally, at the European Level, on the basis of a plan following the "From Farm to Fork" principle, a 50% reduction of antibiotics used in livestock farming by 2023 has been envisaged (EU, 2020, Zigo et al., 2022). In Switzerland, starting in 2019, farm-level antibiotic prescriptions must be recorded in an electronic register for the listing of antibiotic use in veterinary medicine called IS ABV. Consequently, only from 2020, data regarding antibiotics are available to provide an estimate of the antibiotic use at the farm level (FSVO 2022a, FSVO 2022b).

In the EU, from 2006, the use of antibiotics in animal husbandry as growth promoters is not allowed (European Commission, Regulation (EC) No 1831/2003). General education for farmers and veterinarians could involve the correct use of antibiotics exclusively, when necessary, as a measure to prevent a possible emergence of antibiotic-resistant bacteria (Talebi et al., 2019). Several factors must be considered when considering the best therapy to treat mastitis, including the spectrum of activity of the antibiotics, the intrinsic antibiotic resistance of the bacteria themselves, and, finally, the characteristics of the cow itself (Ruegg, 2021). When considering a therapy to treat Gram-negative bacteria infection, a naturally resistance to penicillin antibiotic should be known before thinking about the correct treatment (Ruegg, 2021).

Regarding to the antibiotics authorized for the intramammary use, in Switzerland, sales of the products have declined slightly. Penicillin remains the predominant antibiotic use at the intramammary level; despite an increase use of first and second generation cephalosporins was observed (Federal Office of Public Health and Federal Food Safety and Veterinary Office, 2022). The use of penicillin as the main antibiotic remains the best choice for all European countries, according to a surveillance study published in a paper by De Briyne and co-authors (De Briyne et al., 2014). Recently, a European meeting including eight mastitis experts shared their experience regarding the use of antibiotics in their countries (Preine et al., 2022). The conclusion was, also in this paper that penicillin remains the first choice for the antibiotic treatment (Preine et al., 2022).

The widespread use of antibiotics to treat infections at the human level and the misuse and abuse of antibiotics at the agriculture level, could increase the selection of antibiotic resistant bacteria (Manyi-Loh et al., 2018, Mann et al., 2021). No evidence was observed at the herd level, but a trend in the possible isolation of resistance bacteria, or at least a possible distribution of ARGs among different animals, can be observed (Hanselman et al., 2006, Hershberger et al., 2005). Antimicrobial resistance is an increasingly important problem;

according to the report of the UN Ad hoc Interagency Coordinating Group on Antimicrobial Resistance, a future estimate of 10 million human deaths each year caused by antibiotic-resistant bacteria can be expected, which also implies global financial problems (World Health Organization, 2019). However, based on different studies, the number of antibiotic-resistant mastitis pathogens is not increasing; resistant strains can only be detected after antibiotic treatment, with a return to a normal situation after the treatment (Garcia et al., 2019).

With the introduction in the 2004 of the One Health concepts, the health of humans, animals and the environment are interconnected, highlighting that the best strategy is to consider the health of the planet as a unique entity (Destoumieux-Garzón et al., 2018). The approach must consider the consequences of the hazard and the standards to be applied in order not to compromise the environment and human health with a holistic approach (Sora et al., 2022). In particular, the One Health concept could be applied to dairy production and food safety right from the farm for generally improve the overall health, safety and quality of dairy products (Garcia et al., 2019). The One Health perspective also includes the welfare and health of cows and the effort for the proper use of the antimicrobial therapy (Trevisi et al., 2014, FIL-IDF 2019, Sora et al., 2022).

1.5 Actual knowledge about the resistome

As the number of studies in which the bacteriome is involved increased recently, so have the number of studies concerning the resistome. The risk identification based on an estimation of the antimicrobial resistant genes (ARGs) in the dairy industry and thus in the food chain and herd environment is a topic of public interest (Rubiola et al., 2020). This chapter provides an overview of the latest studies involving the resistome.

The term "antibiotic resistome" was first introduced by Gerry Wright and co-authors in 2006 referring to the "resistance determinants present in the soil" meaning the presence of multi resistant bacteria in soil (D'Costa et al., 2006). After this first definition, an increasing of the studies involve the resistome were performed (Kim and Cha, 2021). Currently, the term antibiotic resistome includes the determination of antimicrobial resistance genes (acquired and intrinsic resistance genes) and the resistance system used by the bacterial that has involved modifications and evolutionary changes to provide resistance (Kim and Cha, 2021). In a study by Liu and co-workers, retail milks from different USA states, raw and milk samples process during pasteurization, were collected and analyzed by using cultured-based methods, 16s rRNA and finally, metagenomic sequencing (Liu et al., 2020). In raw milks, the results showed a high

percentage of aerobic bacteria and, with the time passes in the tank, a possible increase in bacteria with a consequent change in the resistome as well. A study by Tóth et al., analyzed two samples of raw milk. What they found, in line with other previous results, was the presence of a different bacteriome between the samples and the presence of different ARGs, presuming that the results were related with a different amount of antibiotics treatment performed at the herd level based on the different number of cows per herds (Tóth et al., 2020).

A study Rubiola and co-workers, based on sequencing meta-genomic analysis of the milk production environment, revealed the presence and, consequently, the circulation of ARGs (Rubiola et al., 2020). The ARGs that were detected were mainly multi-drug resistance genes and resistance genes to rifampin, aminoglycosides, tetracyclines, sulfonamides, macrolides, lincosamides, streptogramins, and β-lactams (Rubiola et al., 2020). Subsequently, the same group performed a study sampling including the bulk tank milk filters belonging to different herds (Rubiola et al., 2022). The filters analysis showed a high number of Gram-negative bacteria, mainly of the genus *Enterobacter*, *Acinetobacter*, *Escherichia* and, *Pseudomonas* that carried a high number of ARGs. Additionally to the bacteriome and the resistome, the detection of mobile genetic elements was carried out (mobilome). Of the 37 closed plasmids detected, 14 were positive also for the presence of ARGs and classified mobilized, meaning that they could be shared by horizontal gene transfer with other bacteria (Rubiola et al., 2022). Although the detection of ARGs is a wake-up call, a lack of phenotypic results must be taken into account when referring only to metagenomic data.

Another study by Nikoloudaki et al. showed that the ARG genes detected in raw bulk tank milk from healthy cows were mainly related to the presence of Gram-negative bacteria, in particular to *Pseudomonas* spp. (Nikoloudaki et al., 2021). No statistical correlation was found between the herds condition and the presence of predicted ARGs. As the authors state, the further raw pasteurization of milk would reduce the presence of the *Pseudomonas* spp. but, intrinsic resistance to antibiotics and the possibility of transferring them and other virulence genes, must be taken into account. A low percentage of Gram-positive (1-2%) with corresponding harbored resistant genes to the antibiotic's aminoglycoside, β-lactams, macrolide, and tetracycline were identified (Nikoloudaki et al., 2021). Warder et al., analyzed 12 bulk tank milk for the detection of the bacteriome and resistome. With regards to the resistome, also in this case, a high amount of tetracycline resistance genes was found. In addition to this resistance, resistance to aminoglycosides and β-lactams resistance was detected.

An important result of this experiment is the final conclusion that each bulk tank milk carries a specific bacteriome and resistome. The presence of antimicrobial resistant genes, related to antibiotics of great or medium relevance for humans were observed (Warder et al., 2021).

A major concern regarding the presence of ARGs in raw milk may be the correlation between these and the further presence of ARGs in the cheese resistome. Regarding this topic, several factors need to be considered such as the microbiome, the herd environment, human handling of the product, and the interaction between the bacteria involved throughout the cheese production process (Nikoloudaki et al., 2021). Further studies are needed to understand the implication of the raw milk resistome in cheese production.

2. Hypothesis and aim of the study

In recent years, more and more sequenced-based studies have been carried out, while culture-based studies with a final clinical application are lacking. The aim to the doctoral research was to establish whether or not a possible healthy bacteriome could be defined through a cross-sectional field study involving nine herds from one Swiss region **(Chapter 3).** Differently from other studies in which the bulk tank milk was analyzed and consequently more contamination could be rolled out, aseptically milk samples when collected from each cow's quarter.

After a selection of the prevalent species, a group of representative isolates was subjected to whole genome sequencing (WGS). The WGS technology was performed to detect the presence of antimicrobial resistance genes (ARGs) and plasmid genes. In addition, a phenotypic analysis of the antimicrobial resistance using the minimum inhibitory concentration (MIC) was performed. An increasing number of studies involved the identification of ARGs, but the only presence of the gene is not sufficient to provide real insight into antibiotic resistance without phenotypic results. A work carried out in our laboratory has shown how discrepancy between phenotypic and genomic results regarding penicillin can was explained by a defectiveness in the operon promotor sequence (Ivanovic et al., 2023). Based on this findings, phenotypic analyses are important for finalized the resistance to the antibiotics.

The questions involved in our study were:

1. Is there possible an identification of a healthy bacteriome based on a cultured-based methodology? (Identification of the bacteriome in healthy cows)
2. Is it possible to define the resistome of the healthy bacteriome based on WGS analysis of the isolates derived from the bacteriome analysis?
   (Identification of the resistome in healthy cows)
3. What is the correlation between the bacteria that we find in the healthy bacteriome and the bacteria that we found in the herd's environment?
   (In deep analysis of the two main bacterial species isolated in the bacteriome of the milk, *S. xylosus and M. sciuri*, including phylogenetics analysis of *M. sciuri*)

The outcomes should make an important contribution to the field of clinical veterinary, defined when or not, the treatment against the bacteria isolated from the milk should be done. The possible bacteria isolated from the milk if not belonging to the common pathogen causing

mastitis, are bacteria that are less studied, in which, gap of knowledges about the antibiotic resistance (AMR) and their intrinsic resistance is possible to define. This PhD research would fill the gaps in the knowledge of this less common bacteria species identify in the milk whose epidemic role is still unknown or controversial based on previous studies. Characterization of less common bacterial species is important for our increased understanding of their role in the udder health. It is hoped that this research will contribute to a deeper understanding of the udder bacteriome of healthy cows with the identification of a wide group of bacteria present in the udder and an overview regarding the AMR detected with phenotypic and genomic tools.

3. Results

3.1 Elucidation of the Bovine Intramammary Bacteriome and Resistome from healthy cows of Swiss dairy farms in the Canton Tessin

Title: Elucidation of the Bovine Intramammary Bacteriome and Resistome from healthy cows of Swiss dairy farms in the Canton Tessin

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Own contribution: Herd sampling; Execution of the experiments; Data analysis; Graphic design of tables and graphics; Writing the draft of the manuscript, Review of the manuscript.

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Elucidation of the Bovine Intramammary Bacteriome and Resistome from healthy cows of Swiss dairy farms in the Canton Tessin

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Healthy, untreated cows of nine dairy herds from the Swiss Canton Tessin were
analyzed three times within one year to identify the most abundant species of
the intramammary bacteriome. Aseptically collected milk samples were cultured
and bacteria identified using MALDI-TOF. Of 256 cows analyzed, 96% were
bacteriologically positive and 80% of the 1,024 quarters were positive for at least
one bacterial species. 84.5% of the quarters were healthy with somatic cell counts
(SCC) < 200,000 cells/mL, whereas 15.5% of the quarters showed a subclinical
mastitis (SCC ≥ 200,000 cells/mL). We could assign 1,288 isolates to 104 different
bacterial species including 23 predominant species. Non-*aureus* staphylococci
and mammaliicocci (NASM) were most prevalent (14 different species; 73.5%
quarters). *Staphylococcus xylosus* and *Mammaliicoccus sciuri* accounted for
74.7% of all NASM isolates. To describe the intramammary resistome, 350 isolates
of the predominant species were selected and subjected to short-read whole
genome sequencing (WGS) and phenotypic antibiotic resistance profiling. While
complete genomes of eight type strains were available, the remaining 15 were
de novo assembled with long reads as a resource for the community. The 23
complete genomes served for reference-based assembly of the Illumina WGS
data. Both chromosomes and mobile genetic elements were examined for
antibiotic resistance genes (ARGs) using in-house and online software tools.
ARGs were then correlated with phenotypic antibiotic resistance data from
minimum inhibitory concentration (MIC). Phenotypic and genomic antimicrobial
resistance was isolate-specific. Resistance to clindamycin and oxacillin was
most frequently observed (65 and 30%) in *Staphylococcus xylosus* but could not
be linked to chromosomal or plasmid-borne ARGs. However, in several cases, the
observed antimicrobial resistance could be explained by the presence of mobile
genetic elements like *tetK* carried on small plasmids. This represents a possible
mechanism of transfer between non-pathogenic bacteria and pathogens of
the mammary gland within and between herds. The-to our knowledge-most

extensive bacteriome reported and the first attempt to link it with the resistome promise to profoundly affect veterinary bacteriology in the future and are highly relevant in a One Health context, in particular for mastitis, the treatment of which still heavily relies on antibiotics.

1. Introduction

Bovine mastitis, also referred to as bovine intramammary infection (IMI) caused by pathogens, is the most important and costly disease of dairy cows worldwide (Seegers et al., 2003). Severe economic losses in dairy cattle herds are caused by four main factors: reduced milk yield, unsuitability of the milk for consumption, antibiotic treatment costs, and culling of animals in case of treatment failure (Ruegg, 2017). In the frame of the One Health concept, which advocates a general view of human, animal and environmental health, research aimed at describing the bacterial diversity of both commensals and potential pathogens in animal food production systems is highly relevant (Aslam et al., 2021). Such research is expected to considerably reduce the use of antibiotics and thereby the associated dangers of transfer of antibiotic resistance genes (ARGs) and spreading of multi-resistant strains (McEwen and Collignon, 2018). While IMI by pathogens is well understood, very little is known about the bacteria present in the mammary gland of healthy and untreated cows and their antimicrobial resistances (AMR) (= intramammary resistome, IR). A few studies of the milk microbiota, relying both on culture-dependent and culture-independent approaches have been carried out in recent years [reviewed in Quigley et al. (2013) and Oikonomou et al. (2020)]. They arrived at the preliminary conclusion that milk of healthy udders is not a sterile matrix, but instead, harbors a complex microbial community composed of different microorganisms (Addis et al., 2016).

In subclinical IMI, non-*aureus* staphylococci (NAS) and *Streptococcus uberis* are the most frequently isolated bacteria (De Visscher et al., 2015). Despite NAS being considered less pathogenic than *Staphylococcus aureus*, they can carry virulence factors, toxins and antibiotic resistance genes and are able to generate biofilms (Vanderhaeghen et al., 2014; Taponen et al., 2016). However, their potential pathogenicity needs to be further clarified and evaluated in more detail. Potentially, they could just represent commensal microorganisms of the normal flora in the mammary gland (De Buck et al., 2021). A recent Swiss bacteriome study by Sartori et al. (2018) reported that NAS were the main bacteria colonizing healthy cows with *Staphylococcus xylosus* and *Staphylococcus chromogenes* representing the most frequent isolates from the milk of the selected herds. However, the IR of healthy cows was not assessed in that study.

Only recently in 2020, five species belonging to the NAS were reclassified in the novel genus *Mammaliicoccus* due to an evolutionary study based on 16S sequencing conducted by Madhaiyan et al. (2020). Nowadays, the staphylococci and mammaliicocci are indicated with the acronym of non-*aureus* staphylococci and mammaliicocci (NASM) (Rosa et al., 2022).

In Switzerland, the application of antibiotics (AB) in agriculture has been decreasing over the last years (2010–2019), evidenced by a 52% reduction of the sales of ABs used in livestock animals since 2010 (Federal Office of Public Health and Federal Food Safety and Veterinary Office, 2020). In part, this reduction can be attributed to the fact that critical antibiotic classes for human medicine (fluoroquinolones, macrolides, and 3rd/4th generation cephalosporins) were banned to be given for stocks due to the Ordinance on Veterinary Medicinal Product, in line with the aims of the One Health approach (Federal Office of Public Health and Federal Food Safety and Veterinary Office, 2018). However, in contrast to this overall reduction, the use of antimicrobials licensed for treatment of IMI was relatively stable during 2010–2019 (Switzerland in fact has the highest use of intramammary products in Europe) (European Medicines Agency, European Surveillance of Veterinary Antimicrobial Consumption, 2021). Overall, 70% of all antimicrobials administered concern antibiotics for the treatment of mastitis during lactation. The main antibiotic is penicillin followed by aminoglycosides and cephalosporins. This high rate of applications could represent an important reason for the development of resistances in pathogens inducing mastitis episodes and in bacteria colonizing the healthy mammary gland (Oliver and Murinda, 2012).

Unfortunately, at the European level only resistance data of NASM strains responsible for clinical mastitis are available which displayed a high resistance to penicillin G and oxacillin (29.1 and 43.9%, respectively) (El Garch et al., 2020). In contrast, no phenotypic resistance data are available regarding the intramammary bacteriome of healthy cows except for one Swiss study from Frey et al. (2013). In this study, NASM were isolated from control milk, i.e., milk from healthy cows previously positive to mastitis, subjected to treatment, with the characteristic to have somatic cell counts (SCC) <150,000 cells/mL. The results of this study showed a prevalence of phenotypic resistance between 17 to 40% to the antibiotics oxacillin, fusic acid, tiamulin, penicillin, tetracycline, and streptomycin. Increasing research on comparing the resistance profiles of isolates from different countries could provide relevant insights into treatment strategies of affected herds.

Advances in whole genome sequencing (WGS) and the availability of online tools supporting researchers in the identification of antimicrobial resistance genes (ARGs) are important pre-requisites for studying not only the abundance and dissemination of AMR (Hendriksen et al., 2019), but also the potential transfer of ARGs from species colonizing animals to species infecting humans (Wendlandt et al., 2015). The transfer of ARGs commonly involves mobile genetic elements (MGEs). The most prevalent ones are plasmids, i.e., extrachromosomal DNA molecules that encode genes that play a role,

among others, in virulence, antibiotic resistance, tolerance to heavy metals, and metabolism of carbon sources (Malachowa and Deleo, 2010). The classification of plasmids based on the replicon protein (Rep) is an important approach that can be used to examine the distribution of such MGEs in the environment (Orlek et al., 2017).

In the present study, more than 1,200 bacterial isolates were identified allowing to describe 23 predominant species of the intramammary bacteriome of healthy cows. Furthermore, WGS and phenotypic profiling was carried out for 350 isolates from the 23 most abundant species to infer the resistome (IR) at the phenotypic and the gene level and attempt to link phenotype and genotype information. The results are discussed under the aspects of diagnostic and clinical importance as well as of the One Health approach.

2. Materials and methods

2.1. Study design and sample collection

Nine different herds were randomly selected in the Swiss Canton of Tessin. Quarter milk samples were collected aseptically 3 times during winter 2017–2018 (time point 0), late spring 2018 (time point 1, sampling was performed before the cows were sent for common pasturing on alps during the summer season), and winter 2018–2019 (time point 2) from at least 10 randomly selected lactating cows (unless stated otherwise; Table 1), following the guidelines of the National Mastitis Council [National Mastitis Council (NMC), 2016]. Prerequisites for inclusion in the study were that the cows (i) did not receive any antibiotic therapy within the previous five days, (ii) did not show any clinical signs of mastitis or teat injuries, (iii) appeared visually normal, and (iv) their milk was suitable for human consumption according to Swiss legislation (VHyMP 2020[1]). Data regarding age, lactation number, and stage of lactation of the cows were collected. Considering the stage of lactation, we referred to three different stages divided in early (14–100 days after calving), mid (100–200 days after calving), and late lactation (>200 days after calving). For lactation number, we divided the cows into three different groups: (i) 1st lactation (primiparous), (ii) 2nd and 3rd lactation, and (iii) >3 lactations. For herd 6, only samples from the 1st and 2nd sampling could be collected as the farm was given up later.

2.2. Analysis of somatic cell counts

Somatic cell counts (SCC) in individual quarter milk samples (identical to those used for bacteriological analyses, see below) were used to differentiate between healthy quarters and those with subclinical mastitis (cows and quarters with clinical forms of mastitis were strictly excluded from the study). According to the International Dairy Federation (IDF), a quarter was considered healthy if SCC were < 200,000 cells/mL, independent on number and stage of lactation (International Dairy Federation, 2022). Values above were considered as a quarter with subclinical mastitis. Total SCC were analyzed in frozen milk samples using the recently published flow

cytometry method by Widmer et al. (2022). For analysis, the samples were defrosted at room temperature and gently mixed by inversion. The impact of freezing on SCC using this method was tested with 120 raw milk samples and the average decrease in cell numbers was 6.3%.

2.3. Bacteriological analyses and identification

Bacteriological analyses were performed following the *Laboratory Handbook on Bovine Mastitis* of the National Mastitis Council [National Mastitis Council (NMC), 2017]. In brief, 10 µL of each milk sample was streaked out on sheep blood agar (BA) plates (Biomèrieux Suisse SA, Geneva, Switzerland) and bacterial colonies obtained after 24 and 48 h of aerobic incubation at 37°C were evaluated based on their morphology. Samples were considered "contaminated" and not included in the study, if more than 3 morphologically different bacterial colonies could be identified (Wyder et al., 2011). Representatives of each colony type were selected for bacterial identification using MALDI-TOF MS according to the protocols of the manufacturer (Bruker Daltonics GmbH, Bremen, Germany). Analysis was performed by a Microflex LT MALDI-TOF instrument using the MALDI Biotyper (MBT) Compass Library 7,311 (both Bruker Daltonics GmbH). Isolates with a score ≥ 2.2 were identified at the species level. All distinct isolates were conserved in skim milk at-20°C for subsequent analyses.

2.4. Selection of isolated bacterial species for resistome analysis

By analyzing all morphologically distinct colonies for each milk sample by MALDI TOF MS, a total of 1,288 isolates were obtained. To reduce the number of isolates and to restrict it to the most relevant bacteria for each herd and sampling, the following selection procedure was performed: (1) For each BA plate, all morphologically different colonies with an abundance ≥5 were taken, identified, and the corresponding bacteria registered in a frequency table. The table was then sorted in descending order according to the observed frequencies. Starting from top, bacteria were selected until the sum of their frequencies resulted in ≥85% of the total frequency. (2) For each of these selected, relevant bacteria, 5 isolates (if available) were then randomly chosen resulting overall in 350 isolates that were later subjected to phenotypic AMR testing and bioinformatic ARGs analysis after whole genome sequencing (WGS).

2.5. Phenotypic antimicrobial susceptibility testing

Minimum inhibitory concentrations (MIC) were determined for all 350 isolates using different antibiotics panels that accounted for the respective characteristics of the bacterial species. All tests were performed according to the manufacturer's instructions of the Microscan System (Beckman Culture Microbiology, West Sacramento, CA). For details see the Supplementary Methods Section in the Supplementary materials.

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1 https://www.fedlex.admin.ch/eli/cc/2005/824/de

TABLE 1 Overview of the within-herd bacterial positivity for the three sampling time points.

Number of quarters positive per farm and sampling																											Total
	Farm 1			Farm 2			Farm 3			Farm 4			Farm 5			Farm 6		Farm 7			Farm 8			Farm 9			
Sampling	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2	0	1	0	1	2	0	1	2	0	1	2	
Type of Bedding	Straw, sawdust			Straw, sawdust			Straw			Straw, ferus			Straw			Straw		Straw			Straw			Manure			
N. cow sampled	11	10	10	9	8	9	10	10	10	10	10	10	10	10	10	9	10	10	10	10	10	10	10	10	10	10	256
N. cow bacteriologically positive	11	10	10	9	8	8	10	10	10	10	10	7	10	10	9	9	10	10	10	10	10	10	9	10	10	10	250
N. quarter sampled	44	40	40	36	32	36	40	40	40	40	40	40	40	40	40	36	40	40	40	40	40	40	40	40	40	40	1,024
N. quarter bacteriologically positive	43	31	31	32	31	15	38	34	34	39	37	12	29	37	23	27	35	30	36	26	39	40	24	36	31	34	824
Quarters positive only with one bacterial species	18	24	18	4	22	6	15	17	18	26	15	10	19	14	15	17	15	20	18	17	20	15	18	21	19	19	440
Quarters positive with 2 bacterial species	24	5	10	23	8	7	17	11	12	11	13	1	9	17	6	8	17	10	15	8	14	24	5	11	8	11	305
Quarters positive with 3 bacterial species	1	2	3	5	1	2	6	6	4	2	9	1	1	6	2	2	3	0	3	1	5	1	1	4	4	4	79
Quarters positive only by one species NASM	18	17	15	4	22	4	14	15	16	10	8	6	9	7	10	7	12	14	13	8	15	14	17	7	1	4	287
Quarters positive only by two species NASM	24	4	10	23	8	6	17	11	11	10	12	1	5	12	4	5	12	7	15	7	13	23	5	7	2	3	257
Quarters positive only by three species NASM	1	2	3	5	1	2	6	6	3	1	8	1	1	6	1	2	3	0	3	1	5	1	1	3	2	1	69
NASM and *S. aureus*																											
Staphylococcus xylosus	30	14	25	20	9	6	21	17	11	4	20	7	10	19	8	6	22	21	29	10	2	0	1	3	3	5	323
Mammaliicoccus sciuri	18	6	8	29	24	10	7	23	0	2	9	0	2	15	0	0	5	1	16	3	31	38	4	11	2	1	265
Staphylooccus succinus	17	2	1	5	1	1	17	1	5	1	2	0	0	0	0	3	6	2	0	3	3	0	0	2	1	0	73
Staphylococcus equorum	0	0	0	0	0	0	0	0	7	0	0	0	3	0	7	1	0	0	0	0	1	0	13	0	0	0	32
Staphylococcus aureus	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2	7	3	2	3	0	0	0	11	2	0	32
Mammaliicoccus vitulinus	0	0	0	0	0	1	9	0	15	0	0	1	0	0	0	0	0	0	0	0	0	0	2	0	0	0	28

(Continued)

TABLE 1 (Continued)

Number of quarters positive per farm and sampling

	Farm 1			Farm 2			Farm 3			Farm 4			Farm 5			Farm 6		Farm 7			Farm 8			Farm 9			Total
Sampling	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2	0	1	0	1	2	0	1	2	0	1	2	
Type of Bedding	Straw, sawdust			Straw, sawdust			Straw			Straw, ferus			Straw			Straw		Straw			Straw			Manure			
Staphylococus chromogenes	0	1	1	0	0	0	0	2	3	0	0	0	0	0	3	0	0	0	0	0	2	0	2	1	1	1	17
Staphylococcus haemolyticus	0	1	2	0	0	0	0	2	0	0	0	2	0	0	0	1	0	1	0	4	0	0	2	0	0	0	15
Staphylococcus warneri	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	13
Others[1]	1	0	0	3	0	0	1	3	0	3	2	0	0	0	0	3	0	1	0	2	0	0	2	0	0	0	21
Bacillus cereus group[2]	0	3	2	0	3	0	2	3	2	1	13	1	1	13	4	0	3	1	2	0	5	18	0	17	12	9	115
Others *Bacillus*[3]	0	2	0	0	0	1	0	1	0	0	2	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	9
Acinetobacter spp.[4]	0	0	4	0	0	0	0	0	1	2	1	0	1	0	0	1	3	0	0	0	0	2	1	1	4	2	23
Aerococcus viridans	0	0	0	0	0	3	1	0	0	2	0	0	4	0	0	4	1	2	0	0	2	0	2	0	0	0	21
Arthrobacter spp.[5]	0	0	0	0	0	0	0	1	1	0	3	1	4	1	4	0	0	0	0	0	0	0	0	2	0	7	24
Enterococcus spp.[6]	0	0	0	0	1	0	1	0	0	0	3	0	1	0	0	0	0	0	0	1	2	0	0	4	8	21	42
Escherichia coli	0	1	0	3	0	0	0	3	0	0	0	1	0	2	0	0	1	0	0	1	5	4	0	0	7	1	29
Lactococcus spp.[7]	0	0	0	0	0	0	0	0	0	1	2	0	4	2	0	10	6	0	0	0	0	0	0	0	0	0	25
Streptococcus spp.[8]	0	0	1	0	0	2	2	0	0	8	6	1	0	0	4	0	0	1	3	6	1	0	0	1	1	0	37
Others[9]	1	6	1	2	0	0	5	0	6	14	5	1	5	11	3	5	4	3	2	2	7	4	0	1	1	3	92
Not reliable identification	2	4	2	3	3	2	1	0	3	3	0	1	5	2	0	1	0	4	3	1	2	0	2	1	4	3	52
Total																											1,288

Additional relevant information is provided, including the type of bedding used by the herds, the number of cows and the number of quarters. [1]*Staphylococcus epidermidis* (2), *Staphylococcus fleuretti* (2), *Staphylococcus gallinarum* (5), *Staphylococcus hyicus* (1), *Staphylococcus lentus* (3), *Staphylococcus simulans* (2), *Staphylococcus spp.* (6). [2]In this group are included *Bacillus cereus, Bacillus mycoides, Bacillus thuringiensis* (115). [3]*Bacillus altitudinis* (1), *Bacillus licheniformis* (2), *Bacillus pumilus* (3), *Bacillus simplex* (1), *Bacillus subtilis* (1), *Bacillus spp.* (1). [4]*Acinetobacter towneri* (1), *Acinetobacter calcoaceticus* (4), *Acinetobacter guillouiae* (6), *Acinetobacter johnsonii* (3), *Acinetobacter lwoffii* (8), *Acinetobacter spp.* (1). [5]*Arthrobacter arilaitensis* (4), *Arthrobacter gandavensis* (8), *Arthrobacter histidinolovorans* (1), *Arthrobacter ilicis* (1), *Arthrobacter mysorens* (2), *Arthrobacter nicotinovorans* (1), *Arthrobacter protophormiae* (5), *Arthrobacter spp.* (2). [6]*Enterococcus faecium* (2), *Enterococcus faecalis* (5), *Enterococcus gallinarum* (1), *Enterococcus saccharolyticus* (34). [7]*Lactococcus garvieae* (16), *Lactococcus lactis* (9). [8]*Streptococcus agalactiae* (11), *Streptococcus dysgalactiae* (1), *Streptococcus parauberis* (7), *Streptococcus uberis* (17), *Streptococcus spp.* (1). [9]*Aeromonas spp.* (3), *Carnobacterium maltamaticum* (2), *Citrobacter spp.* (5), *Enterobacter cloacae* (3), *Erwinia persicina* (1), *Klebsiella spp.* (10), *Kluyvera spp.* (2), *Kocuria spp.* (2), *Lelliotta amnigena* (2), *Lysinibacillus sphaericus* (3), *Microbacterium arboriscens* (1). *Micrococcus luteus* (1), *Moraxella spp.* (1), *Neisseria macacae* (1), *Paenibacillus spp.* (1), *Pantoea agglomerans* (8), *Proteus spp.* (9), *Providencia rettgeri* (2), *Pseudomonas spp.* (21), *Rahnella aquatilis* (1), *Raoultella spp.* (4), *Serratia marcescens* (5), *Trueperella pyogenes* (2), *Vagococcus fluvialis* (1), *Vibrio cincinnatiensis* (1).

2.6. Whole genome sequencing

Details concerning the extraction of genomic DNA for short and long read sequencing platforms to then first *de novo* assemble, polish and annotate complete genomes of type strains (15 of 23 that lacked a complete genome at NCBI) are described in detail in the Supplementary Methods, along with the respective strategies to assemble the reads even from highly repeat-rich and complex strains (Schmid et al., 2018). The complete type strain genomes served as a basis for reference-based assembly for the 350 WGS sequenced isolates (Illumina HiSeq platform) (Supplementary Tables S1–S4).

The 350 isolates were plated on sheep blood agar (BA) plates (Biomèrieux Suisse SA, Geneva, Switzerland) and incubated aerobically at 37°C for 18 h (h). Two to four single colonies were picked, resuspended in 5 mL BHI (Brain Heart Infusion Broth, Merck KGaA, Darmstadt, Germany) and incubated under the same conditions for 18 h. Subsequently, 200 μL of the pre-cultures were added to 100 mL of fresh BHI and incubated aerobically at 37°C for 18 h under constant shaking, before 50 mL were collected and centrifuged (18,000 × *g* for 5 min at 4°C). The supernatant was discarded, the pellet resuspended in 600 μL of buffer A1 (NucleoSpin® 8 Plasmid kit, Macherey-Nagel AG, Oensingen, Switzerland) and DNA isolated according to the manufacturer's protocol. The total amount and quality of DNA were evaluated by spectroscopy assessing the OD_{260}/OD_{280} ratio (QuickDrop; Molecular Devices, San Jose, CA) and a quantitative analysis (Qubit assay; Thermo Fisher Scientific, USA).

2.7. WGS and assembly

DNA samples ($n = 350$) were sequenced by Eurofins Genomics GmbH (Ebersberg, Germany) on the HiSeq sequencing platform (Illumina, San Diego, CA). The reads were first assembled using the complete genome of the type strain of the corresponding species as reference (Supplementary files *Illumina and long reads sequencing (PacBio/ONT) of type strains*) using SeqMan NGen 16 software (default settings) from the DNASTAR Lasergene 16 software package (DNASTAR Inc., Madison, WI). The unassembled reads were *de novo* assembled with the *de novo* task (with the parameters deactivated 'repeat handling' option, minimum read overlap match of 93%, and contigs longer than 1,000 nucleotides). The assembled chromosomes and contigs were next annotated with the RAST pipeline.[2]

2.8. *In silico* identification of antimicrobial resistance genes

The assembled genomes (chromosomes and contigs) of the 350 isolates were analyzed for the presence of antimicrobial resistance genes (ARGs) using three different approaches: an in-house manually curated fasta database for AMR genes of *Staphylococcus* spp. together with CM software (Supplementary file), and two online software tools,

i.e., ResFinder[3] (Bortolaia et al., 2020) and Resistance Gene Identifier RGI[4] (Alcock et al., 2020, Comprehensive Antibiotic Resistance Database, CARD, 2019). An additional manual check to evaluate the functionality of the ARGs was performed (Clone Manager v9.51; CM9; Sci Ed Software, Westmister, CO) by comparing the reference gene to that of the respective isolate. To leverage the benefits of curated databases (SIB, Swiss Institute of Bioinformatics Members, 2016) all ARGs were compared with CARD to establish an association between the genes and the respective antimicrobial compounds against which their encoded products act.

2.9. Analysis of mutations in the *mecA1* gene of *Mammaliicoccus sciuri* isolates

For 20 of the 83 *Mammaliicoccus sciuri* isolates, the promoter region of the *mecA1* genes was analyzed manually using Clone Manager v9.51 software (CM9Sci Ed Software, Westmister, CO). A sample was considered positive, when a *mecA1* promoter mutation was detected at position-10 as reported in previous studies (Wu et al., 2001; Frey et al., 2013).

2.10. *In silico* analysis of plasmids

The assembled chromosomes and contigs of all bacterial species were analyzed for the presence of plasmids using the PlasmidFinder[5] software (Carattoli et al., 2014); if positive, they were further analyzed using the curated database PLSDB[6] (Schmartz et al., 2022).

2.11. Additional analysis of tetracycline AMR

All reads from isolates that exhibited matches to the *tetK* gene were mapped against the *tetK* reference gene (1,380 bp; NCBI, GenBank: S67449.1) downloaded from the CARD database using SeqMan NGen 16 (default settings). Alignments were manually checked using the Clone Manager 9.51 software (CM9; Sci Ed Software, Westmister, CO). Additionally, plasmid SPADES and Unicycler were used to circularize the plasmids of four randomly selected isolates that carried the *tetK* gene. Subsequently, all reads of isolates positive for the *tetK* gene were assembled with the closed plasmids as references and compared with Clone Manager 9.51.

2.12. Statistics

Descriptive statistics for bacterial prevalence at the cow and quarter level were performed using Microsoft Excel 2016 (Microsoft Corporation). Additionally, descriptive statistical analyses were achieved to evaluate the percentage of healthy and inflamed

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2   https://rast.nmpdr.org

3   https://cge.cbs.dtu.dk

4   https://card.mcmaster.ca

5   https://cge.food.dtu.dk/services/PlasmidFinder

6   https://ccb-microbe.cs.uni-saarland.de/plsdb

(subclinical mastitis) quarters that showed a monoinfection with *S. xylosus* and *M. sciuri*, respectively.

A Fisher's exact test was used to test if there was non-random association between the bacteria and the distribution in different herds. Additionally, a generalized version of the Fisher's exact test for k x m tables was performed to test the association between herds and resistance to the main antibiotics involved (azithromycin, clindamycin, oxacillin, penicillin, and tetracycline). The same test was further performed to evaluate a possible association between milk sampling and lactation stage of the sampled cows.

To assess the impact of the stage of lactation on the intramammary presence of *S. xylosus* and *M. sciuri*, for each bacterium a loglinear model was computed, both at the cow and at the quarter level. The models included the factors bacterium, stage of lactation, farms, and their interactions. The very same procedure was used to assess the impact of the lactation number on intramammary *S. xylosus* and *M. sciuri*. For both *S. xylosus* and *M. sciuri*, their overall presence was evaluated including mono-and co-cultures with other bacteria.

For all statistical analyses except stated the Systat 13.1 software (Systat Software, San Jose, CA) was used. A value of $p < 0.05$ was considered significant.

2.13. Data availability

The complete genomes sequences (and sequences of the plasmids) for 15 type strains that were *de novo* assembled and used as reference genomes are available under Bioproject PRJNA936091.[7] The raw Illumina reads obtained from WGS of 350 isolates are publicly available from NCBI GenBank under Bioproject PRJNA859642.[8] Our manually curated database of *Staphylococcus* spp. ARGs is released as Supplementary Word file.

3. Results

The results are organized along the main two themes of the present study, i.e., the intramammary bacteriome and resistome, respectively (Figure 1).

3.1. Composition of the intramammary bacteriome

The composition of the intramammary bacteriome and distribution of individual species of each single herd at the three sampling time points is summarized in Table 1 and Figure 2. An additional figure showing the distribution of the different bacteria during sampling time (T0, T1, T2) is included in the Supplementary material (Supplementary Figure S1). Overall, a total of 1,024 milk samples (from 256 cows) collected aseptically from each quarter were analyzed. For each herd and time point, 10 randomly selected healthy cows were sampled (unless otherwise stated, see

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Materials and methods). The average age of the cows ranged between 4.4 to 8.9 years. 78.4% ($n = 200$) of the cows were multiparous, while 21.6% ($n = 55$) were primiparous. No data was available for two cows. Another parameter collected was the lactation stage; 108 cows were in late lactation, 76 in mid, and 70 in early lactation. These data are listed in a table integrated in the Supplementary material (Supplementary Table S5).

To assess the impact of the stage of lactation on the intramammary presence of *S. xylosus* and *M. sciuri*, the most frequently observed bacteria in this study, a loglinear model was computed for each bacterium, both at the cow and at the quarter level. The models included the factors bacterium, stage of lactation, farms, and their interactions. The variable sampling time (T0, T1, T2) was omitted from the model as a strong association was observed between this variable and stage of lactation ($p < 0.001$). Indeed, at T1 most cows were in mid (29%) and late lactation (62%). The 4 loglinear models were also used to assess the impact of the lactation number on intramammary *S. xylosus* and *M. sciuri*. The results showed that at the cow level the intramammary presence of *S. xylosus* was independent on the stage of lactation ($p = 0.840$) but dependent on the farm ($p < 0.001$). Different was the situation at the quarter level where a dependency for stage of lactation was observed ($p = 0.004$). Regarding *M. sciuri*, intramammary presence at the cow level was farm dependent ($p < 0.001$), but was independent on the stage of lactation ($p = 0.515$). At the quarter level, intramammary *M. sciuri* positivity was farm ($p < 0.001$) and lactation stage dependent ($p = 0.001$). Considering lactation number, it did not affect intramammary presence of *S. xylosus* at the cow level ($p = 0.652$), whereas an effect was observed at the quarter level ($p = 0.015$). For *M. sciuri*, the loglinear model showed no dependency as well as at the cow ($p = 0.131$) as at the quarter level ($p = 0.076$).

The SCC data demonstrated overall a high number of quarters (84.5%) with SCC below of 200,000 cells/mL and were, therefore, considered healthy. The remaining 15.5% were considered as quarters with subclinical mastitis. For *S. xylosus* explicitly, 81% of the quarters showed values below 200,000 cells/mL, with a median SCC of 36,740 cells/mL, while for *M. sciuri*, 92% of the quarters were considered healthy (SCC < 200,000 cells/mL) with a median SCC of 18,920 cells/mL.

250 of the 256 cows were bacteriologically positive for at least one quarter (98%): nineteen cows were positive for one quarter (7%), 28 cows for 2 quarters (11%), and 64 (26%) and 139 (56%) for 3 or 4 quarters, respectively. At the quarter level, 824 of the overall 1,024 milk samples were bacteriologically positive (80%). The prevalence of positive quarters in the different herds ranged from 30 to 100% (Table 1). The median values for the sampling time point T0, T1, and T2 were 87, 89, and 63%, respectively. Overall, 440 quarters were colonized by one bacterial species (53%), 305 by 2 bacterial species (37%), and 79 (10%) by 3 different species. In 613 quarters (74%) *Staphylococcus* spp. or *Mammaliicoccus* spp. were detected: 287 quarters were positive for one species (47%), 257 (42%) for 2 different species, and 69 (11%) for three different species (Table 1). The percentage of bacteriologically positive quarters differed between the herds. For the one farm that used extracted and compressed manure particles, 5 of 31 quarters (16%) were positive for NASM in the second sampling. For the other 8 farms that used straw and sawdust for bedding, the median prevalence for NASM at the quarter level was 79%.
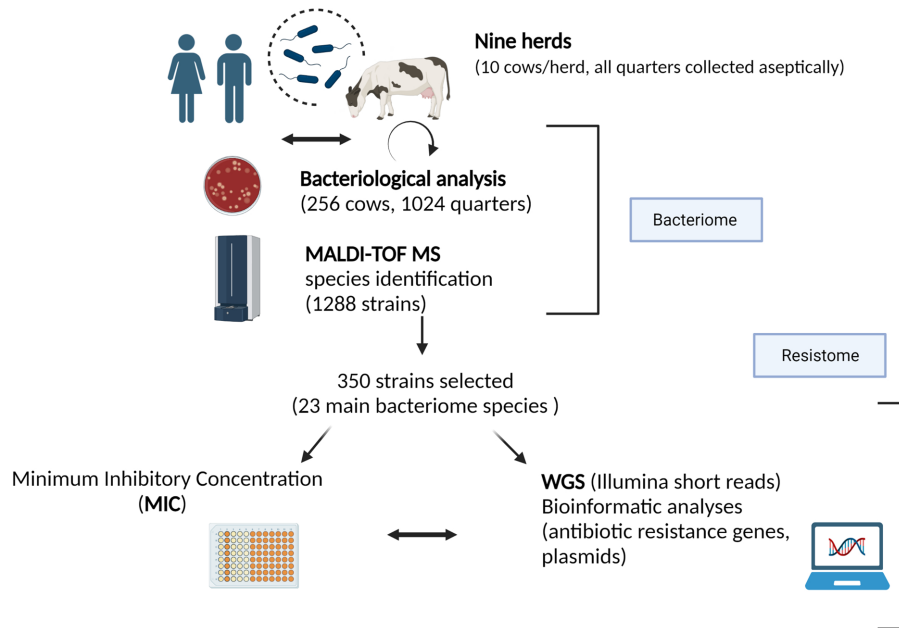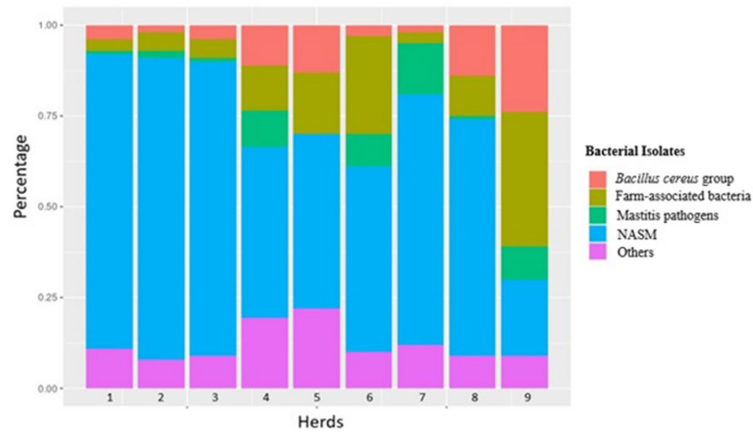
FIGURE 1
Graphical abstract representing an overview of the on-farm sampling (nine dairy cow herds from the Canton Tessin) and the experimental laboratory procedure to describe the bacteriome and resistome of healthy cows (created in Biorender.com). Confirmation of Publication and Licensing Rights.

FIGURE 2
Distribution of the main bacterial groups identified in the nine different herds over the three sampled time points.

S. xylosus was the most frequently identified isolate; it was detected in all herds and sampling time points except for herd 8 at T2. In herds 8 and 9, a much lower prevalence compared to the other herds was recorded (Table 1). In herds 1, 5, and 7, *S. xylosus* represented the main species in all three samplings, while in herd 2, *M. sciuri* was predominantly detected. For the other herds, different patterns were found. In herd 3, NASM were the main bacteria, mainly represented by *S. xylosus*, *M. sciuri* and *Mammaliicoccus*

vitulinus with differences within the samples. In herd 4, the first sampling was colonized by *Staphylococcus warneri*, while in the second and third sampling, *S. xylosus* was mainly identified. Farm number 6 was sampled only twice. The first sampling included mainly *Lactococcus* spp. and the second was mainly composed of *S. xylosus*. At farm number 8, the first 2 sampling included *S. xylosus*, while in the third *Staphylococcus equorum* was mainly detected. Cows from herd 9 displayed a completely different pattern of

isolates. In the first and second sampling mainly bacteria from the *Bacillus cereus* group were isolated. In the third sampling *Enterococcus* spp. was predominantly detected. To evaluate a non-random association between the herds and groups of isolated bacteria (*S. xylosus*, *M. sciuri*, *Bacillus cereus* group, farm-associated bacterial, and mastitis pathogens), a Fisher exact test was performed. The Exact test uncovered a statistically significant association ($p < 0.05$) between the herds and the bacteria isolated (*S. xylosus*, *M. sciuri*, *Bacillus cereus* group, farm-associated bacterial, mastitis pathogens) implying a distinct distribution of the groups in the different herds.

The 1,288 isolates that were isolated and identified, belonged to 104 different bacterial species (Table 1). The intramammary bacteriome compositions displayed a herd-specificity with an overall very high prevalence for NASM (Figure 3). The percentage of bacteria known to cause mastitis [*S. aureus* (2.5%), *S. uberis* (1.3%), and *Streptococcus agalactiae* (0.85%)] was lower in relation to the other categories listed above. *S. xylosus* (323 isolates) and *M. sciuri* (265 isolates) were by far the most prevalent detected bacteria in milk (for a total of 46%, 588 isolates) (distribution of the different NASM explained in the Supplementary Figure S2), followed by the *Bacillus cereus* group (9%). *S succinus* (6%) and *Enterococcus saccharolyticus* (3%), and *Escherichia coli* (2.2%) represented further potentially pathogenic bacteria. 52 isolates (4%) could not be identified by MALDI-TOF MS typing. Figure 3 shows a graphical distribution of all bacterial isolates.

3.2. Intramammary resistome

Among all isolates, 350 isolates were selected to be analyzed in more detail with respect to their genomic sequence and phenotypical antimicrobial resistance profile. To study the AMR genes and the phenotypical resistome, we chose a subset of isolates that represented the most abundant species as described in the Materials and Methods section. The data which species were identified in the study and the corresponding number of isolates are listed in Table 2.

3.2.1. Phenotypic AMR

Clindamycin and oxacillin resistance were most often observed among the 350 isolates with a total of 227 isolates (65%) resistant to clindamycin and 105 isolates (30%) to oxacillin (Table 3). The main resistant isolates were assigned to the NASM, and *Bacillus cereus* families. In total, 18% of the isolates were sensitive to all tested antibiotics, 25% of the isolates (88) were resistant to one and 28% (97) to two different antibiotics. Fifteen percent of the isolates (54) displayed AMR to more than four antibiotics. These isolates mainly belonged to the multi-resistant *Bacillus cereus* group. The distribution of the antibiotic resistances and percentage of the phenotypic resistances are listed in Tables 3, 4 (Graphical representation heatmaps Figure 4). The data of the phenotypic results for all species analyzed are listed in Supplementary Table S7.

3.2.1.1. *Staphylococcus* spp.

For 17 of the 31 antibiotics tested in this study, we identified at least one isolate that was resistant. Eighteen percent of the *Staphylococcus* spp. isolates were sensitive to all tested antibiotics, 34% were resistant to one, 30% to two antibiotics, and the remaining 18% showed multiple resistances for up to six antibiotics. Resistance to clindamycin (116, 73%), oxacillin (31, 20%), tetracycline (26, 16%), azithromycin (25, 16%), and penicillin (14, 9%) were detected most often. In detail, *S. xylosus* were phenotypically resistant to clindamycin in 83% of the isolates, oxacillin (31%), and tetracycline (25%). Fifteen percent of the *S. xylosus* isolates were multi-drug resistant (MDR) for more than 3 classes of antibiotics. For *Staphylococcus succinus* 41% of the isolates showed resistance to clindamycin and 36% to penicillin. *Staphylcoccus equorum* isolates mainly exhibited resistance to azithromycin and erythromycin (macrolides) (69%), and 50% of the isolates were resistant to clindamycin. *S. warneri* isolates only showed resistance to fosfomycin. *Staphylococcus haemolyticus* isolates were resistant to azithromycin and teicoplanin. All *S. chromogenes* isolates showed resistance to clindamycin; interestingly, one isolate was also resistant to macrolides (azithromycin and erythromycin) and tetracycline. In summary, in *Staphylococcus* spp., a high resistance rate to clindamycin, oxacillin, tetracycline and azithromycin was detected.
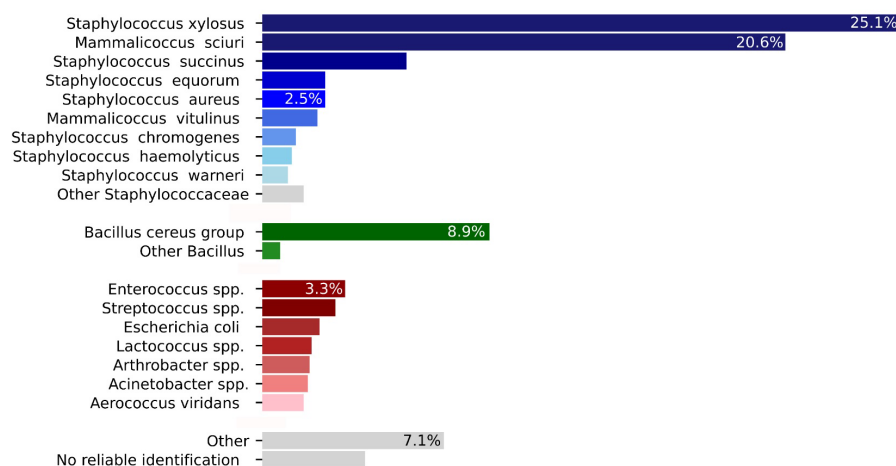

FIGURE 3
Graphical summary of the distribution of the 1,288 bacterial isolates from Table 1 included in the main text (total number and percent). The bacterial species are divided accordingly with the main table.

TABLE 2 Bacteria selected for Illumina-based WGS and phenotypic antibiotic analysis.

Bacterial species isolates	Milk T0	Milk T1	Milk T2	Sum per species
Staphylococcus xylosus#	35	38	28	101
Mammaliicoccus sciuri#	27	40	16	83
*Staphylococcus succinus**	13	4	5	22
*Staphylococcus equorum**	3	0	13	16
Staphylococcus aureus#	4	0	5	9
*Mammaliicoccus vitulinus**	5	0	4	9
*Staphylococcus warneri**	5	0	0	5
*Staphylococcus haemolyticus**	2	0	1	3
*Staphylococcus chromogenes**	0	0	3	3
*Acinetobacter lwoffii**	2	0	0	2
*Aerococcus viridans**	6	0	3	9
*Arthrobacter gandavensis**	2	0	0	2
Bacillus cereus group#	10	25	0	35
*Enterococcus faecalis**	0	1	0	1
*Enterococcus faecium**	0	2	0	2
*Enterococcus saccharolyticus**	3	3	0	6
Escherichia coli #	4	9	0	13
*Lactococcus garvieae**	5	2	0	7
*Lactococcus lactis**	4	3	0	7
Streptococcus agalactiae #	6	5	0	11
Streptococcus uberis #	0	0	4	4
Total number				350

The respective frequency of the bacterial species isolated at three different sampling time points is listed. Hash-tag symbols denote species for which a complete type strain genome was available at the NCBI. Asterisks denote species for whose type strain a *de novo* assembly was carried out combining data from 3rd generation long read sequencing technologies (PacBio and/or ONT) and the Illumina short read platform (see Supplementary Table S2).

3.2.1.2. *Mammaliicoccus* spp.

M. sciuri and *M. vitulinus* were mainly resistant to clindamycin (87%) and oxacillin (52%). Of 83 *M. sciuri* isolates, 91% were resistant

TABLE 3 Distribution of the overall number of phenotypic antibiotic resistances exhibited by the different bacterial isolates analyzed in this study.

Bacteria isolates	No R	R 1 AA	R 2 AA	R 3 AA	R 4 AA	R > 4 AA
Staphylococcus xylosus	11	**37**	34	15	4	
Mammaliicoccus sciuri	4	27	**35**	14	1	2
Staphylococcus succinus	7	7	**8**			
Staphylococcus equorum	2	2	5	**6**	1	
Staphylococcus aureus	**4**	1	1	1		2
Mammaliicoccus vitulinus	**6**	3				
Staphylococcus warneri	**3**	2				
Staphylococcus haemolyticus	1	**2**				
Staphylococcus chromogenes	**2**				1	
Acinetobacter lwoffii	**2**					
Aerococcus viridans	1	1	2	1		**4**
Arthrobacter gandavensis	**2**					
Bacillus cereus group					1	**34**
Enterococcus faecalis						**1**
Enterococcus faecium			**2**			
Enterococcus saccharolyticus			**6**			
Escherichia coli	**10**	2				1
Lactococcus garvieae			1			**6**
Lactococcus lactis		2				**5**
Streptococcus agalactiae	**11**					
Streptococcus uberis	**3**		1			
Total	69	86	95	37	8	55

The table shows the number of resistances observed for the isolates, which could range from resistance to one antibiotic (AB) up to resistance to over four ABs. R, resistance; AB, antibiotics. The meaning of the bold values represent the majority of the samples resistance to the antibiotics.

to clindamycin, 54% to oxacillin, and 8% to tetracycline. Twenty-nine isolates presented a resistance to both clindamycin and oxacillin (35%), and 32% were limited to clindamycin. Additional AMRs to daptomycin (6 isolates: 7%), azithromycin (4 isolates: 5%) and

TABLE 4 Percentage of phenotypic resistance observed against different antibiotics (based on the MIC assays).

Bacteria isolates species	Gm[1]	To[1]	Etp[2]	Imp[2]	Mer[2]	Cpe[3]	Cft[3]	Crm[3]	Cax[3]	Cp[4]	Mxf[4]	NA[4]	Nxn[4]	Tei[5]	Cd[6]	Lin[6]	Dap[7]	Azi[8]	E[8]	Mup[9]	Fd[10]	Lzd[11]	Am[12]	Aug[12]	Ox[12]	P[12]	C[13]	Fos[14]	Cl[15]	Rif[16]	Sts[17]	Prs[17]	Syn[18]	Min[19]	Te[19]	T/S[20]	Total N. isolates
ST_XY			1												83		1	7	2	2	1				31	7	3	1		1					25		101
MA_SC	1						1			1	1				98		7	5	2	4					54	1	2	1		2					8		83
ST_SU														9	41		9	9							36												22
ST_EQ															50		13	69	69									6							6		16
ST_AU	11														22			33	11				22		22												9
MA_VI															33				11					22													9
ST_WA																														40							5
ST_HA														33				33																			3
ST_CH															100			33	33																33		3
AC_LW																																					2
AE_VI					11	33	22	33																	55	22				11				22	44	11	9
AR_GA																																					2
BAC_CER_GR	6	9	6	3	6	100	100	100							91		40	20		100			97	14	86	100	3			6					9	6	35
EN_FAECALIS																100			100												100	100	100		100		1
EN_FAECIUM																100														100							2
EN_SA																100																	100				6
ES_CO												8	8						8			8							15								13
LA_GA						71	86	86	86						57				29				43		86					100				29	29		7
LA_LA						14	71	71	71														57							100							7
STR_AG																																					11
STR_UB																																		25	25		4
Total isolates																																					350

The table displays the percentage of the resistant isolates, i.e., compared to the total number of isolates per species. The antibiotics were arranged according to their classification into different classes (superscript numbers 1 to 20; see legend). Bacterial species listed: ST_XY: *Staphylococcus xylosus*, MA_SC: *Mammaliicoccus sciuri*, ST_SU: *Staphylococcus succinus*, ST_EQ: *Staphylococcus equorum*, ST_AU: *Staphylococcus aureus*, MA_VI: *Mammaliicoccus vitulinus*, ST_WA: *Staphylococcus warneri*, ST_HA: *Staphylococcus haemolyticus*, ST_CH: *Staphylococcus chromogenes*, AC_LW: *Acinetobacter lwoffii*, AE_VI: *Aerococcus viridans*, AR_GA: *Arthrobacter gandavensis*, BAC_CER_GR: *Bacillus cereus group*, EN_FAECALIS: *Enterococccus faecalis*, EN_FAECIUM: *Enterococcus faecium*, EN_SA: *Enterococcus saccharolyticus*, ES_CO: *Escherichia coli*, LA_GA: *Lactococcus garvieae*, STR_AG: *Streptococcus agalactiae*, STR_UB: *Streptococcus uberis*. Classification of different antibiotics: 1 Aminoglycoside (Gm: gentamycin, To: tobramycin), 2 Carbapenem (Etp: ertapenem, Imp: imipenem), 3 Cephalosporins (Cpe: cefepime, Cft: cefotaxime, Crm: cefuroxime, Cax: ceftriaxone) 4 Fluorochinolones (Cp: ciprofloxacin, Mxf: moxifloxacin, NA: nalidixic acid, Nxn: norfloxacin) 5 Glycopeptides (Tei: teicoplanin), 6 Lincosamides (Cd: clindamycin, Lin: lincomycin) 7 Lipopeptides (Dap: daptomycin), 8 Macrolides (Azi: azithromycin, E: erythromycin), 9 Mupirocins (Mup: mupirocin), 10 Nitrofurantoin (Fd: nitrofurantoin), 11 Oxazolidinones (Lzd: linezolid), 12 Penicillin (Am: ampicillin, Aug: amoxacillin/ K clavulanate, Ox: oxacillin, P: penicillin) 13 Phenicols (C: chloramphenicol), 14 Fosfomycins, (Fos: fosfomycin)15 Polymyxins (CI: colistin), 16 Rifampicins (Rif: rifampin), 17 Streptogramins (Sts: streptomycin, Prs: pristamycin), 18 Synercyd (Syn), 19 Tetracyclines (Min: minocycline, Te: tetracycline), 20 Trimethoprim/sulfamethoxazole (T/S).
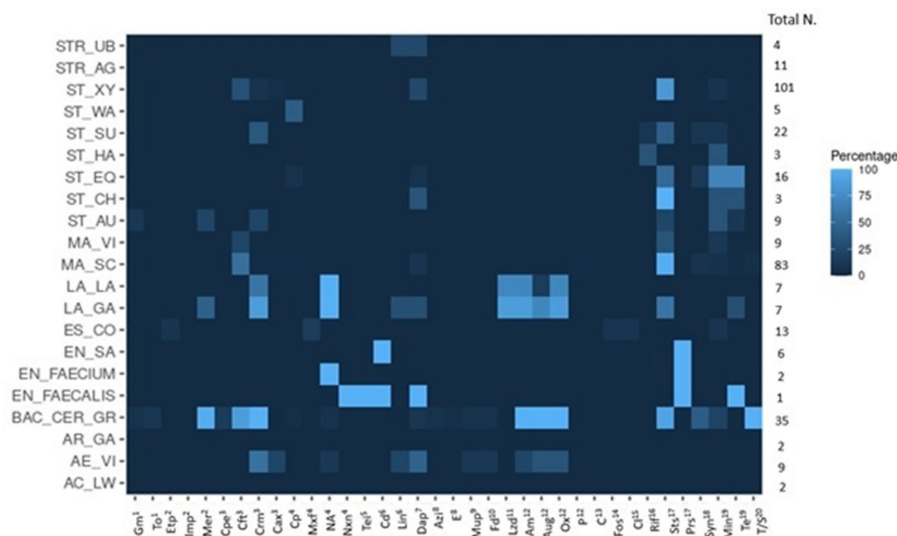
FIGURE 4
Graphical heatmap showing the results displayed in the Table 4. The different color scale represents the percentage of isolates resistant to the different antibiotics checked.

mupirocin (3 isolates: 4%) were observed. 5% of the *Mammaliicoccus* spp. isolates were sensitive to all antibiotics analyzed in this study. For *M. vitulinus*, five out of nine isolates were sensitive to all antibiotics (56%), one isolate was resistant to azithromycin and two displayed a resistance to oxacillin.

The Exact test revealed a highly significant association between the NASM species (*S. xylosus, M. sciuri, S. equorum, S. succinus*) and the five main antibiotic resistances to azithromycin, clindamycin, oxacillin, penicillin, and tetracycline; for all species analyzed ($p < 0.001$). For *S. xylosus* and *M. sciuri*, the two most prominent species, the Exact test showed only a significant association for *S. xylosus* between the herds and tetracycline resistance ($p < 0.001$). A resistance to tetracycline was detected in six out of the nine herds.

3.2.1.3. *Bacillus cereus* group

The isolates of the *Bacillus cereus* group were mostly multi-drug resistant isolates. All isolates were resistant to more than six antibiotics, except one isolate, which was only resistant to four antibiotics. All 35 isolates exhibited resistances to cefepime, cefotaxime, cefuroxime, and penicillin; additionally, 34 out of 35 were resistant to ampicillin. These results indicated a high resistance to β-lactam antibiotics. Only three isolates were resistant to tetracycline (9%), but a high proportion was resistant to clindamycin (32 isolates, 91%).

3.2.1.4. Farm associated bacteria

Further research on antibiotic resistance was performed with bacterial isolates from *Acinetobacter lwoffii, Aerococcus viridans, Arthrobacter gandavensis, Enterococcus* spp., *E. coli, Lactococcus* spp., and *Streptococcus* spp.

For *Acinetobacter lwoffii* and *Arthrobacter gandavensis*, no resistance to any of the tested antibiotics was detected. Regarding *Aerococcus viridans*, the breakpoints were not defined for all tested antibiotics. Based on the EUCAST guidelines for *Aerococcus* spp., only the resistance to amoxicillin/K clavulanate, ampicillin, levofloxacin, meropenem, penicillin, rifampin, and vancomycin could be defined. Based on this observation, we isolated one isolate resistant to meropenem and another one to rifampin. Additionally, five isolates from two different farms were resistant to penicillin (55%). We identified additional resistances to cephalosporins, chloramphenicol, tetracycline (44%) and trimethoprim/sulfamethoxazole. All bacteria of the *Lactococcus* spp. displayed resistance to rifampin and to different categories of β-lactam antibiotics.

All *Enterococcus* species were resistant to lincomycin. Two isolates of *Enterococcus faecium* were additionally resistant to rifampin, while six isolates of *Enterococcus saccharolyticus* were also resistant to synercid. The isolates of *Enterococcus faecalis* displayed multi-resistances to erythromycin, gentamicin, pristinamycin, streptomycin, synercid, and tetracycline.

Ten out of 13 analyzed *E. coli* isolates were sensitive to all the antibiotics tested; two isolates were resistant to colistin and one isolate was resistant to aztreonam, nalidixic acid, nitrofurantoin, and norfloxacin.

3.2.1.5. Mastitis pathogens

Among the nine *S. aureus* isolates, two isolates showed only resistance to azithromycin and clindamycin, one isolate to azithromycin and the β-lactams ampicillin and penicillin. Additionally, one isolate was MDR. Considering the 15 *Streptococcus* spp. (11 *S. agalactiae* and four *S. uberis*), 14 were sensitive to all antibiotics (93.3%). One isolate of *S. uberis* was exclusively resistant to tetracyclines (minocycline and tetracycline).

3.3. Whole genome sequencing

We set out to study the resistome using a collection of 23 type strains for the most abundant species that were uncovered by our extensive bacteriome analysis. For fifteen of these strains, no complete genome was available, and we thus set out to *de novo* assemble their complete genomes as a reference for the community and to avoid missing antibiotics resistance-relevant genes in fragmented Illumina assemblies (Varadarajan et al., 2020). A combination of long reads from third generation long read sequencing platforms (PacBio or ONT) and short read Illumina sequences (for polishing and to identify potential small plasmids) was used and *de novo* assembled (Supplementary Methods). All 350 isolates were sequenced with Illumina HiSeq (Supplementary Methods). A first reference-based assembly was done using the 23 complete genomes of the respective type strain of each species as a reference. The median, minimum, and maximum coverage and median total sequence lengths are listed in Supplementary Table S6. Importantly, for the type strains, we also tried to assemble plasmid sequences (Supplementary Methods; Supplementary Table S2).

3.4. Prevalence of antibiotic resistance genes

To assess the presence and prevalence of ARGs, three different bioinformatic methods were applied. Combining the results of all methods, 66% of the 350 isolates carried at least one ARG. In total, 96 different ARGs were detected; based on the literature, 53 genes were classified as specific for resistance against one antibiotic molecule, 43 were implied in causing resistances to more than one molecule. The high number of identified ARGs largely results from the big number of genes detected by the CARD database for *S. aureus* and *E. coli* species. The complete results for the ARGs, including the functionality of the main ARGs detected in the isolates (Supplementary Tables S8, S9), can be found in the Supplementary material. The main ARGs detected in the 350 isolates are summarized in Table 5 and Figure 5.

3.4.1. *Staphylococcus* spp.

For *S. xylosus*, 64 isolates (63%) were negative for ARGs, while in the 37 remaining isolates (37%) at least one ARG was detected. The *tetK* gene was the most prevalent gene observed in 25 isolates (25%). Other less prevalent genes were *fosD* (6 isolates), *str* (6), *cat* (4), *mphC* (4), *erm* (44) (1), and *msrA* (1). One isolate carried the ARGs *blaZ* and *mecC2*. The isolates of *S. chromogenes*, *Staphylococcus haemolyticus*, and *S. succinus* did not carry any known ARG. For *S. equorum*, most of the isolates (13%) harbored the *mphC* gene, in three isolates (19%) the *str* gene was detected. In one isolate *mphC*, *str* and *tetK* were observed simultaneously. *S. warneri* isolates were always positive for *gyrB*.

3.4.2. *Mammaliicoccus* spp.

In all 83 *M. sciuri* isolates subjected to WGS, the *mecA1* and *salA* genes were detected. Additionally, two isolates (~2%) carried *cat*, *lnu (A)*, *str*, *tetK* and *tetL*. Only one isolate carried 6 ARGs: *mecA*, *salA*, *aac (6′)-aph (2″)*, *lnuA*, *str* and *tetL*. Regarding *M. vitulinus,* seven out of nine isolates (77.8%) were *mphC* positive. One isolate additionally carried the *msrA*. Additional bioinformatic promoter analyses for the

mecA1 gene did not reveal any mutation in the promotor region in any of the 20 analyzed isolates when compared to the promoter region of the *M. sciuri* type strain (Wu et al., 2001; Frey et al., 2013).

3.4.3. *Bacillus cereus* group

A high prevalence of β-lactams antibiotic resistance genes was detected in the *Bacillus cereus* group. The *Bc* gene was found in 24 isolates (68.6%) and the *BcII* gene that encodes a zinc metallo β-lactamase was found in all the isolates. In addition, most of the isolates were positive for *fosB1* (77.1%). In one case, the tetracycline resistance gene, *tetL*, was also detected.

3.4.4. Farm-associated bacteria

Further analyses of the ARGs were done with the other 11 bacterial species, which were less prevalent in the milk samples. For *Arthrobacter gandavensis* and *Enterococcus saccharolyticus* bacteria, no ARGs were detected. The investigation of the *E. coli* isolates showed different results depending on the bioinformatics tools used. The analysis done with the ResFinder software revealed the presence of *mdfA* in all of the isolates. In one isolate, this gene was present together with *fosA7*. Notably, the analysis performed with the CARD database showed a different picture; more than 30 different genes were found in all 13 *E. coli* isolates and they seemed to be an intrinsic part of the *E. coli* genome but, based on our phenotypic profiling, not always expressed in the bacterial isolates.

For the other bacteria that belong to the bacteriome but that were less prevalent, a more detailed analysis for look regarding the presence of ARGs is explained in the Supplementary Table S8.

3.4.5. Mastitis pathogens

For all streptococci, at least one ARG was present. All the *S. agalactiae* isolates carried *mre(A)* and *mprF*. All isolates of *S. uberis* carried the *patB* gene and in one isolate *tetM* was additionally observed. Some *S. aureus* isolates carried at least four resistance genes *mepR*, *mgrA*, *arlR*, and *glpT* (three isolates). All nine *S. aureus* isolates were positive for *arlR*, *mgrA* and *mepR*. In the remaining six isolates, more than four ARGs were detected. Only two isolates were positive for *blaZ*, while three different ARGs were detected specifically for fosfomycin resistance (*fosB*, *glpT*, and *murA*).

A graphical heatmap shows the discrepancy between phenotypic resistant and the presence of ARGs in the same isolates (Figure 6). The results showed for several classes of antibiotics a low correlation between phenotypic and genomic results (aminoglycosides and β-lactams). Differently, in all the phenotypically resistant isolates the presence of the tetracycline ARGs was detected.

3.5. ARGs and plasmids

After *de novo* assembly, 104 (30%) out of the 350 isolates examined were positive for at least one plasmid-based replicon (*rep*) gene, in 40 cases (39%) the gene was located on a contig harboring at least one ARG (Table 6). In *S. xylosus*, *rep7* was mainly found in contigs carrying ARGs for tetracycline (*tetK*), chloramphenicol (*cat*), and streptomycin (*str*). Association of *rep7* with ARGs was also detected in *M. sciuri* (*str*, *cat*, *tetK*, and *lnuA*), in *S. equorum* (*str*, *str* plus *tetK*), and in *S. warneri* (*tetK*). Additionally, *rep7* was detected in three of nine *Aerococcus viridans* isolates that carried as well the *str* and *cat* gene. In *M. sciuri*, the gene *lnuA* was associated with 3 different *rep* types: *rep7*, r*ep13*, and

TABLE 5 Main antibiotics resistance genes (ARGs) identified.

Bacteria	aac(6')-aph(2'')	aac(6')-Ii	Bc or BcII	blaZ	cat	eatAv	erm(44)	fosB1	fosD	gyrB	lnuA	lmrD	lsa(D)	mecA1	mecC2	mre(A)	mphC	mprF	msrA	msrC	OXA-362	patB	salA	str	tetk	tetL	tetM	tetS	Total N. Strains
ST_XY				1	4		1		6						1		4		1					6	**25**				101
MA_SC	1				3						3			**83**									**83**	5	5	2			83
ST_EQ																	**11**							4	1				16
MA_VI																	**8**	1											9
ST_WA													**5**												1				5
AC_LW																						2							2
AE_VI					2						1													4			4		9
BAC_CER_GR			35	1				28																		1			35
EN_FAECIUM		2					1											2											2
LA_GA												3															1	1	7
LA_LA													**4**																7
STR_AG																	**11**	**11**											11
STR_UB																							**4**				1		4

The corresponding phenotypic antibiotic resistance correlated with the genes are listed in Supplementary Table S7. The isolates *Staphylococcus succinus*, *Staphylococcus haemolyticus*, *Staphylococcus chromogenes*, *Arthrobacter gandavensis*, and *Enterococcus saccharolyticus* were negative for the detection of ARGs. The isolates with more than three ARGs [*Staphylococcus aureus* (ST_AU), *Enterococcus faecalis* (EN_FAECALIS), and *Escherichia coli* (ES-COLI)] are described in detail in the Supplementary Table S7. The bold values represent the majority of the strains carried the antimicrobial resistance genes.
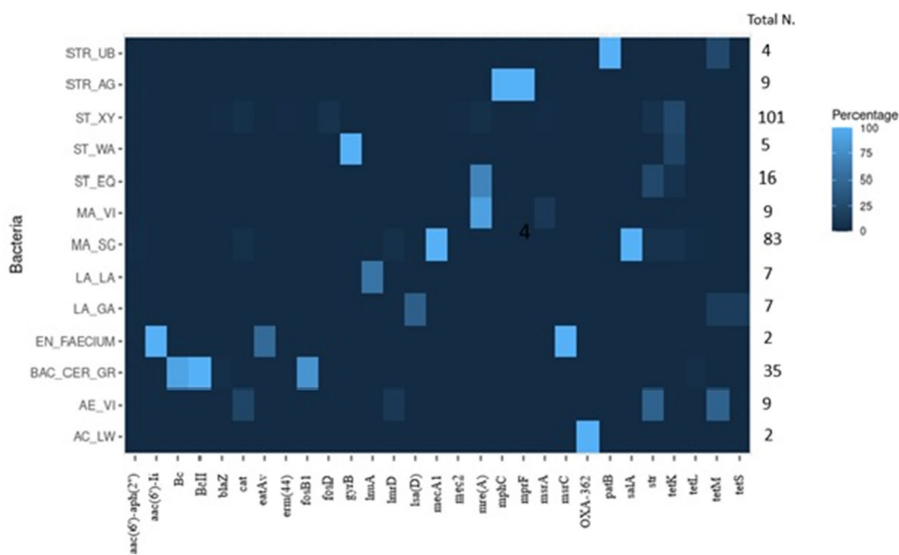
FIGURE 5
Graphical heatmap showing the results displayed in the Table 5. The different color scale represents the positivity of isolates resistant to the different ARGs.
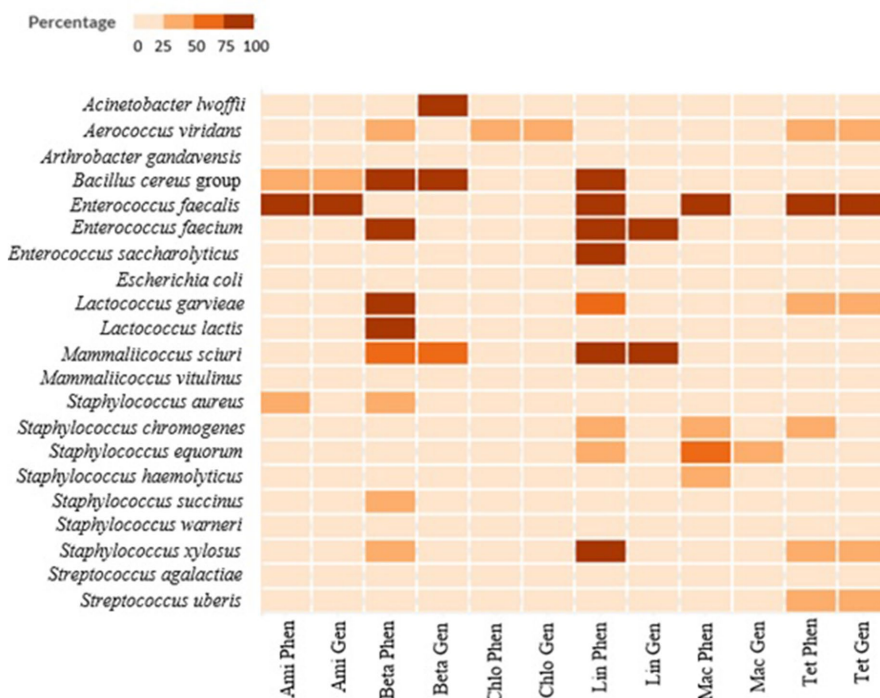

FIGURE 6
Correlation between phenotypic AMR resistance (in percent) and underlying genomic information for six classes of antibiotics (aminoglycosides, β-lactams, chloramphenicol, lincosamides, macrolides, and tetracyclines). The table displays the percentage of resistant isolates (phenotypic analysis) compared with the number of isolates where the ARGs correlated to the resistance to the antibiotics were identified. Ami Phen, Aminoglycoside phenotypic percentage; Ami Gen, Aminoglycoside genomic percentage; BetaPhen, β-lactams phenotypic percentage; Beta Gen, β-lactams genomic percentage; Chlo Phen, Chloramphenicol phenotypic percentage; Chlo Gen, Chloramphenicol genomic percentage; Lin Phen, lincosamides phenotypic percentage; Lin Gen, lincosamides genomic percentage; Mac Phen, Macrolides phenotypic percentage; Mac Gen, Macrolides genomic percentage; Tet Phen, tetracycline phenotypic percentage; Tet Gen, tetracycline genomic percentage.

TABLE 6 Results from the plasmid identification with PlasmidFinder (Center for Genomic Epidemiology, 2019).

Bacterial isolates	N. isolates positive (% on the total)	N. replicons	N. Isolates	Replicon proteins (number of isolates identified)
Staphylococcus xylosus	30 (30%)	1	27	*rep7a* (15), *rep19c* (9), *rep 21* (3)
		2	3	*rep7a*, *rep19c* (2),*rep7a*, *rep21* (1)
Mammaliicoccus sciuri	17 (20%)	1	14	*rep7a* (8), *rep13* (4), *rep21* (2)
		2	3	*rep7a*, *rep13* (3)
Staphylococcus succinus	10 (45%)	1	9	*rep16* (5), *rep19c* (2), *rep20* (1), *rep21* (1)
		2	1	*rep16*, *rep21* (1)
Staphylococcus equorum	7 (43%)	1	7	*rep7a* (4), *rep16* (1), *rep19* (2)
Staphylococcus aureus	6 (67%)	1	5	*rep7a* (4), *rep13* (1)
		2	1	*rep16*, *rep19c* (1)
Mammaliicoccus vitulinus	1 (11%)	1	1	*rep19b* (1)
Staphylococcus warneri	1 (20%)	1	1	*rep7a* (1)
Aerococcus viridans	3 (33%)	1	3	*rep7a* (3)
Bacillus cereus group	1 (3%)	1	1	*rep22* (1)
Enterococcus faecalis	1 (100%)	4	1	*rep2*, *rep8b*, *repUS11*, *repUS41* (1)
Enterococcus faecium	2 (100%)	1	1	*rep1* (1)
		2	1	*rep1*, *repUS15* (1)
Enterococcus saccharolyticus	5 (83%)	1	4	*rep1* (2), *repUS21* (2)
		2	1	*rep1*, *repUS21* (1)
Escherichia coli	10 (77%)	1	2	*IncFCI* (2)
		2 3	4 4	*IncFIA*, *IncFIB* (2), *IncFIA*, *IncFII* (1), *IncX1*, *IncFIC* (1) *IncFIA*, *IncFIB*, *IncFII* (3), *IncFIB*, *IncFIC*, *Inc1-I* (1)
Lactococcus garvieae	3 (43%)	1	3	*rep32* (1), *rep33* (2)
Lactococcus lactis	5 (71%)	1	5	*repUS33* (5)
Streptococcus uberis	1 (25%)	1	1	*repUS43* (1)

rep21. Rep13 was also observed in one contig (each) of *M. sciuri* and *S. aureus* carrying the *str* and *ermT* genes, respectively. The search for the *rep* genes was performed using PlasmidFinder. For comparison, identical analyses were also executed using PLSBD. In this case, 37 (35.6%) out of the 104 *rep* genes detected by PlasmidFinder were found.

For all NASM isolates phenotypically resistant to tetracycline including *S. xylosus* (*n* = 21), *M. sciuri* (*n* = 5), *S. equorum* (*n* = 1), and *S. warneri* (*n* = 1), a small plasmid could be identified always carrying the same *tetK* gene (100% similarity among strains) and the same *rep7a* replication initiation factor gene (Supplementary Table S10). Two of four plasmids circularized with plasmid Spades and Unicycler were, with 4,440 bp and 4,439 bp, very similar in size and were both composed by the three genes *rep7a*, *tetK*, and a plasmid recombination gene (5′-3′). The similarity was 99.7%. The other two circularized plasmids were larger in size amounting to 4,666 bp and 4,804 bp. They showed the same gene structure as the smaller plasmids, but the non-coding part was larger. The remaining 24 plasmids matched best with the 4,440 bp circularized plasmid (= reference) and showed a size between 4,435 bp and 4,448 bp; 19 of them exhibited a size of 4,440 bp with a similarity between 98.7 and 100.0% (toward the reference).

Interestingly, 13 of those plasmids showed an identical similarity of 99.7% when compared to the reference. They were mostly observed in *S. xylosus* (*n* = 11), but also in *M. sciuri* (*n* = 1), and *S. warneri* (*n* = 1) and were repeatedly found in isolates within different farms.

4. Discussion

The composition of the intramammary bacteriome of healthy, untreated cows is extremely relevant in order to assess the prevalence of commensals and potential pathogens and to determine what kind of antibiotics resistance traits are encoded by these species. Enabled by large advances in next generation sequencing, we here describe the most extensive bovine intra-mammary bacteriome study to date, which we expect to have far-reaching implications for veterinary bacteriology practice and for diagnostics (Figure 1). These aspects are becoming increasingly important in the context of the One Health concept (McEwen and Collignon, 2018), where a large reduction of the use of antibiotics is envisaged, including their wide-spread use in animal husbandry and livestock production. Moreover, knowledge

about the routes of transmission of mobile antibiotics resistance elements from animals to humans is also critical. To extend on the very detailed bacteriome, we thus also analyzed 350 isolates from the most abundant species by WGS [informed by a collection of 23 type strains, 15 of which we here assembled *de novo* relying on our expertise (Schmid et al., 2018)] and the knowledge that, compared to complete genomes, Illumina assemblies can miss genes relevant for antibiotics resistance (Varadarajan et al., 2020) and phenotypic profiling. This was done in order to (i) determine their resistome and (ii) to attempt to link phenotypic profiling data with antibiotics resistance genes, an area that requires further developments including more comprehensive and better curated databases of ARGs.

The current study highlights the presence of mainly NASM bacteria in mammary glands from healthy cows of nine Swiss herds. Overall, the bacterial isolates were found to be highly resistant to clindamycin and oxacillin. Genomic analyses revealed some consistent patterns regarding the presence of antibiotic resistance genes, for example the presence of *mecA1* and *salA* in all *M. sciuri* isolates. The tetracycline resistance was related to *tetK*, encoded on a small plasmid, which implicated a possible horizontal gene transfer between different NASM. For various phenotypic AB resistances observed, however, no ARGs were detected. As a consequence, further analyses should be performed to identify new ARGs or *in vitro* studies regarding the antibiotic resistance of veterinary bacterial isolates to actualize the current breakpoint of the MIC broth microdilution.

4.1. Bacteriome

In recent years, a few studies have investigated the bovine intramammary bacteriome using culture-dependent and culture-independent approaches (metagenomics) (Oikonomou et al., 2014; Cremonesi et al., 2018; Metzger et al., 2018; Sartori et al., 2018; Al-Harbi et al., 2021). Based on their findings, the bovine mammary gland is considered to contain a spectrum of different bacterial species. The results of the present study, using a culture-dependent approach, support these recent findings, but they show a much broader and more complex diversity of bacteria than expected. Bacteria from the NASM (61.1%) and the *Bacillus cereus* group (9%) were most frequently identified. Bacteria commonly present in the farm environment were less abundant [*Acinetobacter* spp. (1.8%), *Aerococcus viridans* (1.6%), *Arthrobacter* spp. (1.9%), *Enterococcus* spp. (3.2%), *E. coli* (2.2%), and *Lactococcus* spp. (1.9%)]. Further, a low percentage of known mastitis pathogens were detected in healthy cows [*S. aureus* (2.4%), *S. agalactiae* (0.8%), and *S. uberis* (1.3%)]. Indeed, in the present study 15.5% of the analyzed quarters showed a subclinical mastitis as defined by IDF (SCC ≥ 200,000 cell/mL) and these pathogens definitely contributed to the prevalence although it was also observed in quarters with monocultures of *S. xylosus* (19%) and *M. sciuri* (8%) (International Dairy Federation, 2022). Rarely, increased SCC were also observed for other NASM and bacteria and for co-cultures (data not shown). For *S. xylosus*, its prevalence is substantial, whereas for *M. sciuri* it is lower.

The present study further demonstrates that the intramammary presentence of *S. xylosus* and *M. sciuri* is highly farm dependent but is independent on the cow's stage and number of lactations at the cow level. At the quarter level, however, a significant association was established for *S. xylosus* and both variables indicating that quarters of older cows and in progressed lactation are more susceptible to

intramammary *S. xylosus*. For *M. sciuri*, a significant association between stage of lactation and intramammary presence was observed at the quarter level demonstrating that intramammary *M. sciuri* is more common during later lactation.

4.1.1. Non-*aureus* staphylococci and mammaliicocci

Staphylococcaceae represented by *S. xylosus* (25%) and *M. sciuri* (20.6%) were the most frequently detected bacteria, followed by *S. succinus* (5.7%). Interestingly, *S. xylosus* and *M. sciuri* were frequently (40%) co-isolated from the same quarter. These results confirm previous studies where *S. xylosus* and *M. sciuri* were also the most frequently isolated bacteria (Malinowski et al., 2011; Frey et al., 2013; Xu et al., 2015; De Visscher et al., 2016; Condas et al., 2017; Sartori et al., 2018; Valckenier et al., 2020). In the present study, however, with 73.5% of milk quarter samples being positive for NASM, the detection rate was higher, particularly when compared to studies that have analyzed milk samples from healthy cows (Porcellato et al., 2020; Al-harbi et al., 2021). It has been shown that the prevalence and distribution of NASMs is influenced by regional and environmental factors (Vanderhaeghen et al., 2015; Rowbotham and Ruegg, 2016; Alanis et al., 2021). Indeed, we detected, a clear association between intramammary bacteria and the bedding: mammary glands of cows kept on straw contained a higher number of NASM; while in one farm, in which manure was used as bedding, *Enterobacteriaceae* and *Enterococcaceae* were predominant. However, this finding needs further investigations.

4.1.2. *Bacillus cereus* group

An important aspect is the high prevalence of bacteria belonging to the *Bacillus cereus* group in milk samples of healthy cows (9%). This bacterial group, including twelve closely related species, is commonly found in environmental and food products accounting for between 11 to 47% of isolates, particularly in raw milk from cows and buffalo (Liu Y. et al., 2015; Owusu-Kwarteng et al., 2017; Baldwin, 2020; Radmehr et al., 2020; Zhao et al., 2020; Bartoszewicz and Czyżewska, 2021). Due to heat-resistant spores, these bacteria survive the pasteurization process and could cause spoilage of dairy products and even intoxication of human consumers (Gopal et al., 2015). All of these strains carried multiple ARGs.

4.1.3. Farm associated bacteria

The prevalence of these bacteria was low and accounted for a range between 1.6 to 3% of all isolates. They included *Acinetobacter lwoffii, Arthrobacter gandavensis, E. coli, Enterococcus faecalis, Enterococcus faecium, Enterococccus saccharolyticus, Aerococcus viridans, Lactococcus garvieae* and *Lactococcus lactis. E. coli* and enterococci are typical fecal representatives [National Mastitis Council (NMC), 2017], whereas lactococci are commonly isolated from raw milk with the ability to persist in a farm environment and the cows (Werner et al., 2014). In addition, *Lactococcus garvieae* and *Lactococcus lactis* were previously identified as pathogens inducing chronic subclinical mastitis in cows, mostly during late lactation (Wyder et al., 2011). *Acinetobacter lwoffii* is mainly isolated from human skin and infections, from soil and plants, but also from other sources (Adewoyin and Okoh, 2018). In contrast, *Arthrobacter gandavensis* and *Aerococcus viridans* are largely found on bovine teat skin and in milk (Wyder et al., 2011; Verdier-Metz et al., 2012). These results

demonstrate that farm-associated bacteria can be part of the intramammary bacteriome, but compared to NASM, they play a minor or even a negligible role.

4.1.4. Mastitis pathogens

With an observed frequency of 2.8%, mastitis pathogens were rarely observed. Their presence was farm specific (especially for Farm 4) and included *S. aureus*, *S. agalactiae*, and *S. uberis*. For the streptococci, our findings were very similar to a previous Swiss study that had screened whole herds for *S. aureus* (Moret-Stalder et al., 2009). The low presence of pathogens in the present study is not astonishing as only healthy cows with healthy udders were included, whose milk was suitable for human consumption according to Swiss legislation. Nevertheless, they were detected, raising the question why they were observed. All observed bacteria are known to be frequently involved in subclinical bovine mastitis [National Mastitis Council (NMC), 2017], meaning that the udder and milk are grossly normal so that the farmer was unaware that an IMI was present. Alternatively, it may be possible that the isolates represent apathogenic subtypes, as has been previously shown for *S. aureus* (Fournier et al., 2008).

4.2. Clinical and diagnostic implication

For decades, veterinarians have been convinced that the bovine mammary gland is sterile, and all bacteria isolated from milk have been considered a result of an intramammary infection and mastitis. This has been increasingly questioned in recent times, when it turned out that particularly NASM could regularly be isolated from milk samples of the same, healthy quarter over time (Sartori et al., 2018). So far, NASM have been considered as minor pathogens causing subclinical forms of mastitis (Sears and McCarthy, 2003; Taponen and Pyörälä, 2009; Condas et al., 2017) and are the most common bacteria isolated from clinical milk samples sent in for diagnostic analysis by veterinarians [National Mastitis Council (NMC), 2017]. NASM infections are regularly treated with AB, at least in Switzerland, based on the assumption that the bovine mammary gland is free from bacteria. In contrast to almost all other studies, and certainly in contrast to the clinical work in the field where milk samples are only taken and bacteriologically examined after an udder health problem has been detected, the present study, however, focuses on healthy quarters. Importantly, the same standard diagnostic culturing methods were used as they are routinely applied. And suddenly the very same NASM were found at the species level, a fact that definitely questions the role of NASM in the context of bovine mastitis. Are they really minor pathogens or do they have a more protective function? Even the finding that NASM are found together with increased amounts of inflammatory cells in the milk does not necessarily mean that they are the cause of the observed inflammation. It is well known that inflammation of the mammary gland can also result from inappropriate milking procedures leading to mechanical tissue irritation (Giesecke et al., 1986), the NASM could merely be a by-product as, at the time of sampling, they were in the quarter anyway. As a consequence, and clearly based on the present study, it is no longer possible to interpret the finding of NASM isolated from a diseased quarter in clinical terms. This is particularly true for *S. xylosus* and *M. sciuri* which first of all had been both isolated from healthy quarters. Under field conditions, a positive culture for these bacteria

should no longer be interpreted in the way that a subclinical mastitis is present in the corresponding quarter. This is only possible if the milk of the quarter shows SCC ≥ 200,000 cells/mL or a positive California mastitis test.

Indeed, the clinical significance of NASM present in the mammary gland remains to be further elucidated. Potentially, the most abundant NASM such as *M. sciuri* and *S. xylosus* need to be further subtyped to tease out some relevant differences.

4.3. Resistome

In Switzerland, between 2018 and 2019 approximately 70% of all antimicrobials used for IMI were products applied for the treatment of mastitis during lactation. Penicillin followed by aminoglycosides are the most predominantly used antibiotics according to the Swiss Antibiotic Resistance Report 2020 (Federal Office of Public Health and Federal Food Safety and Veterinary Office, 2020). Over the last years, an increase of antimicrobial resistant bacteria has been reported which is at least in part due to the misuse of antibiotics in agriculture (Manyi-Loh et al., 2018; Mann et al., 2021). Concomitantly, ARGs were detected in different environments including milk samples (Pol and Ruegg, 2007; Saini et al., 2013).

4.3.1. *Staphylococcus* spp.

Although their pathogenicity and epidemiology are still under debate (Nyman et al., 2018; De Buck et al., 2021), IMI caused by NASM are regularly treated with antibiotics in Switzerland (BLV, 2019). This requires a good understanding of AB resistance patterns and the mechanisms of action to offer an optimal therapy. In this study, they exhibited the highest resistance to clindamycin and oxacillin, which has increased compared to a previous Swiss study (Frey et al., 2013). The reason of the clindamycin resistance increase could be the recent decrease and change in the resistance breakpoint (from >0.5 to >0.25 mg/mL) in the EUCAST guidelines [The European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2022]. The percentage of penicillin resistant isolates was higher (23.3%) than the percentage for the *Staphylococcus* spp. isolates in the Frey work (8%). Both studies from Switzerland recognized a high number of isolates resistant to oxacillin, 20 and 47%, respectively. A tetracycline resistance rate between 12 to 38% was detected in *S. xylosus* bacteria in a previous Swiss study of different food products such as fermented sausages, cheeses, and meat starter cultures (Leroy et al., 2019). Our study, with a percentage of 25% *S. xylosus* isolates, confirm a high resistance prevalence for this antibiotic.

4.3.2. *Mammaliicoccus* spp.

Previous studies involving *Mammaliicoccus* spp., showed a high variability of tetracycline resistant isolates. In Switzerland, two previous studies analyzed the presence of *M. sciuri* in pigs, cattle, poultry, and different food matrixes as bulk tank milk, minced meat, and abattoir employees demonstrated the presence of resistant *M. sciuri* (Huber et al., 2011; Nemeghaire et al., 2014). The low resistance to aminoglycosides found in our study, agrees with a study by Hauschild et al. (2007) where the resistance of 204 *M. sciuri* isolates was evaluated. Recently, Lienen et al. (2022), revealed a high resistance prevalence of 26 *M. sciuri* isolates against five to twelve antimicrobial substances including methicillin. Additionally, a high prevalence of

penicillin resistance was detected (90%), much higher than in our study (52%). The high number of resistant isolates can be attributed to a selection of the *Mammaliicoccus* isolates belonging to herds where MRSA isolates were detected. Interestingly, 15% of *S. xylosus* and 21% of *M. sciuri* were MDR. The prevalence of multiple resistant isolates was considerable and can become problematic, if the ARGs would be transferred to a pathogenic bacterium such as *S. aureus.*

4.3.3. *Bacillus cereus* group

The *Bacillus cereus* group showed a high prevalence of resistance to β-lactams, in accordance with a recent paper (Bartoszewicz and Czyżewska, 2021) that had isolated *Bacillus* isolates from raw milk and which showed more often resistance than isolates from natural environments; the authors hypothesized that the higher resistance could be due to residual amounts of antibiotics in the milk. To our knowledge, and as described before, a high antibiotic resistance could be associated with intrinsic phenotypic resistance (Owusu-Kwarteng et al., 2017; Mills et al., 2022).

4.3.4. Farm-associated bacteria

The two *Acinetobacter* isolates analyzed in our study were sensitive to all antibiotics and did not show any known ARGs. In contrast, Gurung et al. (2013) found that *Acinetobacter* were highly resistant to different antibiotics tested.

The nine *Aerococcus viridans* isolates were mainly resistant to β-lactams (5) and tetracycline (4). These results partially agree with those of Sun et al. (2017) showing only a partial resistance for tetracycline and no resistance for the β-lactams. A high resistance prevalence to trimethoprim/sulfamethoxazole was found in previous works (Martín et al., 2007; Liu G. et al., 2015; Sun et al., 2017) while in this study, only one isolate was resistant to this antibiotic. Notably, the differences between the studies could be due to the different methods used; the earlier studies mainly used disk diffusion while our study used the microdilution broth (MIC).

The Enterococci of the present study (*Enterococcus faecalis, Enterococcus faecium, Enterococcus saccharolyticus*) were commonly resistant to lincomycin. This is in line with the results described by Różańska et al. (2019). All *Enterococcus saccharolyticus* and all *Enterococcus faecalis* were resistant to synercid. No isolates were resistant to vancomycin, which represents an example of an actual increasing clinical problem in humans with infections caused by *Enterococcus* spp. Our enterococci isolates showed less antibiotic resistant isolates compared to those isolated from humans (Rogers et al., 2021). This is most likely due to the fact that the majority of enterococci found in our study were *Enterococcus saccharolyticus,* which were susceptible to all AB tested except to lincosamides and synercid.

A general low resistance prevalence to antibiotics was found in *E. coli* isolates in this study (three isolates out of 13). Colistin resistance, a last resort antibiotic, was found in two isolates. This is in contrast to the Swiss antibiotic report (Federal Office of Public Health and Federal Food Safety and Veterinary Office, 2020) where all *E. coli* isolates causing bovine mastitis were susceptible to this AB.

Most *Lactococcus* spp. isolates, were multidrug-resistant. This result was in contrast to the Swiss study by Walther et al. (2008), who demonstrated that *Lactococcus lactis* isolates were sensitive to all 17 antibiotics tested. In that study, the main resistances were observed to tetracycline, clindamycin, and erythromycin, respectively (14.6, 7.3, and 7.3%). In contrast, our work showed a high resistance to rifampin

(100%), penicillin, and cephalosporins (71%). Another *Lactococcus* species, *Lactococcus garvieae*, exhibited resistance to clindamycin (four out of seven isolates). Additionally, resistance to rifampin and β-lactam AB was prominent, disagreeing with previous Swiss, and international studies (Walther et al., 2008; Devirgiliis et al., 2013). However, the higher β-lactams resistance observed in the current study could well be associated with the large use of this class of antibiotics at the intramammary level.

4.3.5. Mastitis pathogens

The resistance profile of bovine streptococci has been shown to be strongly influenced by the geographical origin of the sample and the species (Saed and Ibrahim, 2020; Kabelitz et al., 2021). In our study, all *S. agalactiae* isolates were sensitive to all the antibiotics used in the MIC assay. This supports the results of a recent study of bovine mastitis isolates in which the resistance to antibiotics in *S. agalactiae* isolated from different European countries were studied (El Garch et al., 2020). The tetracycline resistance rate of *S. uberis* agreed with the results obtained by El Garch et al. (2020) and with Swiss data (Federal Office of Public Health and Federal Food Safety and Veterinary Office, 2020). Interestingly, although penicillin is the most frequently used antibiotic against *Streptococcus* causing mastitis, no resistant isolates were found. This observation agrees with the work of Käppeli et al. (2019). The percentage of penicillin resistance of *S. aureus* isolates is comparable to results reported in two other European studies (El Garch et al., 2020; Ivanovic et al., 2023).

4.4. *In silico* analysis of ARGs and association with phenotypic results

Advances in DNA sequencing technologies and the availability of various bioinformatics tools and curated databases of ARGs have revolutionized diagnostic microbiology and microbial surveillance (Hendriksen et al., 2019). In our study, we used bioinformatics tools to try to find the ARGs causing the observed resistance. In particular, the genomes were evaluated for ARGs using two of the most popular online tools, Comprehensive Antibiotic Resistance Database (CARD) and ResFinder (Center for Genomic Epidemiology, 2019); they are very effective and have sustainable curation strategies (Lal Gupta et al., 2020). Additionally, an in-house, BLAST based program (GBlast) together with a manually curated database for *Staphylococcus* spp. containing 105 ARGs was used to specifically search for fragmented genes.

The present study demonstrates that there still is a big gap between the phenotypic and genotypic findings. In fact, for many bacteria and many ABs tested, no ARGs were found that could explain the observed phenotype. Obviously, the genetic basis of the mechanisms leading to phenotypic AMR in the bacteria present in the surrounding of cows is less well understood compared with the main pathogens causing mastitis. Nevertheless, *S. xylosus* plays an important role as a fermentative agent in food industry and *Bacillus cereus* has been known for its role in food poisoning for a long time. Particularly striking is the poor association between phenotype and genotype for NASM although recent genomics methods and databases were used. From an evolutionary point of view, NASM are close to *S. aureus*, which has been intensively investigated for AMR during the last decades (Madhaiyan et al., 2020). Based on these considerations,

we expected to find the NASM chromosomal ARGs orthologous to those of *S. aureus* that contribute to intrinsic AMR. Furthermore, we expected to find the same plasmid based ARGs that were previously observed in *S. aureus,* as a possible plasmid transfer between the two species was observed (Fišarová et al., 2019). Except for *tetK* (see below), few of these assumptions turned out to be correct. The reasons for this discrepancy remain largely unclear. For orthologous genes, the similarity between those of NASM and *S. aureus* used as the target genes is probably too low so that the NASM genes cannot be detected by the software tools applied. Considering the plasmid transfer, it is rare between *S. aureus* and NASM in the environment of cows although *S. aureus* is a major mastitis pathogen and is commonly observed on dairy farms (Leuenberger et al., 2019). From a technical point of view, the discrepancy between phenotypic and genotypic findings is hardly the result of inappropriate genomic methods, since the same were successfully applied in our recent publication to predict phenotypic penicillin resistance in *S. aureus* by WGS (Ivanovic et al., 2023).

Importantly, the present work demonstrates that only using genomic approaches might not be adequate to infer phenotypic AMR. Additional genomic and wet laboratory investigations are necessary, and a more comprehensive overview of the mechanisms of actions of different antibiotics.

4.4.1. *Staphylococcus* spp.

Except for tetracycline, no or little genomic information was found explaining the observed resistances in *Staphylococcus*. This is particularly true for β-lactam AB (penicillins and cephalosporins), lincosamides, and in part for macrolides (limited explanation for *S. equorum*). These results demonstrate that NASM may have developed their own mechanisms of resistance. Maybe some of these mechanisms rely on yet undiscovered genes orthologous to known ARGs, but it is also possible that they are based on completely distinct and so far unknown mechanisms and genes. Tetracycline resistance could be fully explained in all resistant isolates by the presence of the *tetK* gene. It was always present on a small plasmid (approx. 4,440 bp after closing) and displayed a 99% similarity to the plasmid pSX10B1, previously found in *S. xylosus* isolated from fermented sausage and linked to a possible, albeit low, transfer of this resistance to other *S. xylosus* strains (Leroy et al., 2019).

The missing association between phenotypic and genotypic results for β-lactam ABs has largely to do with the fact that no known ARGs were found explaining penicillin and oxacillin resistance in *S. xylosus* and *S. succinus*. In fact, no *blaZ* or *mecA, mecA1* or *mecC* genes were observed except in one case. The lack of these genes in *S. xylosus* agrees with the PCR findings reported by Frey et al. (2013), who were also unable to detect them. In the case of *S. equorum*, 75% of the isolates were positive for the *mphC* gene, explaining their observed resistance to macrolides. Although this association is substantial, still 30% of the isolates rely on genes other than *mphC*. Interestingly, this gene was located on the chromosome and was not associated with a mobile element suggesting that it is an intrinsic part of the *S. equorum* chromosome.

4.4.2. *Mammaliicoccus* spp.

The *mecA1* and s*alA* genes were observed in all isolates analyzed. Both were located on the chromosome and were not associated with

a mobile element. This means both genes are part of the core genome of *M. sciuri* and contribute to the intrinsic AMR of this bacterium. A high association was observed between clindamycin resistance (lincosamides) and the presence of *salA,* as all resistant isolates (93%) also carried the gene. In contrast, as previously found by Cai et al. (2021), although 100% of the isolates carried the *mecA1* gene, only 55% of the isolates showed resistance to oxacillin. For *M. sciuri* it is known that high-level *mecA1* expression is required to observe oxacillin resistance in these bacteria. Overexpression is associated with a *mecA1* promoter mutation at position-10 (Wu et al., 2001; Frey et al., 2013). In the present study, however, none of the 20 isolates analyzed showed the indicated mutation independent whether they were oxacillin resistant or not. Furthermore, all 20 genes could be translated *in silico* into a full-length protein suggesting that the protein function was not harmed by a mutation. These findings suggest that a different mechanism than the previously observed overexpression of *mecA1* accounts for the observed oxacillin resistance.

Even more controversial was that all oxacillin resistant isolates were penicillin susceptible although the low-affinity penicillin-binding proteins (PBP2A) encoded by the *mec* genes including *mecA1* cause resistance to all β-lactam AB (Schwendener and Perreten, 2022). All these findings suggest to re-consider the function of *mecA1* and its expression in future studies. The fact that resistance to β-lactam AB at the genomic level is still incompletely understood in *Staphylococcaceae* is further illustrated by our results for *M. vitulinus*: two isolates were oxacillin resistant, but no ARG was found. Considering tetracycline resistance and *M. sciuri*, all phenotypically positive isolates harbored either the *tetK* ($n = 5$) or the *tetL* genes ($n = 2$). The *tetK* gene was always found on the same small plasmid as observed in *S. xylosus*. For the *tetL* gene, a location on a new, larger plasmid or a correlation with a transposon could be assumed but this will require a further, detailed follow-up study.

4.4.3. *Bacillus cereus* group

The majority of the *Bacillus cereus* group isolates contained *fosB1*; confirming previous reports that found this ARG in isolates from vegetables (Fiedler et al., 2019) and humans (Bianco et al., 2021). Just in one case we could find an association with the phenotypic results.

All *Bacillus cereus* isolates carried the gene *BcII*, previously shown to be involved in β-lactam resistance (Bartoszewicz and Czyżewska, 2021). Additionally, in agreement with the study of Fiedler et al. (2019), a lower prevalence of the *Bc* gene was detected (68%). In our study, when at least one of the two genes was present, the resistance to β-lactams could be always detected.

4.4.4. Farm-associated bacteria

A recent review about the resistomes of *Acinetobacter* non-*baumannii* strains demonstrated that penicillin resistance mediating ARGs were described to be the most prominent resistance genes in these bacteria (Baraka et al., 2020). This study supports these results, with the presence of the gene *Oxa-362*.

A perfect agreement between some phenotypic results and the ARGs was found in *Aerococcus viridans*: all isolates were resistant to tetracycline and, concomitantly, the *tetM* gene was detected. The same was true for the chloramphenicol resistant isolates, which encoded the corresponding *catA8* gene. In contrast, no ARGs were

found that could explain oxacillin resistance, and the presence of the *str* gene was not accompanied by streptomycin resistance.

With a total of 49 genes, a rather high number of ARGs were detected in *E. coli*. Thirty-one genes were observed in all isolates, a finding that confirmed the results of Tyson et al. (2015). In addition, 18 ARGs were found in 8 to 92% of the isolates. Despite the large number of ARGs, all isolates except three were susceptible to all ABs tested. This discrepancy could be easily explained by the fact that for clinical use, and implemented on the commercial resistance plate, only those AB classes for which inherent genes and AMR known mechanisms were contained. The lack of clinical relevance is probably also the reason why most ARGs found in CARD for *E. coli* were missing in the ResFinder database (Center of genomic Epidemiology), an observation that had previously also been made by Jeamsripong et al. (2021).

Lactococcus garvieae and *Lactococcus lactis* were mainly positive for the ARGs *lsa(D)* and *lmrD*, respectively. These genes confer resistance to lincosamides, streptogramins, and pleuromutilins (Shi et al., 2021). In the present study, however, both species were susceptible to these ABs. Further discrepancies between phenotype and genotype were also found for other ABs: although all *Lactococcus garvieae* and *Lactococcus lactis* isolates were resistant to rifampicins and the majority to cephalosporins, no ARGs were detected for both species.

4.4.5. Mastitis pathogens

All *S. agalactiae* isolates carried the ARGs *mprF* and *mreA*. Both genes are commonly present in this species and are always found on the chromosome. *MprF* confers resistance to peptide-based AB while *mreA* to macrolides (Clancy et al., 1997; Ernst et al., 2009). However, in the present study, no phenotype antibiotic resistance results were observed. This fact could be correlated with an intrinsic presence of the genes on the chromosome.

In *S. uberis* isolates, a variant of the gene *patB* was found. In contrast, the *patA* gene, which is correlated with the mechanism of resistance to quinolone, was not identified (El Garch et al., 2010). As a consequence, the resistance to fluoroquinolones could not be detected.

In all *S. aureus* isolates, *arlR, arlS, lmrS, mepR, mgrA,* and *norA* were identified. All these ARGs are intrinsically located on the chromosome and are considered to contribute to basic AMR of *S. aureus* (CARD database). In the present study, however, none of the isolates showed the corresponding phenotype. This is emphasized in case of AMR to quinolones as each isolate harbored the ArlR/ArlS and the MgrA/NorA systems. The same was also true for the *lmrS* gene that was present in all isolates, but no AMR was observed for aminoglycosides, linezolid, macrolides, and phenicols. Additionally, four isolates were *fosB* positive, but sensitive to fosfomycin. In contrast, the observed AMR to penicillin/ampicillin and azithromycin/erythromycin could be explained by the presence of *blaZ* and *ermT* genes, respectively. In *S. aureus*, both genes are normally plasmid based (Kadlec and Schwarz, 2010; Ivanovic et al., 2023) leading to the question whether the current MIC methods are appropriate to assess AMR resulting from mechanisms whose ARGs are intrinsically located on the chromosome. Alternatively, the ARG may not be expressed in the media that we used. In general, transcriptomic or proteomic analyses of the expression levels of certain ARGs (and

under different conditions or media) would add additional relevant information for the aim to be able to link genotype and phenotype data.

In summary, the association between phenotype and genotype is missing for various ABs analyzed in the present study. This was observed for β-lactam, lincosamide, and macrolide ABs where the gap can be highlighted. Reasons for this discrepancy may be that the orthologous ARGs of these less commonly studied bacteria were not detected by the current bioinformatics methods, that the corresponding proteins were not expressed, or that, as a consequence of convergent evolution, other AMR mechanisms than the known ones were involved in expression of the observed phenotype. Creating complete genome assemblies for the 350 isolates was beyond the financial possibilities of this study, and might contribute to the fact that some ARGs were missed as well.

4.5. Plasmids and their transfer of ARGs

Plasmids are an important source for the exchange of ARGs between different species and have been reported for *Staphylococcus* species (Malachowa and Deleo, 2010; Mlynarczyk-Bonikowska et al., 2022).

Based on the analyses of circular plasmids, we can contemplate a possible horizontal gene transfer mechanism, when *S. xylosus* and *M. sciuri* co-exist in the same farm. The bacteria apparently transfer small plasmids involved in the antibiotic resistance mechanisms as for tetracycline (*tetK*). The plasmid harboring the *tetK* gene was found in *S. xylosus* (approx. 4,440 bp) and displayed a 99% similarity to the plasmid pSX10B1, previously isolated in *S. xylosus* from fermented sausage (Leroy et al., 2019). In this study, the small plasmid mediated the mechanisms of mobilization, but was rarely transferred to others *S. xylosus* bacteria. The small plasmids are non-conjugative mobilizable, which means that they are not able to be transferred without some helper elements as conjugative plasmids or transposons (Francia et al., 2004; Ramsay et al., 2016). In our study, the completely identical small plasmid found in *S. xylosus* was isolated also from *M. sciuri* and one isolate of *S. warneri* and *S. equorum*. The presence of the same small plasmids circulating in different species could suggest an exchange of the plasmids between the four NASM species. Additional to the small plasmids carrying the tetracycline resistance, other plasmids, carrying *cat* (chloramphenicol resistance) and *str* (aminoglycosides resistance), for instance were detected. These results, although limited to only some of the bacterial species, highlight a possible transfer of ARGs mainly through small plasmids.

In summary, our study finds a high prevalence of bacteria in aseptically collected milk samples from healthy cows. The composition of the intramammary bacteriome displayed a farm-and bedding-dependency: the predominant isolated species were *S. xylosus* and *M. sciuri*, especially in herds that used a straw bedding system. The physiological significance of the NASM in the mammary glands, however, remains to be elucidated in further studies. In contrast, species belonging to the *Bacillus cereus* group or other mastitis pathogens were only rarely detected. It is essential to get more knowledge about the bacteriome of the mammary glands of healthy and diseased cows to understand and preserve the

physiologically normal microbiota, hinder pathogens to gain a foothold and, in the long term, prevent the development and spread of resistances. Further studies addressing the phylogeny of the isolates from milk and herd environment need to be done to understand the origin of the isolates. NASM displayed individual species-specific ARG profiles. Not all phenotypic resistances were based on the presence of known ARGs. WGS represents an important tool for detecting ARGs but still needs to be associated with phenotypic analysis and with gene and/or protein expression analyses. Screening for new genes associated with AMR and an increase of the ARG databases will be essential, especially for the One Health concept.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: All the reads of the different bacteria isolated were uploaded in NCBI under the Bioproject PRJNA859642, https://www.ncbi.nlm.nih.gov/bioproject/859642. The genome and the plasmids of the 15 de novo assembled type strains were uploaded in NCBI under the Bioproject PRJNA936091, https://www.ncbi.nlm.nih.gov/bioproject/936091. Our manually curated database of 105 Staphylococcus spp. ARGs is released as Supplementary material Data Sheet 2.

Author contributions

HUG designed and wrote the initial project application. AR, LS, and MV performed the sampling of the herds. AR, II conducted experiments. JW and LE performed the SCC measurements. TS, MS, and CHA performed *de novo* genome assembly of the type strains (with long read data contributed by DF and JF), identified additional plasmids and closed them. AR and HUG analyzed the data. AR and HUG wrote the first draft of the manuscript with substantial input from CHA. LV, MD, and AS contributed to the conceptualization of the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1183018/full#supplementary-material

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3.2 Distribution of *Staphylococcus xylosus* and *Mammaliicoccus sciuri* isolated from quarter milk and environmental samples in Swiss dairy herds; in depth-analysis of the genomic of *Mammaliicoccus sciuri*

Title: Distribution of Staphylococcus xylosus and Mammaliicoccus sciuri isolated from quarter milk and environmental samples in Swiss dairy herds; in depth-analysis of the genomic of Mammaliicoccus sciuri

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Abstract

Non-aureus staphylococci and mammaliicocci (NASM) are universally recognize as major bacteria involved in bovine intramammary infection. Additionally, NASM colonized the udder microbiome of healthy cows and could express bacteriocins and supplementary factors against the pathogen bacteria. Despite the increase of the studies regarding these bacteria, a clear understanding about their circulation at the herd level remain unclear. To achieve this goal, a research-involved *Staphylococcus xylosus* and *Mammaliicoccus sciuri* isolated from quarter milk samples and environment samples belonging to nine different herds was done. From a selective group of strains whole genome sequencing (WGS) to detect the presence of antimicrobial resistance genes (ARGs) and phenotypic analysis of the minimum inhibitory concentration (MIC) were accomplished. Additional phylogenetic analysis for *Mammaliicoccus sciuri* were done to understand the phylogenetic correlation among the strains originated from different matrixes. The results showed the presence of the same bacteria in both milk and environmental samples. A similar pattern of antibiotic resistance profile was detected independently from the origin of the samples. Oxacillin and Clindamycin antibiotics showed the high rate of resistance for both the species analyzed. For *M. sciuri* excluding a common pattern of ARGs (*mecA1* and *salA*), ARGs to streptomycin (*str*) and tetracycline (*tetk, tetL,tetM*) were detected. A high positivity of ARGs for streptomycin and tetracycline were detected also for the *S. xylosus* strains, with an additional high number of fosfomycin ARGs (*fosD*). For *M. sciuri* a several STs from the different matrixes were detected including new STs. The phylogenetic analyses showed a great variability between and within the different herds but the division of the bacterial species in two different main clades with one udder correlated was strengthened also with the ANI analysis. This works improves our knowledge on the diffusion of NASM in the herd environment and regarding the epidemiology of *M. sciuri*.

Key words: milk quarters, environmental samples, Staphylococcus xylosus, Mammaliicoccus sciuri, WGS, phylogenetics, ARGs

1. Introduction

Non-aureus staphylococci (**NAS**) bacteria are often isolated from quarter milk samples but still their role as commensal or pathogen of minor importance at the herd level is still not completely understood (Hammel et al., 2020, De Buck et al., 2021). Additionally, NAS colonized the udder microbiome of healthy cows and could express bacteriocins and supplementary factors against the pathogen bacterial pathogen (Winther et al., 2022).

NAS included all the staphylococci other than *Staphylococcus aureus*, one of the main pathogens correlated with mastitis and cause of intramammary infection (IMI) at the herd level. Mastitis remains the main cause of economic losses at the herd level due to the decrease in the production of milk, culling of animals and costs for antibiotic therapy (Ruegg, 2017). Recently, the attention to the fact that NAS can also cause IMI increasing due to the correct identification of these bacterial species. Using the old method, NAS were not always correctly identify at the species level; thanks to the development of the MALDI-TOF instrument fast and correct speciation are possible now (Cameron et al., 2018).

NAS were recently re-classified assumed on a phylogenetic study involving 72 selected housekeeping genes (Madhaiyan et al., 2020). The new genus *Mammaliicoccus* was introduced and the bacteria previous called *Staphylococus sciuri*, *Staphylococcus fleuretti*, *Staphylococcus lentus*, *Staphylococcus stepanovicii* and *Staphylococcus vitulinus* were reassigned to the novel genus. *Mammaliicoccus sciuri* was chosen as type species for the

group. Additionally, a new acronym included the *Staphylococcus* and *Mammaliicoccus* genus, non-*aureus* staphylococci and mammaliicocci (**NASM**) (Rosa et al., 2022).

The most prevalent NASM changed establish on the geographical regions. In a recent Swiss paper by Sartori et al., a field study was performed including five herds; the dominant NASM species was recognised as *Staphylococcus chromogenes* (*S. chromogenes*) (Sartori et al., 2018). Regarding the two species analysed in our study, *Staphylococcus xylosus* (*S. xylosus*) and *Mammaliicoccus sciuri* (*M. sciuri*), the first one was identified as prevalent NASM in previous works in Canada, China, and other European countries including Switzerland (Frey et al., 2013, De Buck et al., 2021). In Switzerland, the species was identified in milk originated from clinical and subclinical mastitis and from control samples derived from milk collected from cows after the mastitis treatment (Frey et al., 2013). Regarding *M. sciuri*, the species was predominant in China, Belgium and Switzerland (De Buck et al., 2021). Based on the bibliography, the NASM were not only isolated from the milk but they were additionally isolated from feces (Wuytack et al., 2020), and other environmental samples (teat apex (Mahmood et al., 2018), heifers and body sites (Adkins et al., 2018). Furthermore, NASM were isolated also from the food chain (brining bath cheese (Hammer et al., 2019) and ready-to-eat-food from animal origin (Chajęcka-Wierzchowska et al., 2015). These findings highlight a big variety of matrixes that can be the surface for the growth of these bacteria.

During the last years owing to the improved of the sequences-based technologies, an increased number of works, studying the antimicrobial resistance and in particular the antimicrobial resistance genes (**ARGs**), were reported. Previous phenotypic study, especially in Switzerland empathized a high resistance prevalence of NASM to oxacillin, penicillin and tetracycline resistance. Unfortunately, not in all the strains was found the association between phenotypic and antibiotic resistance results, detected by PCR (Frey et al., 2013). Similar studies detecting the ARGs were done also in Canada where a correlation was observed between resistance strains and the use of the antibiotic at the systematic level more than the antibiotics use at the intramammary level (Nobrega et al., 2018).

Thanks to the last technologies, an increasing number of genomic methods to typing bacterial strains were developed. Previous works differentiate the NASM strains isolated from the milk with the PFGE (Kot et al., 2012). In the Kot and co-workers' study, different *Staphylococcus* spp. species were isolated from the milk and from the cowshed environment showing a unique PFGE pulsotype for the *M. sciuri* and the circulation of different pulsotypes for *S. xylosus* belonging to strains from the milk and the milker's hand strains (Kot et al., 2012). Recently, a newly **MLST** scheme for the genomic typing of *M. sciuri* was developed (Shauer et al., 2021). In this paper, the authors detected 28 different Sequence Type (STs) belonging to 92 strains of *M. sciuri* isolated from nasal swabbing from ruminants including cattle; a high variability of STs were found. Currently, no MLST scheme is available referring to *S. xylosus*.

Regarding phylogenetics, just a few studies involving *S. xylosus* and *M. sciuri* were done. In a recent study, a previous phylogenetic analysis comparing the *M. sciuri* isolated from different farms was done (Lienen et al., 2022). The data showed genomic differences between the farms and closely related strains within distinct farms. Additional studies were done involving phylogenetic analysis including several *Staphylococcus* spp. and highlighted a big phylogenetic variability. Five main clades were correlated with common biological traits as virulence factors, environmental niche, geographical distribution, and host specificity (Naushad et al., 2016). This work highlighted the need of more phylogenetics studies for a better understanding of the epidemiology and farm diffusion of the different NASM species.

Due to the gap of knowledge about NASM, the goal of our study was to characterize *S. xylosus* and *M. sciuri* strains isolated from quarters milk and environmental samples. Phenotypic and genomics antibiotics methods were perfomed. Additionally, thanks to the aid of the whole genome sequencing, a phylogenetic analysis for *M. sciuri* was accomplished to elucidate the epidemiological role of the bacteria in the herd environment.

2 Materials and Methods

2.1 *Staphylococcus xylosus* and *Mammaliicoccus sciuri* bacteria isolation

Additionally to the bacterial isolates from the milk samples already involved in our previous article (Romanò et al., in review), during the second sampling of the research project (TIME 1), environmental samples were collected from different matrixes in the 9 herds. Before and after the milking procedure, a sterile swab was passed through the liners of the milking machine (Liners samples). Before the milking procedure, sterile teat skin swabs were collected from the same cow that were milked (Teat skin samples). Furthermore, the bedding from each farm was collected with the use of sterile glows. The collection of the bedding was done each five cows places of laying. All the samples collected were refrigerated at 4°C.

In the laboratory, bacterial culture from the milk were done as previously described, plating directly on blood agar (Romanò et al., in review). The swabs of the environmental samples were directly inoculated in 9 ml of BHI solution while for the bedding, 1 g was added to 9 ml of BHI. The enrichment cultures were incubated at 37°C for 18 hours. Subsequently, the enrichments were plated on blood agar and the colonies identify by MALDI-TOF. Briefly, a small amount of each colony originating from BA plate was smeared with a toothpick on the dedicated target plate (Bruker Daltonics GmbH, Bremen, Germany) followed by the addition of 1 μl of α-cyano-4hydroxycinnamic acid-matrix (Bruker Daltonics GmbH). The MALDI-TOF MS analyses were done with the Microflex LT instrument using the MBT compass reference Library 7311 (Bruker Daltonics GmbH). The strains collected and identified with a score ≥ 2.2 were identified at the species level according to the manufacturer's guidelines (www.bruker.com). The bacteria species belonging to S. xylosus and M. sciuri were isolated and conserved in skim milk at -20°C for the subsequent analyses. A selection of 15 strains of *S. xylosus* and 60 strains of *M. sciuri* isolated from the environment was randomly performed.

2.2 Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) for the milk samples was already performed in the previous article (Romanò et al., in review). For all the environmental strains, 15 *S. xylosus* and 60 *M. sciuri* selected samples, the minimum inhibitory concentration (MIC) was done. All the tests were performed according to the manufacturer instructions of the Microscan System (Beckaman Coulture Microbiology, West Sacramento, CA) manufacturer instructions. On the strains the MIC for 30 different antibiotics was determined by the microdilution assays using the Microscan System (Beckman Coulter Microbiology, West Sacramento, CA) according to the manufacturer's instructions. The Gram-positive MIC panel Pos MIC 32 was chosen which included the following antimicrobial agents (μg/mL): amoxicillin/K clavulanate (0.5/0.25–8/4), ampicillin (0.5–8), azithromycin (1–2), cefepime (4–8), cefotaxime (1–2), cefuroxime (4–8), chloramphenicol (8), ciprofloxacin (0.5–1), clindamycin (0.25–0.5, 2), daptomycin (0.5–4), ertapenem (0.5–1), erythromycin (1–2), fosfomycin (32), fusic acid (2), gentamycin (1–4), imipenem (2–8), levofloxacin (1–2), linezolid (0.5–4), meropenem (2–8), moxifloxacin (0.5–1), nitrofurantoin (64), oxacillin (0.25–2), penicillin (0.03–0.25, 2), rifampin (0.5–2), synercid (1–4), teicoplanin (1–8), tetracycline (1–2), tobramycin (1–4), trimethoprim/sulfamethoxazole (1/19–4/76), and vancomycin (0.25–8). The breakpoint of

EUCAST were used to determine the susceptibility or resistance of the isolates (EUCAST, 2022). When the breakpoint for an antibiotic were not available, the CLSI breakpoint included in the program of the Microscan were used (CLSI, 2012). For the *M. sciuri* were used the same breakpoint of the *Staphylococcus* spp. The breakpoints are based on strains isolated from humans and not on specific mastitis pathogens.

2.3 DNA extraction and whole genome sequencing by Illumina
The DNA extraction and the whole genome sequencing were performed as our previous work (Romanò et al., in review). Briefly, the total 75 environmental strains (15 *S. xylosus* and *60 M.sciuri*) were plated on sheep blood agar (BA) plates (Biomèrieux Suisse SA, Geneva, Switzerland) and aerobically incubated at 37°C for 18 hours. Two to four colonies were picked up and resuspended in 5 ml BHI (Brain Heart Infusion Broth, Merck K GaA, Darmstadt, Germany) and incubated aerobically at 37°C for 18 hours. Subsequently, 200 µl of the culture were added to 100 ml of fresh BHI and incubated aerobically at 37°C for 18 hours under constant shaking. From this final culture, 50 ml were collected and centrifuged at 18'000g for 5 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 600 µl of Buffer A1 from the NucleoSpin® 8 Plasmid kit (12 X 8 preps) (Macherey-Nagel AG, Oesingen, Switzerland). The complete isolation was performed according to the manufacturer's protocol. The total amount and the quality of the extracted DNA were evaluated by spectroscopy assessing the OD260/OD280 ratio (QuickDrop; Molecular Devices, San Jose, CA). Additional quantitative control was done using the Qubit assay (Thermo Fisher Scientific). The extracted DNAs were sent to Eurofins Genomics GmbH (Ebersberg, Germany) for WGS using the Illumina HiSeq sequencing platform (Illumina, San Diego, CA). The reads were first assembled against the reference type strain chromosomal genome of the correspondence species *S. xylosus* NCTC11043 (NCBI Biosample: SAMEA3539705) and *M. sciuri* NCTC12103 (NCBI Biosample: SAMEA3505362) downloaded from NCBI. The assembling was done using a commercial software SeqMan NGen 16 (default settings) included in the DNASTAR Lasergene 16 software package (DNASTAR, Inc, Madison, WI). The unassembled reads were de novo assembled with the de novo task using the SeqMan NGen 16 software (DNASTAR). The parameters were changed by deactivating the 'repeat handling' option in the software settings, selecting a minimum match for overlapping read segments of 93%, and selecting contigs with lengths bigger than 1000 nucleotides. For further verification, the chromosomes and the contigs were annotated with the RAST pipeline (Aziz et al., 2008) with the goal of recognizing and naming the known genes. Additionally, the strains' genomes were uploaded to the Type (Strain) Genome Server (TYGS), a free bioinformatics platform to verify the identity of the strains based on whole genome-based taxonomic analysis (Meier-Kolthoff and Göker 2019).
The follow subchapters regarding the research of antimicrobial resistance genes and the plasmids identification were performed for both species *S. xylosus* and *M. sciuri*; differently, the phylogenetic analysis were accomplished only for the bacterium *M. sciuri*.

2.4 Researching of antimicrobial resistance genes (ARGs)
The detection of the ARGs was perfomed using the Resfinder tool from Center for Genomic Epidemiology (Bortolaia et al., 2020). In addition, the analyses of the isolates were accomplished also with Resistance Gene Identifier RGI (Alcock et al., 2020, Comprehensive Antibiotic Resistance Database, CARD). The results represent a combination of the two commercial tools.

2.5 Plasmid identification

To analyze in deep the possible correlation between the presence of ARGs and the presence of plasmids, all the strains were submitted to Plasmid Finder (Carattoli et al., 2014).

2.6 Multilocus sequence typing (MLST)

On the total 143 *M. sciuri*, MLST analysis was achieved on raw reads through the Center for Genomic Epidemiology Online Platform (Larsen et al., 2012) or by submitted the assembled genome on PubMLST (Jolley et al., 2018). The new alleles and STs were uploaded on PubMLST and based on analyses performed by the database curators, nominated with a new ID (Schauer et al., 2021).

2.6 Phylogenetic analysis and average nucleotide identity (ANI) calculations

Phylogenetic analysis was performed on all the assembled 143 *M. sciuri* genomes. The genes used for the phylogenetic analysis were 70 genes originated from the previous work of Madhay and co-workers, involved in the reassignment of the family *Staphylococcaceae* (Madhay et al., 2019). The 70 genes were extrapolated from the original assembled chromosome samples with the use of a developed in-house software called Extractor. Subsequently, all the genes were concatenated and the multiple sequences alignment of all the concatenated fasta sequences performed using Clone Manager 16 (Clone Manager v9.51; CM9; Sci Ed Software, Westmister, CO). Additionally, the type strain of Staphylococcus aureus (SA_ST) was included in the phylogenetic analysis. The alignment of the strains was uploaded on Bioedit (BioEdit DNASequence Alignment Editor Software). With this software, the gaps were split and a new alignment save and use for further analyses. The phylogenetic tree was inferred with the software PhyML (Guindon et al., 2010) and the obtained phylogenetic tree visualized by MEGA11 (Tamura et al., 2021). Graphical representation of the tree was displayed by ITOL (Letunic and Bork, 2021). Average nucleotide identity (ANI) calculations were performed using pyani v0.2.9 with the ANIm method (Pritchard et al., 2016).

2.6 Statistics

A ChiSquare was performed to see the statistically association between the two bacterial species numerosity and the presence in the milk or environmental samples. The test was performed used GraphPad (https://www.graphpad.com/quickcalcs/contingency1/).

The degree of similarity between the distributions of ST among milk strains and environmental samples originated from the second sampling was estimated using the Czekanowski index or Proportional Similarity Index (PSI). The PSI was performed by R 4.0.5 using the package "EpiR". The values for PSI range from 1 for identical frequency distributions of the variable of interest to 0 for no similarities between the data sets (Feinsinger et al., 1981).

2.7 Data availability

The original contributions presented in the study are publicly available. The raw Illumina reads regarding the milk samples were already uploaded in NCBI GenBank database under the Bioproject number PRJNA859642 and included in the previous paper (Romanò et al., in review). In addition, the raw Illumina reads belonging to the environmental samples were uploaded in the NCBI GenBank database under the Bioproject number PRJNAXXX.

3 Results
3.1 Isolation of *S. xylosus* and *M. sciuri* from environmental samples

From the total 220 environmental samples, the results regarding the presence of *S. xylosus* and *M. sciuri* were listed in the Table 1. The presence of *S. xylosus* and *M. sciuri* was detected in different range of prevalence between the herds. The prevalence of *S. xylosus* was in a range between 0% (Herd N.9) to 40% (Herd N.3). Differently the isolation of *M. sciuri* was higher with range from 7% (Herd N. 9) to 90% (Herd N. 2). In the farm N. 9, another type of bedding was used (manure). The statistically analysis showed no statistically differences between the number of bacteria isolated from the milk for the two different species (P = 0.6985). Differently, there is a statistically association between the distribution of the environmental samples for the two species (P < 0.0001). A general higher prevalence of *M. sciuri* isolated from environmental samples was showed. Another data that emerged from our study is the high prevalence of *M. sciuri* on the teat skin of the cow sampled. The percentage of correspondence bacteria isolated from the milk and the teat skin collected from the same cow was only 13% for *S. xylosus*. However, a high percentage of *M.sciuri* was identified in both milk and teat skin/orifice (93%). Another interesting finding is that also in the liners of the milking machine before the milking procedure, strains of *S. xylosus* and *M. sciuri* were isolated. On the total 42 swab from the liners before milking procedure, 17 samples (40.5%) were positive for *M. sciuri* and 2 (5%) for *S. xylosus*.

3.2 Whole genome sequencing analyses

In addition to the previous strains isolated from milk samples involved in the recent work (Romanò et al., in review), different strains from the environment were selected. The samples isolated from the environment originated from different sites (liners of milking machine, teat skin, teat orifice, bedding). The total 15 *S. xylosus* strains, isolated from 6 herds originated from 5 strains of the liners of the milking machine, 5 of the bedding and 5 of the teat skins of the cows analyzed. Regarding *M. sciuri*, 25 strains from the teat skin/orifice, 22 from the liners of the milking machine and 13 from the bedding were sequenced. In the Supplementary table 1 are listed the data regarding the whole genome sequencing of *S. xylosus* and *M. sciuri* environmental samples. Additionally, the number of contigs not assembled with the reference strains were listed in the Supplementary Table 1.

3.3 Phenotypic antibiotic characterization

Referring here to the phenotypic antibiotic resistance, as for the milk samples, *S. xylosus* and *M. sciuri* were highly resistant to clindamycin and oxacillin.

More in detail, *S. xylosus* were mainly resistant to clindamycin (n=98; 84%), oxacillin (n= 36; 31%) and tetracycline (n=26; 22%). Additionally, the resistance to azithromycin was of the 8.6%. The wide spread resistance to clindamycin was detected in both milk and environmental samples. The 10% of the strains (n=12) were susceptible to all the antibiotics tested.

The main antibiotics resistance in *M. sciuri* were to clindamycin (n=136; 95%), oxacillin (n=80; 56%). In a small percentage *M. sciuri* was resistant also to azithromycin (n= 12; 8%) and tetracycline (n=15; 10%). The strains that were susceptible to all the antibiotics were 5, 3.5% on the total.

3.4 ARGs identified in *Staphylococcus xylosus*

The total number of ARGs isolated in the *S. xylosus* strains were nine, belonging to seven different drug classes (aminoglycoside, β-lactams, fosfomycin, macrolides, phenicols, streptogramins, and tetracycline). Up to the total 116 strains isolated, 77 showed no ARGs (66%). In 39 strains (33.6%) at least one ARGs was identified. Concerning the strains positive to ARGs, the major of the remaining were positive only for the tetracycline resistance gene,

tetK (n=26). The *tetK* gene was found in six on the total nine herds sampled. The heatmap N.1 (**Figure 1**) shows all the ARGs found in the correspondence 39 strains of *S. xylosus* isolated.

3.5 ARGs identified in *Mammaliicoccus sciuri*

The total number of ARGs isolated in the *M. sciuri* strains was 11, belonging to eight different drug classes (aminoglycosides, β-lactams, lincosamides, pleuromutilins, streptogramins, and tetracycline). The total 143 strains isolated have in common the presence at the chromosomal level of the genes *mecA1* and *salA*. These two genes were isolated in both milk and environmental samples, and for three herds are the only ARGs isolated. The *mecA1* is a gene carried the resistance to β-lactams. The resistance to lincosamide and streptogramin was carried by *salA* as a general efflux unspecific pump. 11 strains carried the *str* gene and 15 *tet* resistance genes (*tetK, tetL, tetM*). The *tetK* ARG was found in four herds on the total nine herds involved in the study while *tetL* and *tetM* were isolated correspondent in herd N.1 and 2. In the herd number 5, we observed contemporary the presence of all the three genes. The genes for the resistance to phenicols *fexA* and *fexB* were isolated only from strains belonging to environmental samples. The heatmap N.2 (**Figure 2**) shows all the ARGs isolated excluding all the isolates that carried only the *mecA1* and *salA* ARGs.

3.6 Detection of plasmids

A higher number of *rep* gene was detected in milk compared with the environmental strains. This fact may be correlate with a general low number of environmental strains isolated of *S. xylosus*. For *M. sciuri*, six strains up to the 60 were positive for *rep* genes. Two strains showed the presence of *rep7* and the tetracycline resistant gene *tetK*. Comparing these plasmids with the reference tetracycline strains, a similarity of the 99% was found. Different was the situation for the contigs were *rep* and *str* were detected. The plasmids carried the *str* gene, in addition to the replication protein and the ARGs included genes correlated with the mobilization of the plasmids (plasmid mobilization relaxome protein, *Mob*).

3.7 MLST characterization

Regarding the *M. sciuri* strains, MLST analyses were done for all the strains to identify the distribution of STs. In total, 78 different STs were identified, the majority of the samples belonged to new MLST profiles. The two mainly STs identified were ST107 (11 samples) and ST177 (12 samples). The PSI calculated was 0.37 highlight a very low correlation between the distribution of the STs belonging to milk and environmental samples. More in details, the PSIs for the different environmental matrix (bedding, liners, teat skin) was calculated: 0.208, 0.28 and 0.218.

3.8 Phylogenetics analysis involving M. sciuri isolated from milk and environmental samples

Phylogenetic analysis (**Figure 3**) showed the presence of two main branches originating from a common *M. sciuri* ancestor. One of the resulting clades included most of the taxa (126) whereas the clade with fewer taxa harbored the type strain of *M. sciuri* (ST30). Within each clade, the distances were generally small demonstrating little evolution. Nevertheless, a considerable number of different peripheral subclades were observed with only some taxa forming larger common clades. Most of these clades were comprised of taxa from different herds. Within some of these clades, taxa of two different isolation sources were found, namely from bedding and teats, liners and milk, and from milk and teats. The ANI analysis, as the phylogenetics analysis performed, showed that all the *M. sciuri* belonging to the same bacterial

species. Although, as displayed in the phylogenetic analysis, two different groups could be distinguished (**Figure 4**).

4 Discussion
4.1 Presence of NASM in milk and environmental samples
The results from the culturing experiments showed the presence of *S. xylosus* and *M. sciuri* not only in the milk samples but also in the environmental samples including liners of the milking machine, teat skin, teat orifice, and bedding. Our results agreed with previous experiment showing the presence of both bacteria not only in the milk, but also in the dairy environment (De Visscher et al., 2014, Patel et al., 2019). In previous Swiss studies, *S. xylosus* was detected in a high percentage of mastitis clinical milk samples (Frey et al., 2014, Dolder et al., 2017). Another study performed by our group showed a high percentage of *Staphylococcus chromogenes* and *S. xylosus* in milk of cow involve in the sanitation program for the eradication of the *S. aureus* GTB (Sartori et al., 2018). Based on previous bibliography, *S. xylosus* is mainly associate with the mammary gland besides *M. sciuri* was also observed in the environment of the farm (De Visscher et al., 2014). These previous thoughts are in line with our results highlight a statistically significant higher prevalence of *M. sciuri* in the environment compared to *S. xylosus*. Our study suggested that when *M. sciuri* was present in the teat skin in the 93% of the cases, was present also in the milk. Another important fact is the detection of the bacteria also in the liners of the milking machine before milking. Despite the hygienic protocol that are daily use for the cleaning of the liners, a low percentage of NASM was detected. This fact could be explained by bad hygienic measure at the farm level or specific intrinsic characteristics of the bacterial species able to form a biofilm layer that facilitate the persistence of the bacteria in the environment as showed in a previous paper by Lee and Lee analyzing NASM isolated from bulk tank milk (Lee and Lee, 2022). Although, in this study the potential of biofilm formation was considered possible but weak for both the species involved in our research (Lee and Lee, 2022). Further analysis to clarify this important issue needs to be consider for a better understanding of the persistence of the NASM.

4.2 Species-specific intrinsic resistance and wild type resistance to tetracycline, phenicols, aminoglycosides, and lincosamides
Concerning antibiotic resistance, *S. xylosus* and *M. sciuri* were highly resistant to clindamycin and oxacillin. There were no differences between phenotypic resistance of the milk strains and environmental strains. However, at least for *S. xylosus* just a few strains from the environment were isolated. Nowadays, penicillin remains the main antibiotic used for the mastitis treatment (Preine et al., 2022). Despite the high use of this antibiotic, a low percentage of antimicrobial resistance to penicillin was detected *S. xylosus* 7%, *M. sciuri* 2%. In *M. sciuri*, the searching for ARGs uncovered the presence of *mecA1* and *salA* genes on the chromosomes. The presence of the same pattern of these two was already detected in other strains isolated from bovine mastitis (Frey et al., 2013, Fergestad et al., 2021). The MecA1 protein is assumed to cause oxacillin resistance whereas SalA is a pump causing resistance to lincosamides and streptogramins. Furthermore, in 12 strains (20%) genes coding for resistance to aminoglycosides (*str*), lincosamides (*lnuA*) and tetracyclines (*tetK, tetL, tetM*) were identified on the de novo contigs. Genes coding for resistance to phenicols (n=1 *fexA*, n=1 *fexB*) were only identified in two strains obtained from environment samples. A high correlation between phenotypic and genomic results was observed for tetracycline and lincosamide resistance. However, no association was found for oxacillin resistance indicating that a mechanism different from MecA1 is likely involved in oxacillin resistance of *M. sciuri*.

Differently from other studies were methicillin resistant *M. sciuri* (MRMS) were detected, our study did not show strains MRMS. This difference could be explained by the fact that in the previous study, a pre-selection of farm positive to MRSA was done and a specific enrichment was performed to detect them the MRMS (Lienen et al., 2022).

4.4 Detection of plasmids in milk and in lower amount in the environmental samples

Despite the low number of plasmids detected, the possibility to have the presence of mobile genetic elements in the milk and in the environmental samples was detected.

In particular as previous showed in our previous work, the *tetk* gen was always related with the presence of a small plasmid (~ 4 Kb). As previous showed, this plasmid was detected not only in one bacterial species of NASM, but also in different NASM that were circulating in the same farm.

4.5 MLST showed a high variability of STs between and within the herds. Phylogenetic showed the presence of two main clades for *M. sciuri*.

Due to the development of the *M. sciuri*'s MLST scheme only in 2021, just a few works included the genotyping for this bacterial species were performed (Schauer et al., 2021, Yang et al., 2022, Boonchuay et al., 2023). More in details, the works already published included mainly MRMS and different animal species; not only cow's *M. sciuri* strains from the milk were included. The Schauer study first showed the distribution of the different STs in samples that colonized the camelides, 92 strains isolated regroup in 28 different STS with a majority of ST1 (N. 35) and ST2 (N. 15) (Schauer et al., 2021). In the study by Yang and co-authors, on the total 32 *M. sciuri* isolated from retail pork and slaughterhouse carcasses, three main STs (ST63, ST30, and ST96) and other 13 different new STs were identified (Yang et al., 2022). In Boonchuay and co-workers research, from 11 MRMS strains, 7 different STs were identify (71, 81, 120, 122, 199, and 200) (Boonchuay et al., 2023). What this work has in common with the previous one is the great variability of the STs isolated also if they derived from the same matrix. Nowadays, in the public PubMLST database, 257 different STs were detected. In particular a small percentage of them derived from mastitis cases (6.9%) but the majority belonging to other sources of the strains (carrier-colonization, bacteremia, food/environment, unknown (https://pubmlst.org/bigsdb?db=pubmlst_msciuri_isolates). This fact highlights that in a broad range of matrixes *M. sciuri* could be identify. The strains classified as mastitis strains belonging to different STs.

Considering the STs identified in our study, 78 different types were detected, the majority belonging to new STs. The main STs were ST107 (11 samples) and ST177 (12 samples). The PSI value calculated was 0.37 revealing a very low overlap between the STs observed in the milk and the environmental samples. Phylogenetic analysis showed the presence of two main branches originating from a common *M. sciuri* ancestor. One of the resulting clades included most of the taxa (126) whereas the clade with fewer taxa harbored the type strain of *M. sciuri*. Within each clade, the distances were generally small demonstrating little evolution. Nevertheless, a considerable number of different peripheral subclades was observed with only some taxa forming larger common clades. Most of these clades were comprised of taxa from different herds. Within some of these clades, taxa of two different isolation sources were found, namely from bedding and teats, liners and milk, and from milk and teats. These findings demonstrate that an exchange of *M. sciuri* among different matrices can happen, also between the environment and the mammary gland. Interestingly, the peripheral subclade including the type strain was composed by taxa that had been isolated from all analyzed matrices indicating minor specialization. The ANI calculations showed an

average nucleotide identity above 95%, indicated that all the strains belonged to the same species. Similar to the two clades observed in the phylogenetic analysis, the ANI analysis has shown two distinct clusters with a high average nucleotide identity. This observation providing support to the phylogenetic analysis performed, and the presence of two different group of *M. sciuri* with different genomic characteristics despite their evolutionary proximity. In a recent work, Lienen and co-workers (Lienen et al., 2022) did a previous phylogenetic analysis comparing the *M. sciuri* isolated from different farms. The data showed genomic differences between the farms and closely related strains within distinct farms. Comparable data were obtained with our phylogenetic analysis. Our phylogenetics analysis showed a clear distinction of two subclades originated but far from the *M. sciuri* type strain. A comparison between phylogenetic analysis could be done but an important consideration regarding the fact that different methods were used to performing the analysis needs to be taking into account. Further studies with the possibility of longitudinal studies to genotype and characterize the NASM during short timing needs to be done to detected genomic characteristics that may be related with the persistence.

5 Conclusion

S. xylosus and *M. sciuri* were very commonly isolated from milk and environmental samples, with strains from either source mainly showing antimicrobial resistance to clindamycin and oxacillin. *S. xylosus*, differently from *M.sciuri*, was mainly correlated with the milk showed a higher association with the intramammary udder than *M. sciuri* more farm-environment correlated. *S. xylosus* strains were high resistant to clindamycin but a disagreement between the phenotypic and genomic results was found. While clindamycin resistance could be well explained by the presence of the *salA* gene, no genomic explanation, however, could be found for oxacillin although *mecA1* had been previously postulated to have this property. Differently, a perfect correlation between phenotypic and genomic results was present for the antibiotics tetracycline and lincosamides. A remarkable variability was observed for *M. sciuri* at the ST level. Considering the phylogenetic analyses, a considerable number of different peripheral subclades was observed demonstrating that an exchange of this bacterium among different matrices occurs, also between the environment and the mammary gland.

Table 1: Overview of the environment distribution of *S. xylosus* and *M. sciuri* isolates in the different herd environments.

|	Herd 1	%	Herd 2	%	Herd 3	%	Herd 4	%	Herd 5	%	Herd 6	%	Herd 7	%	Herd 8	%	Herd 9	%	Total
Teat skin samples*	10		8		10		10		10		10		10		10		10		88
Pos samples S. xylosus	Neg		2		4		1		4		1		Neg		Neg		Neg		12
Pos samples M. sciuri	10		7		9		10		10		10		10		9		Neg		75
Liners milking machine samples	9		10		6		9		6		8		14		12		10		84
Before milking procedure	5		5		3		4		3		4		7		6		5		42
Pos samples S. xylosus	Neg		Neg		1		Neg		Neg		1		Neg		Neg		Neg		2
Pos samples M. sciuri	5		5		Neg		1		Neg		Neg		2		4		Neg		17
After milking procedure	4		5		3		5		3		4		7		6		5		42
Pos samples S. xylosus	1		1		1		1		Neg		Neg		Neg		1		Neg		5
Pos samples M. sciuri	Neg		4		3		5		3		4		7		6		2		34
Type of bedding	Straw, sawdust		Straw, sawdust		Straw		Straw		Straw		Straw, ferus		Straw		Straw		Manure		
Bedding samples	4		2		4		4		2		4		9		9		9		47
Pos samples S. xylosus	1		Neg		2		2		1		1		Neg		Neg		Neg		7
Pos samples M. sciuri	4		2		4		4		1		4		7		6		Neg		32
Total environmental samples	23		20		20		23		18		22		33		31		29		219
Pos samples S. xylosus	2	9	3	15	8	40	4	17	5	28	3	14	Neg		1	3	Neg		26
Pos samples M. sciuri	19	83	18	90	16	80	20	87	14	78	18	82	26	79	25	81	2	7	156

* : The number of the teat skin samples corresponding to the correspectively milk samples.

Figure 1: Distribution of the ARGs isolated from the *S. xylosus*. The heatmap graphically represent only the strains in which at least one ARGs was detected.
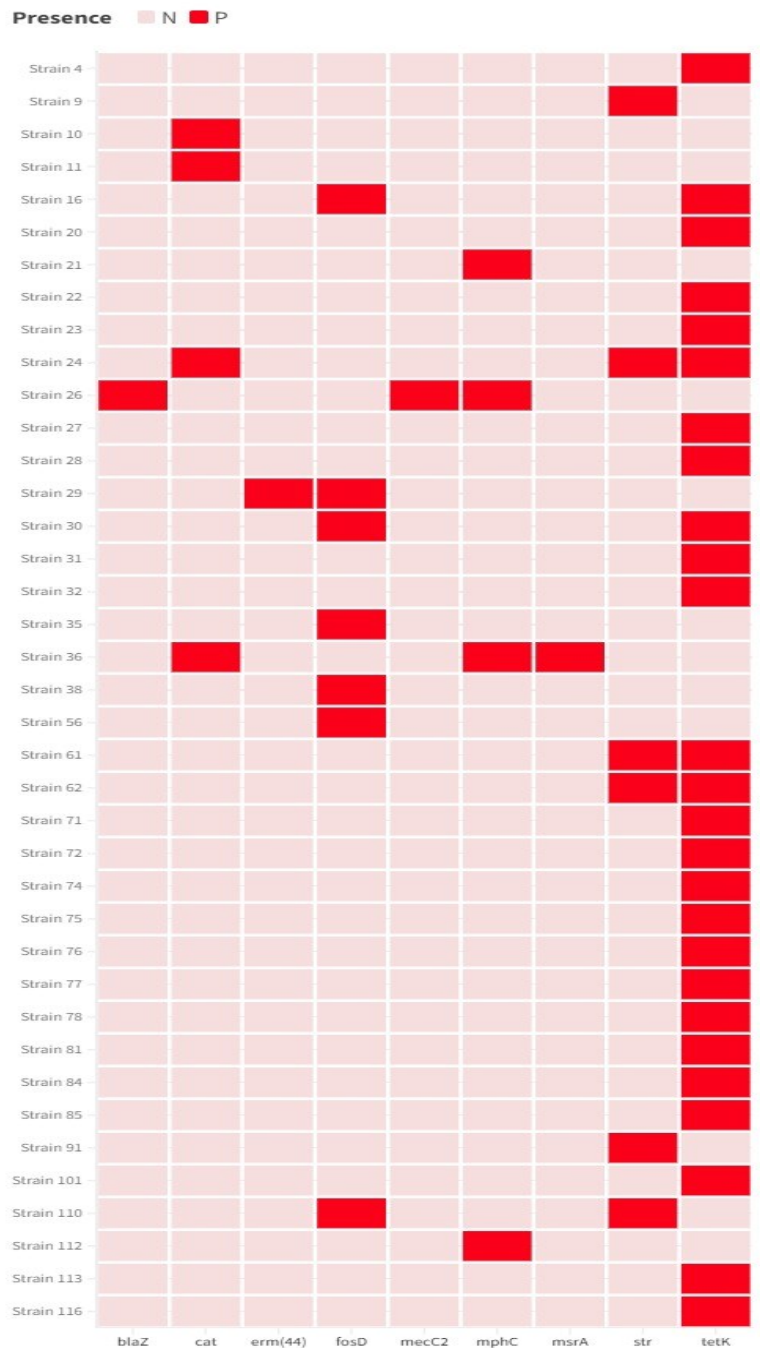
Figure 2: Distribution of the ARGs isolated from Mamm. sciuri isolates excluding the strains positive only for the common pattern found at the chromosomal level *mecA1* and *salA*.
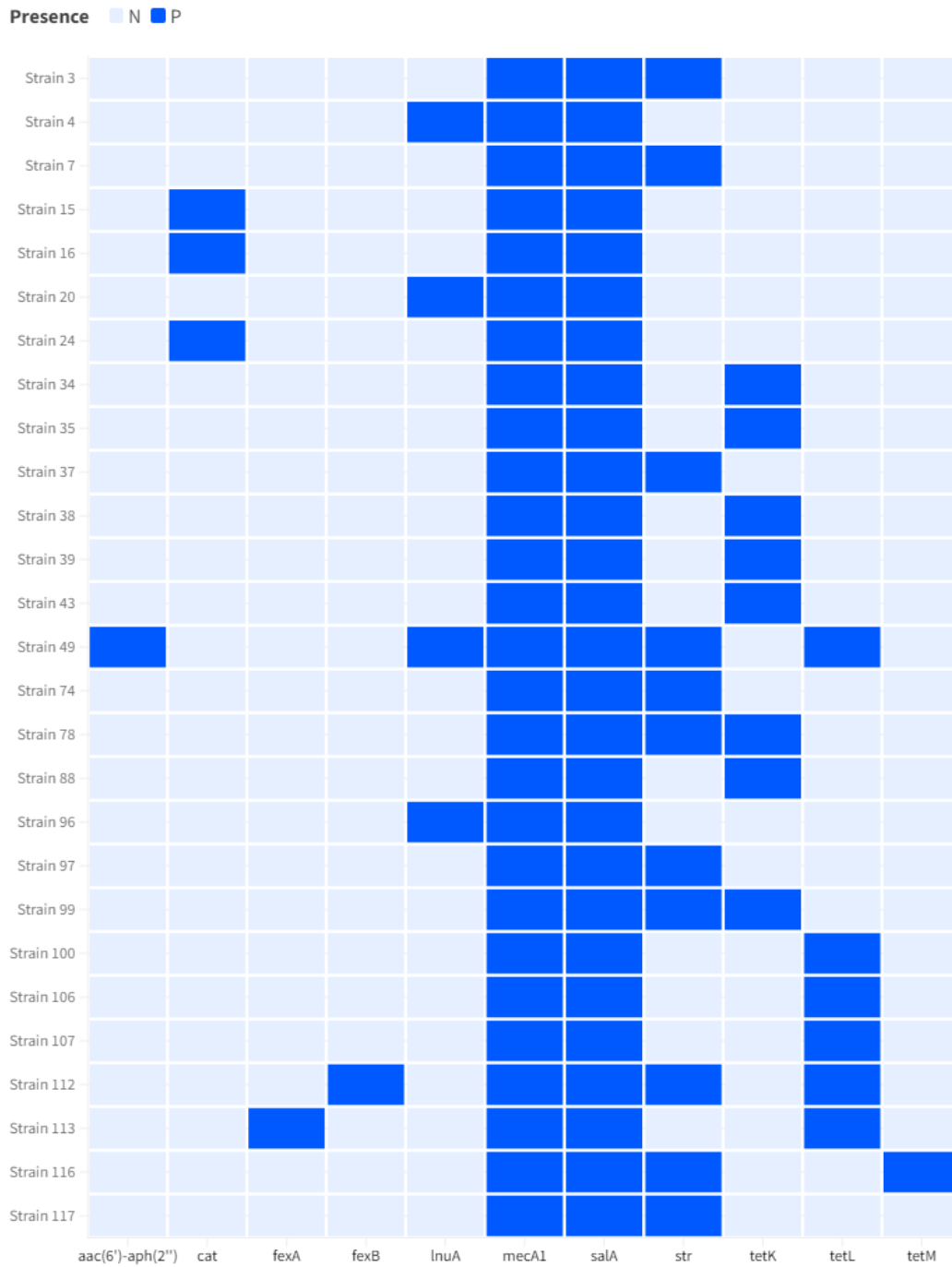
Figure 3: Phylogenetics analysis regarding *M. sciuri* strains isolated from milk (n=83) and environmental samples (n=60). The phylogenetics analysis was perfomed by PhyML; the graphical representation was done by iTOL. In addition, including in the tree, the type strain of *M. sciuri* and the type strain of *S. aureus* were added.
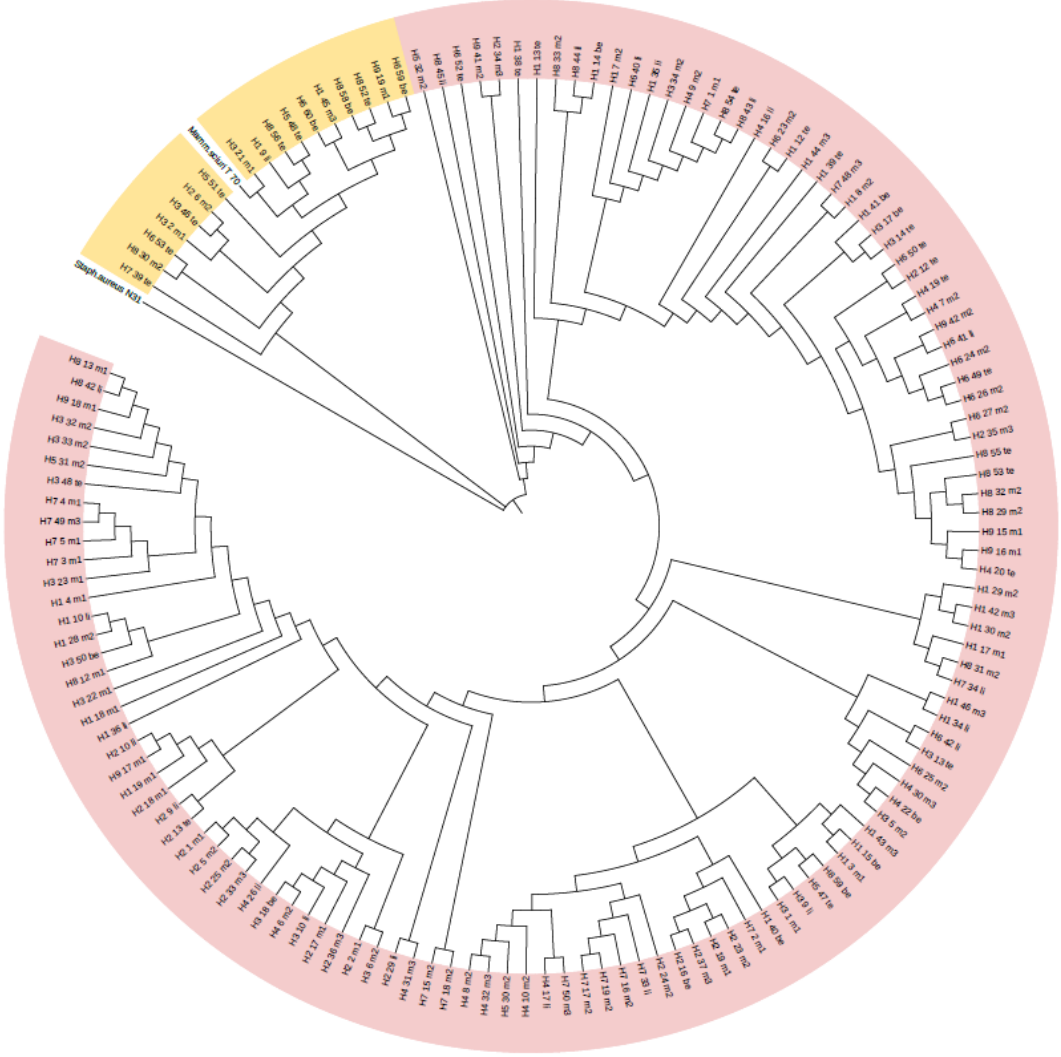
Figure 4: Graphic representing the ANI analysis performed with all the 143 isolates strains from the milk and the environment. In addition, the type strain of *M. sciuri* was included.
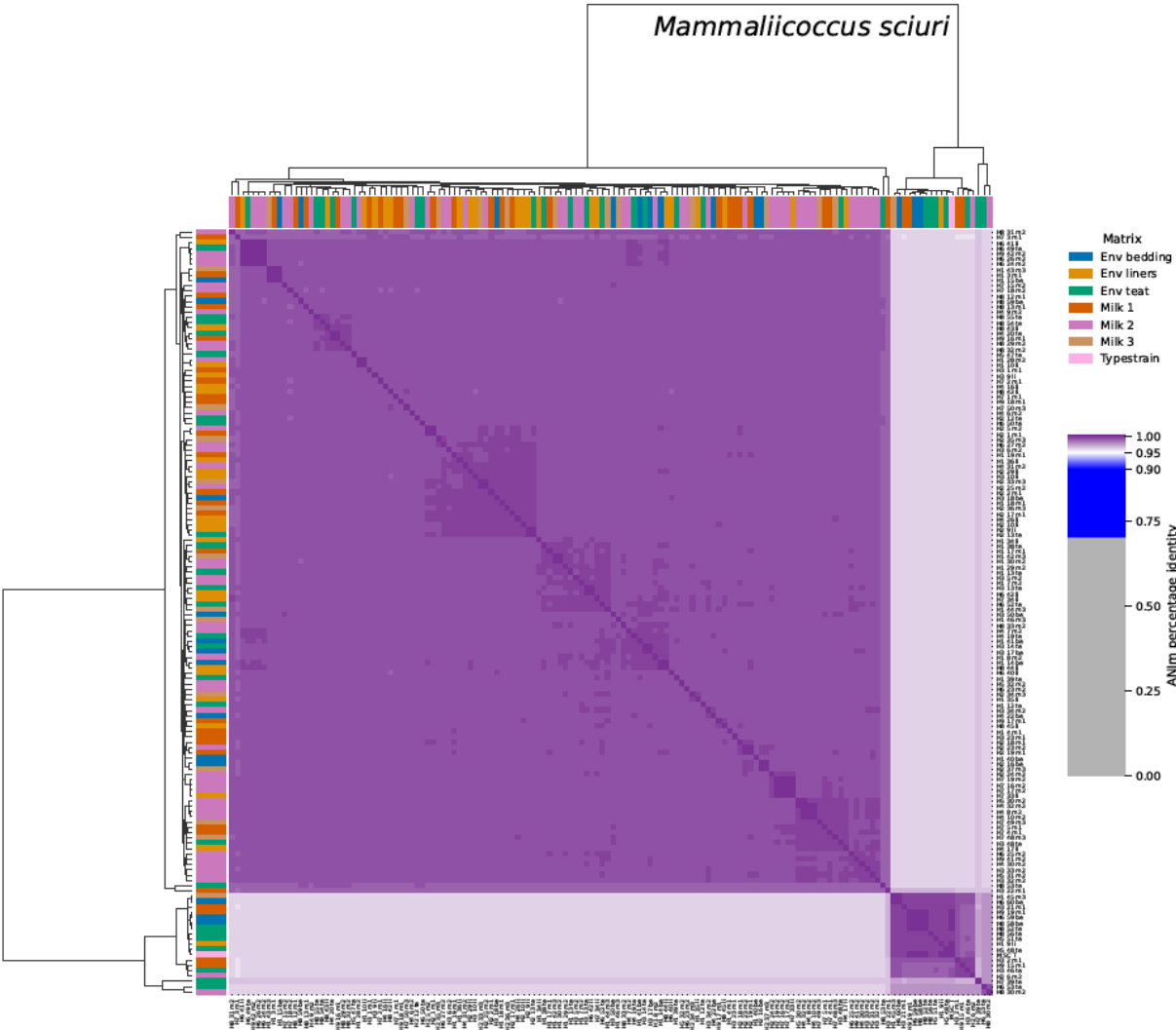
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4. Overall discussion and Outlook

The first question in this research was to detect the presence of a bacteriome in the milk of healthy cows **(Chapter 3)**. Previous studies have attempted to define a possible core bacteriome in healthy cows, but due to differences in samples size, matrices analyzed and different environmental conditions, it is difficult to arrive at a final definition of the bacterial species belonging to the healthy milk microbiota (Guo et al., 2021). Nevertheless, the prevalent bacterium isolated in our research is *Staph. xylosus,* already identified as a bacterial species isolated from healthy cows in international works (Porcellato et al., 2020, Park et al., 2022). Accordingly, it might be possible for this bacterium to belong to the healthy bacteriome, but further methods of genotyping the bacterial species need to be developed. This is because, from previous works, strains of *S. xylosus* have been correlated with mastitis cases with high SCC (De Buck et al., 2021). Also in our study, the 19% of the quarter positive only to *S. xylosus* were above the SCC range of the IDF guidelines, despite the 81% that was below the range of 200'000 cells/ml (IDF, 2022).

Although the common high prevalence of NASM between the different herd analyzed, a statistic significant difference between the bacteriome of the different herds could be address. These results are in line with different bacteriome study involving metagenomics in which a different bacteriome from different herds was detected (Tóth et al., 2020, Parente et al., 2020). In addition, our data are in agreement with the insights found in the previous work of our research group and another study conducted in Switzerland by analyzing clinical mastitis samples and control milk samples (Frey et al., 2013, Sartori et al., 2018).

Despite the increase of bacteria resistant worldwide, the complete non-use of antimicrobial therapy at the livestock level is not feasible due to the consequences that could have on animal health, dairy production capacity and price inflation (Woolhouse et al., 2015). Despite the extensive use of antibiotics in recent decades, and particularly the use of penicillin as the main treatment for mastitis, a low rate of resistance to this antibiotic has been demonstrated in our research. Even antibiotics defined as critical by WHO for their huge increase in antibiotic resistance bacterial infection in humans might not be considered a problem with regard to the resistome detected in our study.

Notwithstanding, the high detection of clindamycin and oxacillin that are consider high important antibiotics for humans according to the WHO report were detected (Collignon et al., 2016); despite this, in our research, these antibiotics were mainly related with intrinsic resistance for *M. sciuri* isolates. Because of their intrinsic antibiotic resistance located on the genome and not on a mobile genetic element, resistance cannot be shared with other bacterial species. However, the detection of plasmids in bacterial strains from the milk and the herd environment could not rule out a possible, albeit low, passage of ARGs from what we consider healthy bacteria to bacteria that are pathogenic to cows and the humans. Although the cost of WGS has been dramatically reduced in recent years and this important method is now available in many countries, additional phenotypic analyses are still needed. For this purpose, identification and isolation of bacterial species remain an important step in defining the best antibiotic treatment. Tetracycline resistance, as reported in other previous studies culture and sequences-based, could be an environmentally related resistance (Gasparrini et al., 2020). Tetracycline resistance was already reported to be associated with the presence of mobile genetic elements as plasmids and transposons, consequently, the resistance by coniugation could be transmitted to other bacteria (Jahantigh et al., 2020). In our study, (**Chapter 3**) the

tetracycline resistance was always related with the presence of small plasmids (~ 4 Kb). Based on the identification of the same plasmid in closely related species, in one case from two species belonging to the same milk quarter, possible transfer of resistance and subsequent circulation at the farm level could be hypothesized. Although a low rate of plasmid transfer with resistance genes between *S. xylosus* and the pathogen *S. aureus* was observed in previous experiments on starter culture and meat products, further study of cloning or involvement of transconiugants might be a possible alternative to study the tetracycline spread at the herd level or directly the plasmids transfer between species at the milk level (Leroy et al., 2019). There is a need to improve the identification of new ARGs correlated with phenotypic outcomes where phenotypic/genomic correlation was not detected. A possible limitation of our study could be the exclusive use of Illumina short reads and not implementation with PacBio sequencing. Due to financial restrictions, only Illumina sequencing was chosen for sequencing with the goal of having several species and a large number of strains sequence. In previous studies, involving the detection of the resistome, a high percentage of ARGs was related with the detection of Gram-negative bacteria (Rubiola et al., 2022). In our study, the bacteria most commonly identified were Gram-positive bacteria and in particular NASM, in which low antibiotics sharing by horizontal gene transfer (HGT) was observed comparing to Gram-negative (Juhas 2015). Despite this, more studies involving HGT among S. aureus and NASM are required. In addition, there is a need for more studies regarding the antimicrobial MIC range of bacteria isolated specifically from mastitis strains and from the animal isolates in general. Currently, only a few antibiotics related to mastitis isolates are available (CLSI, 2023). In vitro tests are needed to further establish the correct range and consequently which therapy is best to be adopted against mastitis strains.

In regard to the role of our two prevalent bacterial species *S. xylosus* and *M. sciuri*, the presence of mainly, *M. sciuri* in the environment was observed. These findings were also previous detected in other studies, empathizing the widespread of this bacterial species all around the herd environment (Vanderhaeghen et al., 2014, Vanderhaeghen et al., 2015). As other papers already suggested, the NASM need to be consider as species with different characteristics; different strains showed different properties as in some cases some are more udder-adapter and others seems more environmental correlated (Traversari et al., 2019, Wuytack et al., 2020). Of particular concern could be the presence of the NASM species also in the liners of the milking machine before the milking process. This fact might suggest that not in all the farms that were sampled, correct hygienic measure were taken or in alternative the formation of biofilm involving NASM for the persistence of this species in the environment as already found in other research (Pedersen et al., 2021, Ruiz-Romero and Vargas-Bello-Pérez, 2023). Regarding the limitation of the **Chapter 4**, the choice of the strains to be subjected to WGS was random and limited due to the costs of the experiment. This second paper could be a good starting point for further characterization of dairy NASM from milk and environment. Nevertheless, more appropriate and economical solutions need to be implemented for large herds.

Important further research should include implementation in the genotyping development for *S. xylosus*. This bacterium that, as in other researches was found in the mammary gland of healthy cows (Porcellato et al., 2020, Park et al., 2022) could have an important role as protector against the infection cause by *S. aureus* and also other mastitis pathogens. Further studies for the involvement of NASM as a measure to prevent or treat mastitis need to involve trials as the bacteria species could be classified as "probiotic drugs" (Rainard et al., 2018). In addition, *in vivo* study and not just *in vitro* studies are needed to clarify, for example, the action of some bacteriocins effects.

Phylogenetic analysis to comparing strains of *S. xylosus* belonging to clinical mastitis case, subclinical mastitis, and healthy cows may be performed to have a look at the epidemiology role of this bacterium. Additionally to the role in colonize the mammary gland, this bacterium is used as starter culture in meat and cheese preparation and is a component of the microbiome of the cheese (Heo et al., 2020, Leroy et al., 2020). During the last decades, an increasing number of studies, in particular WGS were performed involved this bacterium. The studies involved starter culture didn't showed high resistance to antibiotics (Zarkecka et al., 2023) but increasing the number of studies for monitoring the presence of ARGs should be performed.

In spite of the limited number of herds sampled, this works strengthens our knowledge on the bacteriome of healthy cows and antibiotic resistance in particularly for species not belong to the major mastitis pathogen and in general less studied. Additionally, the high number of species and strains sequenced from milk and environmental samples enables to document the AMR mechanisms at the herd level for maintain the efficacy of the antibiotic treatment.

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6. Candidates (co)-authored publications in the area of bovine mastitis

In this chapter, two research studies are added as works finalized during my PhD as secondary contribution. The first paper is a field study performed in Italy regarding the role of *adlb* as marker for a fast screening of high prevalent contagious mastitis genotype of *S. aureus*. The second paper, where I share the co-first author, is a short communication including European strains of different genotypes of *S. aureus* in which the *adlb* gene and the AMR were described.

The first study "*Staphylococcus aureus adlb* gene is associated with high prevalence of intramammary infection in dairy herds of northern Italy: A cross sectional study" is a collaboration work between Agroscope and two Italian Governative Institution IZSLER and CNR. In this study, 60 herds were involved and the detection of *S. aureus* was performed. 75 strains of *S. aureus* were genotyped by RS-PCR, screened for the detection of the gene *adlb* and sequenced by Illumina (WGS). A correlation between the presence of the *adlb* gene and a high prevalence of mammary infection caused by *S. aureus* was highlight. This work is a good example of experimental study that empathized the role of a specific genetic marker related with specific and prevalent genotypes and high contagiousness.

The second work "Bovine *Staphylococcus aureus*: a European study of contagiousness and antimicrobial resistance" was a short communication in which 211 strains of *S. aureus* belonging to ten European countries were analyzed. The analysis involved, also in this case, a screening for the contagious marker *adlb* by qPCR, phenotypic antimicrobial resistance analysis by MIC, and mPCR for the detection of the gene involved in the resistance to penicillin (*bla* operon) (*blaz, blaI, blaRI*). The results of the study showed an association between the genotype of *S. aureus* and the sensitivity/resistance to different antibiotics. This research could be applied in a further study for optimizing the antibiotic therapy against different genotypes of *S. aureus* with the final goal of an increasing the cure rate of the treatment.

Staphylococcus aureus adlb gene is associated with high prevalence of intramammary infection in dairy herds of northern Italy: A cross-sectional study

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ABSTRACT

Staphylococcus aureus is a major mastitis pathogen in dairy cattle worldwide, responsible for substantial economic losses. Environmental factors, milking routine, and good maintenance of milking equipment have been described as important factors to prevent intramammary infections (IMI). *Staphylococcus aureus* IMI can be widespread within the farm or the infection can be limited to few animals. Several studies have reported that *Staph. aureus* genotypes differ in their ability to spread within a herd. In particular, *Staph. aureus* belonging to ribosomal spacer PCR genotype B (GTB)/ clonal complex 8 (CC8) is associated with high within-herd prevalence of IMI, whereas other genotypes are generally associated with individual cow disease. The *adlb* gene seems to be strictly related to *Staph. aureus* GTB/CC8, and is a potential marker of contagiousness. We investigated *Staph. aureus* IMI prevalence in 60 herds in northern Italy. In the same farms, we assessed specific indicators linked to milking management (e.g., teat condition score and udder hygiene score) and additional milking risk factors for IMI spread. Ribosomal spacer-PCR and *adlb*-targeted PCR were performed on 262 *Staph. aureus* isolates, of which 77 underwent multilocus sequence typing. In most of the herds (90%), a predominant genotype was identified, especially *Staph. aureus* CC8 (30%). In 19 of 60 herds, the predominant circulating *Staph. aureus* was *adlb*-positive and the ob-served IMI prevalence was relevant. Moreover, the *adlb* gene was detected only in genotypes of CC8 and CC97. Statistical analysis showed a strong association between the prevalence of *Staph. aureus* IMI, the specific CCs, and carriage of *adlb*, with the predominant circulating CC and presence of the gene alone explaining the total variation. Interestingly, the difference in the odds ratio obtained in the models for CC8 and CC97 suggests that it is carriage of the *adlb* gene, rather than the circulation of these CCs per se, that leads to higher within-herd prevalence of *Staph. aureus*. In addition, the model showed that environmental and milking management factors had no or minimal effect on *Staph. aureus* IMI prevalence. In conclusion, the circulation of *adlb*-positive *Staph. aureus* strains within a herd has a strong effect on the prevalence of IMI. Thus, *adlb* can be proposed as a genetic marker of contagiousness for *Staph. aureus* IMI in cattle. However, further analyses using whole-genome sequencing are required to understand the role of genes other than *adlb* that may be involved in the mechanisms of contagiousness of *Staph. aureus* strains associated with high prevalence of IMI.
Key words: *Staphylococcus aureus*, mastitis, *adlb* gene, dairy cattle

INTRODUCTION

Staphylococcus aureus is one of the most important pathogens causing mastitis in dairy cattle worldwide, and it results in economic losses for dairy farmers in terms of reduced milk yield and quality and increased treatment costs (Hogeveen et al., 2011). The spread of this bacteria within a herd primarily happens during milking, and management factors such as milking rou-

tine and the good maintenance of milking equipment are important to prevent *Staph. aureus* IMI (Dufour et al., 2012). Nevertheless, it has been reported that *Staph. aureus* IMI may be widespread in many herds but not in others, where the infection is limited to a few animals, suggesting a central role of the strain circulating within the herd and involved in the IMI. Several studies have reported that different *Staph. aureus* genotypes are associated with different virulence and pathogenicity properties. In particular, *Staph. aureus* genotype B (**GTB**) is associated with high contagiousness and pathogenicity, leading to high within-herd prevalence of IMI. In contrast, other genotypes are associated with individual cow disease and rarely seem to cause herd health problems (Fournier et al., 2008; Graber et al., 2009; Cremonesi et al., 2015; Cosandey et al., 2016). Moreover, *Staph. aureus* GTB seems to be highly associated with the mammary gland (Leuenberger et al., 2019). Specific sanitation programs based exclusively on the identification and management of *Staph. aureus* GTB-positive dairy herds were successfully carried out in Switzerland (Sartori et al., 2018). Thus, discrimination between different genotypes seems to be important for *Staph. aureus*–targeted control programs (Barkema et al., 2006; Sartori et al., 2018; Exel et al., 2022). Although previous studies investigated the virulence factors and mechanisms that could facilitate *Staph. aureus* colonization of the mammary gland and its establishment and persistence in the host tissue (Monistero et al., 2018; Hoekstra et al., 2020; Pérez et al., 2020; Vaughn et al., 2020), no study to date has clearly identified a single marker or combination of markers capable of predicting *Staph. aureus* contagiousness within a herd. The possible association between the circulating *Staph. aureus* strain (in terms of genotype and virulence factors) with specific farm health parameters, including the incidence of clinical mastitis or the prevalence of subclinical IMI, has been evaluated (Dufour et al., 2012; Luini et al., 2015; Magro et al., 2017). Using a stochastic bio-economic model, Exel et al. (2022) proposed different control strategies based on the described epidemiological and clinical differences between different *Staph. aureus* strains.

Sartori et al. (2017) demonstrated that the single-copy gene *adlb* is strictly related to *Staph. aureus* GTB and may be a potential marker of contagiousness. This gene encodes the adhesion-like bovine protein and is located in the GTB-specific staphylococcal cassette chromosome SCCgtb. A study conducted in northern Italy on bulk tank milk samples confirmed the association between *Staph. aureus* GTB and the presence of *adlb*, even though some non-GTB strains also carry the gene (Gazzola et al., 2020).

Boss et al. (2016) reported that 80% of *Staph. aureus* strains isolated in 12 European countries belonged to only 6 different clonal complexes (**CC**), of which CC8, CC705, and CC97 were the most frequent. Additionally, the distribution of sequence types (**ST**) differs based on the considered country and region of interest (Boss et al., 2016; Cvetnić et al., 2021). Recently, Gazzola et al. (2020) investigated the distribution of multilocus sequence typing (**MLST**) profiles of *Staph. aureus* strains isolated in northern Italy and compared them to their ribosomal spacer-PCR (**RS-PCR**) genotypes. They found 16 CC, the most frequent being CC8, CC97, CC398, and CC1, isolated from bovine milk and reported as livestock-associated lineages (Boss et al., 2016).

Because *Staph. aureus* IMI is mainly chronic and subclinical, its contagiousness is of utmost importance in determining the economic losses for the affected herd. In this work, we aimed to investigate the prevalence of *Staph. aureus* IMI in northern Italian dairy farms and to relate the *Staph. aureus* circulating genotypes (especially the presence/absence of the *adlb* gene) as well as some farm characteristics and milking management factors to the prevalence of IMI within the herds as a marker of contagiousness of the circulating strains.

MATERIALS AND METHODS

This analysis did not require approval by an Institutional Animal Care and Use Committee because it did not involve animals used for scientific purposes as required by Directive 2010/63/EU (European Union, 2010) [Art. 2 ... 5. This Directive shall not apply to the following:... (f) practices not likely to cause pain, suffering, distress or lasting harm equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice.].

Study Design and Herd Data Collection

Between September 2011 and August 2012 and between March 2016 and March 2017, 60 dairy cattle herds with *Staph. aureus* IMI were enrolled in our study. The average size of the herds was 102 milking cows (range: 18 to 417 cows). All farms reared Holstein Friesian cattle and were located in the Lombardy, Emilia-Romagna, or Piedmont regions in northern Italy. These herds were representative, in terms of the number of lactating cows and average milk yield per cow, of a geographical area where more than 70% of Italian bovine milk is produced.

We identified many herds known to be infected with *Staph. aureus* during routine diagnostic activities con-

ducted during the 3 previous months on bulk tank milk samples or individual milk samples by 2 regional public health veterinary laboratories located in northern Italy (i.e., Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna and Istituto Zooprofilattico del Piemonte, Liguria e Valle D'Aosta). We considered only the farms established free from other contagious microorganisms, such as *Streptococcus agalactiae* and *Mycoplasma bovis*. Moreover, we selected only the herds that, at the time of sampling, did not follow any specific *Staph. aureus* mastitis control program and did not conduct a specific sanitation program aimed to control this pathogen. Considering the resources available for our study, the first 60 farms that fulfilled the described criteria and voluntary agreed to participate to the sampling (and eventually to take place specific actions) were enrolled in our study.

At first, composite milk samples were collected cow by cow (first sampling round) from all lactating cows of the herds, and bacteriological analysis was then performed to determine the prevalence of *Staph. aureus* IMI (Maisano et al., 2019). Then, *Staph. aureus*-positive cows were resampled 1 to 3 wk later by collecting quarter milk samples to detect the infected quarters and perform molecular characterization of the isolates (second sampling round). Finally, the proportions of *Staph. aureus*-infected cows and the average number of infected quarters per cows were determined as indicators of strain infectivity. Specific indicators of milking management, such as teat condition and udder hygiene, were evaluated as well. These 2 parameters can be considered risk factors for *Staph. aureus* transmission and, consequently, for within-herd prevalence of IMI (Zadoks et al., 2001; Graber et al., 2009), thus potentially leading to biased conclusions regarding the contagious properties of different *Staph. aureus* strains. Teat condition score (**TCS**) and udder hygiene score (**UHS**) were visually evaluated for each cow during milk sampling and assigned according to Neijenhuis et al. (2001) and Schreiner and Ruegg (2002), respectively. Herd-level TCS and UHS were calculated as the arithmetic mean of individual cow TCS and individual cow UHS. The milking routine was assessed based on a specific checklist of 8 items created by the Italian National Reference Center for milk quality. The checklist is largely in accordance with the recommendations of the National Mastitis Council (NMC, 2016). The checklist was created to specifically address Italian milking practices. For each question (**Q**), scores from 1 to 3 were assigned (Q scores), where 1 was optimal (the goal for the farmer), 2 was acceptable (not the goal but nondetrimental), and 3 was insufficient (dangerous or not allowed). A cumulative milking routine score (**MRS**) was calculated for each herd as the arithmetic mean of the 8 Q

scores. The management factors and the scoring system are listed in Table 1. The TCS, UHS, and Q scores were assigned during milk sampling by 4 veterinarians experienced in mastitis control and specifically trained (by both classroom training before the study and field practice with an expert tutor as a gold standard for the internal validation of the checklist) to reduce intra- and interobserver variability. Furthermore, age of the lactating cows at the time of sampling was also evaluated in the study as a possible risk factor for IMI prevalence. The age (in days) of lactating cows was obtained from the bovine registry of farms and average herd age (**HA**) was calculated. This parameter was considered a risk factor, because older cows are generally more likely to become infected and cure rates decrease with increasing age of the cow (Barkema et al., 2006).

Sample Collection and Bacteriological Analyses

Composite milk samples were collected hygienically (after foaming predipping and drying with disposable paper towels), and the subsequent quarter milk samples were collected aseptically (by thorough disinfection of teat using denatured alcohol). All samples were kept at 4°C and bacteriological assays were performed within 48 h. Milk samples were cultured using standard methods: 10 μL of the sample was plated on esculin blood agar (**EBA**) and Baird Parker with rabbit plasma fibrinogen agar (**BP-RPF**). After incubation at 37°C for 48 h, suspected *Staph. aureus* colonies (hemolytic on EBA or displaying the typical halo on BP-RPF) were confirmed by tube coagulase test. The growth of one colony in 10 μL of inoculated milk (100 cfu/mL) was chosen as the threshold to define a sample as positive (Dohoo et al., 2011) and a cow or quarter as infected.

Molecular Analyses

Genotyping by RS-PCR. For each herd, *Staph. aureus* isolates from quarter milk samples were confirmed by a specific PCR assay targeting the *nuc* gene (Cremonesi et al., 2006) and genotyped by RS-PCR. Specifically, 5 *Staph. aureus* isolates (if present) per herd from different positive cows were randomly selected and genotyped. In case of different cultural morphologies of colonies (i.e., pigmentation and hemolysis on EBA and type of halo on BP-RPF), up to 5 isolates per morphology were selected.

DNA was extracted from strains using the DNeasy Blood and Tissue kit (Qiagen), following the manufacturer's instructions. DNA was then stored at −20°C until use. The RS-PCR was performed according to Fournier et al. (2008), based on amplification of 16S-23S rRNA intergenic spacer region. The PCR products

Table 1. Farm characteristics and specific milking management factors included in the statistical analysis as possible risk factors in the spread of IMI by *Staphylococcus aureus*

Factor	Category	Description
Q1 Hygienic level of the milking parlor	1: Optimal 2: Acceptable 3: Insufficient	Cleaning thoroughly with high-pressure hot water after each milking Cleaning with high-pressure cold water after each milking Rough cleaning with cold water
Q2 Udder and teat preparation	1: Optimal 2: Acceptable 3: Insufficient	Foremilk examination, preparation with predipping, and accurate cleaning of the teats (no signs of dirt) and drying them with disposable material (one for each cow) Preparation with good cleaning of the teats and drying teats with disposable materials (one for more than one cow) Cleaning of udder or teats with water and no drying or no use of disposable materials for each cow
Q3 Use of back-flushing	1: Optimal 2: Acceptable 3: Insufficient	Use of back-flushing system with the use of steam and disinfectants Use of back-flushing system with only hot or cold water Nonuse of back-flushing system
Q4 Post-milking teat disinfection	1: Optimal 2: Acceptable 3: Insufficient	Use of postdipping teat disinfection with a specific film product and frequent cleaning of the cups Use of postdipping teat disinfection with a specific product and occasional cleaning of the cups Nonuse of postdipping teat disinfection
Q5 Management and routine of milking procedures	1: Optimal 2: Acceptable 3: Insufficient	Correct stimulation followed by attaching the cluster within 90 s; control of the milk flow and of cluster during milking; removing the clusters, avoiding machine stripping Correct stimulation followed by attaching the cluster within 90 s; irregular or no control of the milk flow and of cluster during milking but removing the clusters, avoiding machine stripping No correct stimulation followed by rapid attaching of the cluster, no control of the milk flow or of cluster during milking or removing the clusters without attention to machine stripping
Q6 Cleaning and sanitizing of milking equipment	1: Optimal 2: Acceptable 3: Insufficient	Regular cleaning and disinfection procedure program, taking water hardness in account; no residual dirt or biofilm on the inner side of the liners Regular cleaning and disinfection procedure program; no residual dirt or biofilm on the inner side of the liners No coherent or absent cleaning and disinfection procedure program or residual dirt or biofilm
Q7 Hygienic level of milkers	1: Optimal 2: Acceptable 3: Insufficient	Milkers use clean clothing, with clean waterproof apron and disposable gloves Milkers use clean clothing and waterproof apron but use plastic nondisposable gloves Milkers use dirty clothing or no use of gloves
Q8 Maintenance of milking equipment and liner replacement	1: Optimal 2: Acceptable 3: Insufficient	Fully checked by a specialist at least once a year, and liner replacement ≤600 h of use Fully checked by a specialist at least once a year, and liner replacement between 600 and 1,000 h of use Fully checked by a specialist less than once a year or only in case of problems, or liner replacement >1,000 h of use or only replaced when damaged
Milking routine score (MRS)	Cumulative milking routine score	Calculated as the arithmetic mean of the 8 Q scores
Udder hygiene score (UHS)	Average of the scores of all cows	Hygiene of udder, flanks, and legs was scored based on a 4-point scale system, from very clean (score 1) to very dirty skin (score 4; Schreiner and Ruegg, 2002)
Teat condition score (TCS)	Average of the scores of all teats	Callosity of the teat orifice was scored based on a 4-point scale system: absent callosity = 1; a smooth callous ring around the orifice = 2; rough and very rough callous rings = 3 and 4, respectively (Neijenhuis et al., 2001)
Herd age (HA)	Age in days	Average age of cows >21 mo
adlb status of the herd (ADLB)	*Staph. aureus* circulating strain is *adlb*-positive	Yes or no

were analyzed using the miniaturized electrophoresis system DNA 7500 LabChip (Agilent Technologies), and genotypes were inferred from electrophoresis profile using Mahal 2.0 software, which is freely available online (https://mahal.vitech.dev/#/). Ribosomal spacer-PCR allows classification of isolates in several genotypes (**GT**) that can be grouped in clusters (**CL**). Each CL includes the genotype itself and its variants, differing in only one band in the electrophoretic analysis (Syring et al., 2012; Cosandey et al., 2016).

If all tested *Staph. aureus* isolates within a herd or most of them (i.e., 4 of the 5 isolates tested) belonged to the same RS-PCR genotype, this genotype was considered the predominant circulating strain likely responsible for IMI within the herd; in the remaining cases, the infection was considered "mixed" by different genotypes, none individually responsible for the herd problem (Table 2). The number of 5 isolates per herd is based on the previous studies by Fournier et al. (2008) and Cremonesi et al. (2015), which showed that either there is no variation among genotypes within one herd or it is very low, particularly when more than 5 isolates are involved in the IMI.

***adlb*-Targeted PCR.** The *adlb*-targeted real-time PCR was performed on all RS-PCR genotyped isolates according to Sartori et al. (2017).

MLST Analysis. Multilocus sequence typing analysis was performed on a subset of strains, based on the results of RS-PCR. The selection was done as follows: one strain per RS-PCR genotype per herd was analyzed by MLST. Therefore, in the herds with a unique circulating genotype, only one strain was randomly selected; in herds with different genotypes, one strain per genotype was randomly selected.

In detail, the selected strains were subjected to whole-genome sequencing on the Miseq platform (Illumina) as follows: genomic libraries were prepared using Nextera XT DNA Library Preparation Kit (Illumina) and sequenced generating paired-end reads of 250 bp. Raw reads were checked for quality using FastQC (Babraham Bioinformatics, 2018). The MLST analysis was performed on raw reads through the Center for Genomic Epidemiology online platform (Center for Genomic Epidemiology, 2020), or by submitting them to PubMLST (https://pubmlst.org). The original contributions in the present study are publicly available. Illumina raw reads have been deposited in the National Center for Biotechnology Information GenBank database under the Bioproject number PRJNA897860.

Statistical Analysis

Descriptive Analysis. The 60 herds were sorted in ascending order according to their cow prevalence for *Staph. aureus*, and then divided in 3 groups of equal size. Group 0 (herds 1 to 20), group 1 (herds 21 to 40), and group 2 (herds 41 to 60) were considered as low, intermediate, and high cow prevalence groups, respectively. For each group, data of continuous variables (HA, UHS, TCS) were expressed as minimum and maximum, mean, median, standard deviation (**SD**), and standard error, and they were plotted as box plots. The overall comparison among groups and the comparisons between 2 groups were performed by single-factor ANOVA using the Kruskal-Wallis test, and by the Mann-Whitney U test, respectively. Categorical variables [*adlb* presence (ADLB) Q1-Q8, GT, ST, CC] were instead expressed as frequencies or minimum and maximum. For graphical representation of ADLB and Q1-Q8, the mean and standard error of the mean were calculated and plotted. Comparisons among groups were computed by exact χ^2 test. All analyses were performed using Systat 13.0 software (Systat Software Inc.).

Modeling of Staph. aureus Cow Prevalence. Quasi-binomial logistic regression was applied to model the within-herd prevalence of *Staph. aureus* IMI (response variable) as a function of the explanatory variables identified in the study, by using R 3.6.3 (R Core Team, 2020) with "MASS" package. A quasi-binomial distribution was used for describing the error distribution to account for overdispersion. Specifically, we tested the effect on cow prevalence of the binary variable ADLB, the categorical variable predominant clonal complex (**pCC**), and the different continuous variables (i.e., HA, UHS, TCS), in addition to the global milking score (MRS). The MRS, defined as the mean over the variables Q1 to Q8, was introduced in the statistical model to replace the individual Q variables to avoid the problem of collinearity among various Q variables. To evaluate in the model the effect on the prevalence of the major CC observed in the population, the categorical variable pCC was introduced. In detail, to take into account only the main CCs observed in the population (and avoid estimating the effect of rare CCs using a limited amount of data), the categorical variable pCC was built by assigning to each farm its pCC under the following conditions: (1) at least 10% of the farms displayed the pCC, or (2) at least 5% of the farms displayed the pCC and the within-herd prevalence range of farms in which circulated the given pCC does not include the overall prevalence of *Staph. aureus* in the study population (i.e., 20.3%). The farms not fulfilling criterion (1) or (2) were assigned to the group "Other CCs." The "Other CCs" group (which represents a generic CC introduced in the farm) was used as the benchmark to evaluate the effect size of a given CC on within-farm prevalence.

Table 2. Characteristics of the 60 analyzed herds, in ascending order of *Staphylococcus aureus* IMI prevalence[1]

Farm no.	Group	Herd size	RS-PCR (no.)[2]	MLST (ST-CC)[3]	Predominant circulating CC	*adlb* status	*Staph. aureus* IMI prevalence (%)[4]	HA[5]	TCS[5]	UHS[5]	MRS[5]
1	0	285	C (1)	ST151-CC705	CC705	Negative	0.7	1,413	2.8	1.4	1.625
2	0	114	B (1)	ST8-CC8	CC8	Negative	0.9	1,380	1.2	2.2	2.125
3	0	158	R^{I} (2)	ST133-CC133	CC133	Negative	1.3	1,532	1.5	2.0	1.375
4	0	131	R (1)	ST352-CC97	CC97	Negative	1.5	1,477	1.7	1.7	1.625
5	0	119	S (2)	ST398-CC398	CC398	Negative	1.7	1,562	1.4	2.1	2.375
6	0	141	C (3)	ST504-CC705	CC705	Negative	2.1	1,371	2.3	1.6	1.625
7	0	47	S (1)	ST398-CC398	CC398	Negative	2.1	1,607	1.4	1.9	1.375
8	0	157	C (3)	ST504-CC705	CC705	Negative	2.6	1,466	2.2	2.0	1.625
9	0	267	S (5)	ST398-CC398	CC398	Negative	3.7	1,306	1.4	1.4	1.875
10	0	417	BJ (5)	ST1-CC1	CC1	Negative	3.8	1,500	1.7	1.6	1.75
11	0	44	B^{III} (1); C^{II} (1)	ST291-CC398; ST504-CC705	Mixed[6]	Negative	4.5	1,458	1.4	2.2	1.5
12	0	85	S (2); BE (2)	ST398-CC398; ST97-CC97	Mixed	Negative	4.7	1,596	2.1	1.3	2.125
13	0	105	C (4)	ST504-CC705	CC705	Negative	4.8	1,335	1.8	1.5	1.625
14	0	88	R (3)	ST352-CC97	CC97	Negative	5.7	1,461	1.4	1.9	1.25
15	0	52	S (1); Z (1)	ST398-CC398; ST1380-CC479	Mixed	Negative	5.8	1,621	1.3	1.6	1.375
16	0	203	BA (5)	ST398-CC398	CC398	Negative	5.9	1,392	1.4	1.9	1.25
17	0	65	C (4)	ST504-CC705	CC705	Negative	6.2	1,450	1.5	1.9	1.375
18	0	127	R (5)	ST352-CC97	CC97	Negative	6.3	1,482	1.0	2.5	1.5
19	0	134	AO (5); Z (3); C (1); BE (1)	ST97-CC97; ST1380-CC479; ST504-CC705; ST97-CC97	Mixed	Negative	7.5	1,547	1.3	2.1	1.5
20	0	40	F (2)	ST20-CC20	CC20	Negative	7.5	1,590	2.0	1.5	2.125
21	1	24	B (1); R^{III} (1)	ST8-CC8; ST133-CC133	Mixed	Negative	8.3	1,944	1.2	2.0	1.875
22	1	101	F (5)	ST9-CC9	CC9	Negative	8.9	1,539	1.6	1.2	2
23	1	59	AO (3)	ST97-CC97	CC97	Negative	10.2	1,457	1.8	1.9	2
24	1	66	Z (5)	Unknown-CC97	CC97	Negative	12.1	1,607	2.3	1.6	1.625
25	1	63	Y (5)	ST45-CC45	CC45	Negative	12.7	1,386	1.5	1.9	2.125
26	1	54	I^{I} (5)	ST6881-CC97	CC97	Negative	14.8	2,487	1.5	1.1	1.875
27	1	73	F (5)	ST389-CC389	CC389	Negative	15.1	1,488	1.7	1.8	1.5
28	1	18	BN (2)	ST71-CC71	CC71	Negative	16.7	1,979	1.8	1.8	1.625
29	1	38	BI (2); R^{I} (3)	ST97-CC97; ST30-CC30	Mixed	Negative	18.4	1,234	1.5	1.8	1.625
30	1	131	S (5)	ST398-CC398	CC398	Negative	19.1	1,534	1.6	1.5	1.625
31	1	70	F^{III} (4); Y^{I} (1)	ST9-CC9; ST9-CC9	CC9	Negative	20.0	1,485	1.4	1.4	2.25
32	1	96	F^{III} (5)	ST20-CC20	CC20	Negative	21.9	1,431	1.2	2.0	2.125
33	1	73	CJ (5)	ST9-CC9	CC9	Negative	23.3	1,463	2.2	1.3	1.875
34	1	38	B (4)	ST8-CC8	CC8	Positive	23.7	1,367	1.5	2.0	1.5
35	1	70	U (5)	ST20-CC20	CC20	Negative	24.3	1,617	1.3	1.4	1.5
36	1	82	AO (5)	ST97-CC97	CC97	Negative	25.6	1,577	1.3	1.8	2.25
37	1	95	AX (4); I^{I} (1)	ST97-CC97; ST97-CC97	CC97	Negative	26.3	1,557	1.5	2.7	1.75
38	1	45	R (5)	ST352-CC97	CC97	Negative	26.7	1,489	1.6	1.5	1.625
39	1	205	B (5)	ST8-CC8	CC8	Positive	27.3	1,556	2.0	2.6	1.625
40	2	132	BQ^{I} (5)	ST97-CC97	CC97	Positive	28.0	1,416	1.4	2.2	2.125
41	2	166	B (5)	ST8-CC8	CC8	Positive	28.3	1,626	1.5	1.3	1.75

Continued

Table 2 (Continued). Characteristics of the 60 analyzed herds, in ascending order of *Staphylococcus aureus* IMI prevalence[1]

Farm no.	Group	Herd size	RS-PCR (no.)[2]	MLST (ST-CC)[3]	Predominant circulating CC	*adlb* status	*Staph. aureus* IMI prevalence (%)[4]	HA[5]	TCS[5]	UHS[5]	MRS[5]
42	2	111	B (5)	ST8-CC8	CC8	Positive	28.8	1,405	1.2	1.5	1.625
43	2	107	BM (5); BT (1)	ST126-CC126; ST126-CC126	CC126	Negative	31.8	1,502	1.6	2.9	2.125
44	2	122	K (5)	ST6837-CC5	CC5	Negative	35.2	1,426	1.4	1.6	1.75
45	2	30	B (5)	ST8-CC8	CC8	Positive	36.7	1,677	1.4	1.9	1.5
46	2	37	B (5)	ST8-CC8	CC8	Positive	40.5	1,502	1.3	1.9	2
47	2	239	S^II (5)	ST126-CC126	CC126	Negative	41.8	1,317	1.4	1.6	1.375
48	2	100	R^VI (5)	ST97-CC97	CC97	Positive	43.0	1,448	1.3	2.5	1.875
49	2	78	B (4)	ST8-CC8	CC8	Positive	46.2	1,716	1.2	1.9	2.125
50	2	73	B^III (5); B (1); B^I (1)	ST8-CC8; ST8-CC8; ST8-CC8	CC8	Positive	46.6	1,543	1.8	2.8	1.25
51	2	99	B (5)	ST8-CC8	CC8	Positive	49.0	1,539	1.1	1.5	1.875
52	2	62	B (5); B^I (1)	ST8-CC8; ST8-CC8	CC8	Positive	51.6	1,587	1.9	1.4	1.875
53	2	25	B (5); B^III (2)	ST8-CC8; ST8-CC8	CC8	Positive	56.0	1,856	1.2	2.5	2.25
54	2	87	R^VI (5)	ST97-CC97	CC97	Positive	58.6	1,513	2.2	1.9	2.375
55	2	56	B (5)	ST8-CC8	CC8	Positive	60.7	1,513	1.4	1.3	2.125
56	2	142	B (5); B^I (1)	ST8-CC8; ST8-CC8	CC8	Positive	62.0	1,454	2.7	1.1	1.75
57	2	66	B (5); AQ (1)	ST8-CC8; ST1-CC1	CC8	Positive	62.1	1,645	1.9	1.1	2.125
58	2	81	S^II (5)	ST126-CC126	CC126	Negative	66.6	2,589	2.2	1.9	1.625
59	2	49	B (5)	ST8-CC8	CC8	Positive	67.4	1,605	1.4	2.2	1.625
60	2	37	B (5)	ST8-CC8	CC8	Positive	73.0	1,951	1.3	1.2	1.625

[1]The prevalence of both *Staph. aureus*-infected cows and some IMI risk factors are listed, as well as results of molecular analyses performed on the strains isolated in each farm.

[2]The number in parentheses indicates the number of isolates belonging to the corresponding genotype. The sum of the isolates per herd is the total number of isolates analyzed by ribosomal spacer (RS)-PCR in the herd. For example, 10 isolates were genotypes by RS-PCR in herd 19.

[3]Multilocus sequence typing (sequence type-clonal complex).

[4]IMI prevalence is expressed as a proportion of positive cows.

[5]HA = age of cows; TCS = teat condition score; UHS = udder hygiene score; MRS = milking routine score. See Table 1 for specifications.

[6]Mixed = within the same herd different genotypes were identified, of which none was predominant.

The response variable was modeled for dependence on multiple explanatory variables by using a forward stepwise selection procedure (Venables and Ripley, 2002) with a drop-in-deviance test statistic based on quasi-likelihood inference (Roback and Legler, 2021) to define the model providing the best prediction. The drop-in-deviance test comparing Model 1 (with p parameters) and Model 2 (with q parameters, and $q < p$) was performed using the statistic

$$F = \frac{1}{\hat{\phi}} \times \frac{D_2 - D_1}{p - q},$$

where $\hat{\phi}$ represents the overdispersion parameter for the variance, D_1 and D_2 represent the residual deviance for Model 1 and Model 2, respectively, and $p - q$ represents the difference in the number of parameters between the models (Roback and Legler, 2021). Then, the test statistic was compared with an F-distribution with $p - q$ and $n - p$ degrees of freedom (where n represents the sample size). We used the odds ratio (**OR**) as effect size statistics in quasi-binomial logistic regressions. All data included in the statistical analyses are listed in Table 2.

RESULTS

Sample Collection and Bacteriological Analyses

During the first sampling round, a total of 6,079 composite milk samples from as many cows were collected from the 60 selected herds. Overall, 1,233 cows were *Staph. aureus*-positive, and within-herd prevalence ranged from 0.7 to 73%.

Because some cows were slaughtered or dried off between the first and second samplings (an interval of 1 to 3 wk), 1,228 positive cows were resampled during the second round, collecting a total of 4,912 sterile quarter milk samples. All cows resampled were positive for at least one quarter. The proportion of infected quarters per cow within a herd ranged from 1 to 3 in the different herds (Table 2). Teat condition score and UHS ranged from 1.0 to 2.8 and from 1.1 to 2.9, respectively; HA ranged from 1,234 to 2,589 d (Table 2). The results referring to the 8 Q scores of the milking routine check list are shown in Supplemental Table S1 (https://data.mendeley.com/datasets/3z2vnckwg3/1; Romanò et al., 2022); MRS are reported in Table 2.

Molecular Analyses

Genotyping by RS-PCR and MLST. Ribosomal spacer-PCR was performed on 262 *Staph. aureus* isolates from the 60 investigated herds. The number of genotyped isolates per herd ranged from 1 to 10, depending on the number of isolates obtained and on their morphological characteristics, as described in Materials and Methods. A predominant genotype was detected in 54 herds: it was a unique genotype in 46 herds, whereas in 8 herds it was predominant (i.e., 4 of the 5 strains tested belonged to the same RS-PCR genotype). In the 6 remaining herds, up to 4 different genotypes were isolated, of which none was predominant ("mixed" isolates; Table 2).

Seventy-seven out of these 262 strains were also analyzed by MLST; 19 different ST, grouped into 15 CC, were identified, including 2 previously unknown profiles. The results are shown in Table 3. The most frequent MLST profiles were CC8-ST8 (n = 23; 30%), CC97-ST97 (n = 11; 14%), CC398-ST398 (n = 7; 9%), and CC705-ST504 (n = 6; 8%). When a predominant genotype circulated within a herd (n = 54), it was CC8 in 17 herds (31%), CC97 in 12 (22%), CC705 and CC398 in 5 (9%), and CC20, CC9, and CC126 in 3 (6%) herds. In the 6 remaining herds with a predominant genotype, we isolated a CC that was not isolated in any of the other analyzed herds (Table 2). Comparing the results of MLST and RS-PCR, all CC8-ST8, CC398-ST398, and CC705 *Staph. aureus* strains belonged to CLB, GTS (RS-PCR genotype S), and CLC (RS-PCR genotypic cluster C), respectively.

adlb-Targeted PCR. The *adlb*-targeted PCR was performed on the same 262 isolates that underwent RS-PCR. Eighty-five of 87 CLB strains were *adlb*-positive (75 GTB, 7 GTB[III], and 3 GTB[I]), whereas *adlb* was detected in only 15 of the non-CLB circulating strains (10 GTR[VI] and 5 GTBQ[I]; Table 2). Among the 77 strains analyzed with MLST, the *adlb* gene was present in 22 of 23 CC8 (96%) and 3 of 17 CC97 (18%). The remaining CCs did not harbor the gene.

Nineteen herds were considered *adlb*-positive because the predominant strain carried this gene. Conversely, 41 herds were identified as *adlb*-negative, because none of the isolated strains carried the gene. Our results show that IMI prevalence was always considerably higher in *adlb*-positive herds compared with *adlb*-negative herds. The relationship between prevalence of *Staph. aureus* IMI and circulation of *adlb*-positive strains within the herd is displayed in Figure 1 and Figure 2. No *adlb*-positive strain was isolated in herds with an IMI prevalence <23%, and the effect of the carriage of *adlb* gene on IMI prevalence is represented in Figures 1, 2, and 3.

Statistical Analyses

Descriptive Analysis. An association was observed among the 3 groups of prevalence (group 0/low preva-

Table 3. Distribution of clonal complexes (CC) and sequence type (ST), and their relation with ribosomal spacer (RS)-PCR genotypes and the presence of *adlb*, of the 77 strains analyzed with multilocus sequence typing (MLST)

No. of strains	CC[1]	ST	ST (% of total)	Genotype (GT)	*adlb*-positive strains
23	CC8	ST8 (23)	30	B (18), B^I (3), B^{III} (2)	B (17), B^I (3), B^{III} (2)
17	CC97	ST97 (11)	14	AO (3), R^{VI} (2), BE (2), AX (1), BI (1), BQ^I (1), I^I (1)	R^{VI} (2), BQ^I (1)
		ST352 (4)	5	R (4)	
		ST6881 (1)	1	I^I (1)	
		Unknown (1)	1	Z (1)	
8	CC398	ST398 (7)	9	S (6), BA (1)	
		ST291 (1)	1	B^{III} (1)	
7	CC705	ST504 (6)	8	C (5), C^{II} (1)	
		ST151 (1)	1	C (1)	
4	CC9	ST9 (4)	5	CJ (1), F (1), F^{III} (1), Y (1)	
4	CC126	ST126 (4)	5	S^{II} (2), BM (1), BT (1)	
3	CC20	ST20 (3)	4	F^{III} (1), F (1), U (1)	
2	CC1	ST1 (2)	3	AQ (1), BJ (1)	
2	CC133	ST133 (2)	3	R^I (1), R^{III} (1)	
2	CC479	ST1380 (2)	3	Z (2)	
1	CC5	ST6837 (1)	1	K (1)	
1	CC30	ST30 (1)	1	R^I (1)	
1	CC45	ST45 (1)	1	Y (1)	
1	CC71	ST71 (1)	1	BN (1)	
1	CC389	ST389 (1)	1	F (1)	

[1]All strains belonging to the same CC were isolated in different herds.

lence; group 1/intermediate prevalence; group 2/high prevalence) and GT, ST, and CC ($P < 0.001$). In particular, all CC705/GTC strains and most of the herds with various GTs ("mixed") were observed in group 0. *Staphylococcus aureus* belonging to CC398/ST398, and CC97/ST352 were the most prominent in group 0 as well. In contrast, less variability was observed in group 2: *Staph. aureus* strains mainly belonged to CC8/ST8/CLB, and CC126/ST126/GTS^{II} strains were isolated exclusively in this group (Table 2).

The presence of *Staph. aureus* strains carrying the *adlb* gene was highly group-dependent ($P < 0.001$). In fact, they were never observed in group 0, whereas they were found in 3 herds of group 1 (15%) and in 16 herds (80%) of group 2. Based on the exact χ^2 test, the presence of at least one *Staph. aureus* carrying the *adlb* gene in a herd was highly dependent on the GT of the strain itself ($P < 0.001$). In fact, only *Staph. aureus* strains belonging to CC8/ST8/CLB and CC97/ST97 harbored the gene. Among CC97/ST97 strains, *adlb* was found only in strains belonging to GTR^{VI} and GTBQ^I.

As for HA, medians did not differ among groups ($P = 0.105$). Groups 1 and 2 showed increased standard deviation, as a result of one herd in each group having considerably older cows. The UHS ($P = 0.756$) and TCS ($P = 0.759$) values were very similar among the groups (Table 2; Figure 4A; Supplemental Table S2; https://data.mendeley.com/datasets/3z2vnckwg3/1; Romanò et al., 2022).

For some of the Q variables (Q1, Q2, Q3, Q4, Q8), all 3 defined levels (optimal, acceptable, and insufficient) were observed, whereas insufficient values were never detected for the remainder (Q5, Q6, Q7). A significant association was observed between Q7 and the group variable ($P = 0.032$): the hygienic level of the milkers worsened as the prevalence of *Staph. aureus* IMI increased. As for all remaining Q variables, no significant association among groups was observed.

Modeling of Staph. aureus Cow Prevalence. The statistical analysis of factors affecting *Staph. aureus* within-herd prevalence, performed through quasi-binomial logistic regression, revealed that the pCC present in the farm and ADLB (the presence/absence of the *adlb* gene in the circulating strains) were the only explanatory variables included in the best model obtained from the forward stepwise selection (see Table 4). In Supplemental Table S3 (https://data.mendeley.com/datasets/3z2vnckwg3/1; Romanò et al., 2022), we show the effect size (expressed as OR) associated with the parameters estimated in the 1-variable and 2-variable best models selected through the model selection process. The model selection process identified the pCC as the main explanatory variable in explaining the observed difference in *Staph. aureus* within-herd prevalence (see the 1-variable best model in Table 4 and Supplemental Table S2; https://data.mendeley.com/datasets/3z2vnckwg3/1). Specifically, the 1-variable best model predicted a significantly different OR with respect to the benchmark for 4 of 5 of the main CC observed in the study (i.e., CC8, CC97, CC705, and CC126; see Supplemental Table S2). However, the 2-variable best model (which provided the best fit in
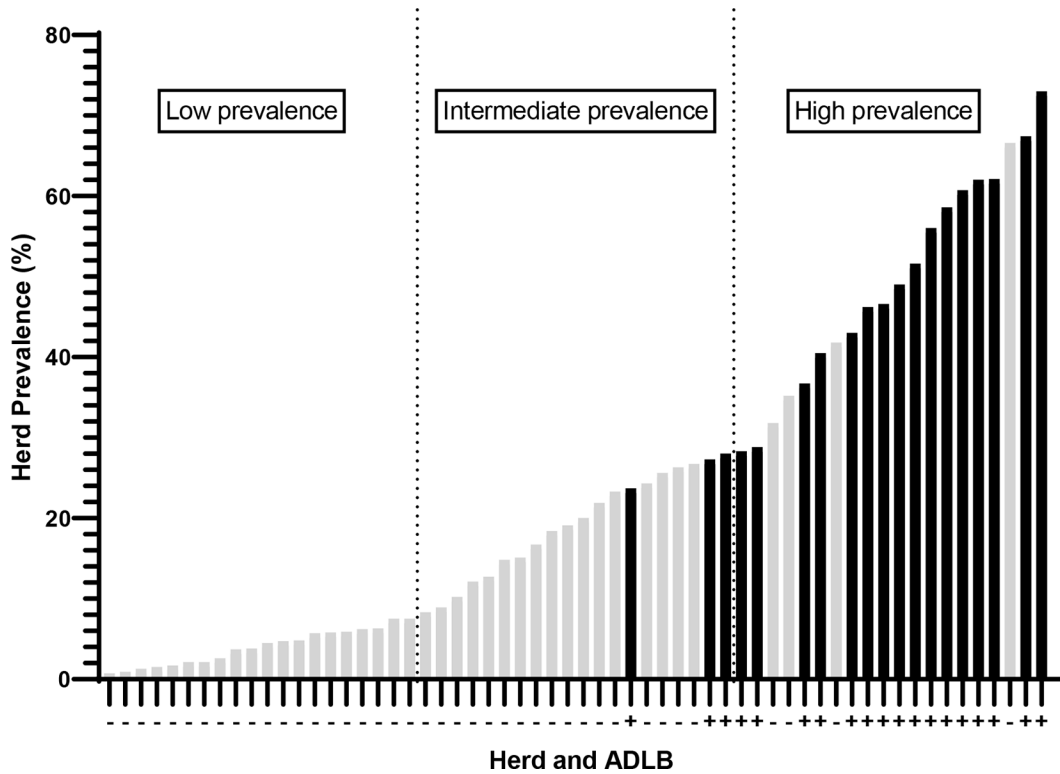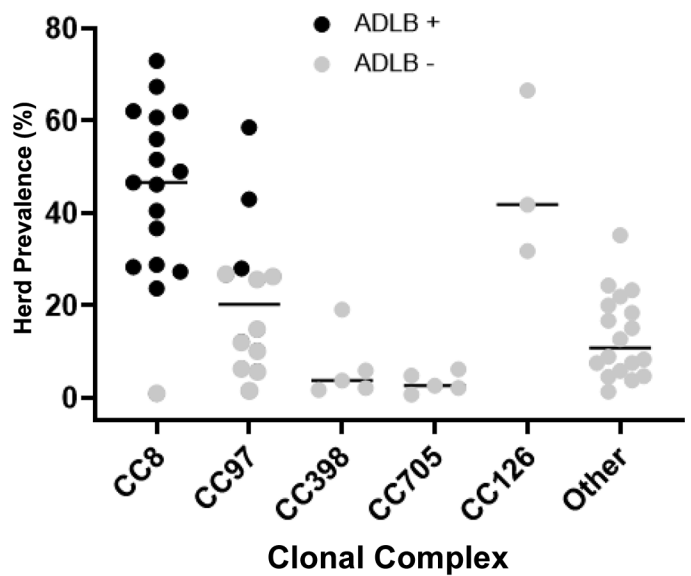
Figure 1. Distribution of *Staphylococcus aureus* IMI prevalence in the 60 farms in relation to their *adlb* status. The vertical lines show the 3 groups of 20 farms, arbitrarily defined as characterized by low (group 0), intermediate (group 1), and high (group 2) prevalence for statistical analysis. Gray (−) = *adlb*-negative farms (gray); black (+) = *adlb*-positive farms.
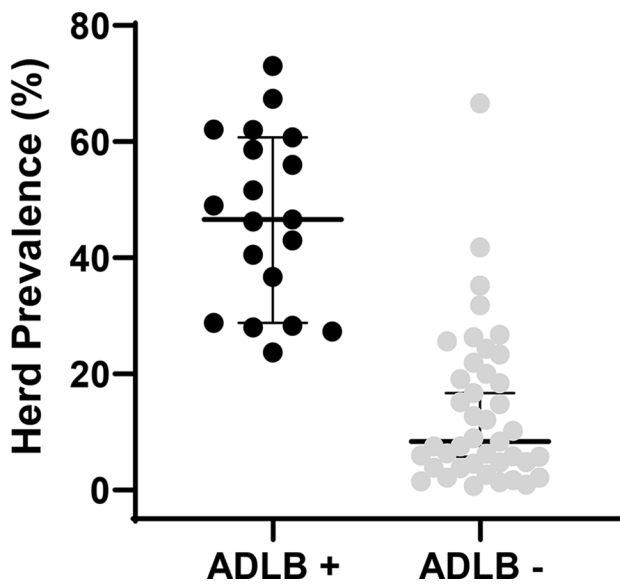
Figure 2. *Staphylococcus aureus* IMI prevalence in the 60 herds based on their *adlb* status. Gray = *adlb*-negative farms; black = *adlb*-positive farms. The central line is the median and the whiskers are 95% CI.

Figure 3. Distribution of *Staphylococcus aureus* IMI prevalence in the 60 farms in relation to the clonal complex and *adlb* status of the predominant circulating strains. Gray = *adlb*-negative farms; black = *adlb*-positive farms. Lines indicate the median.
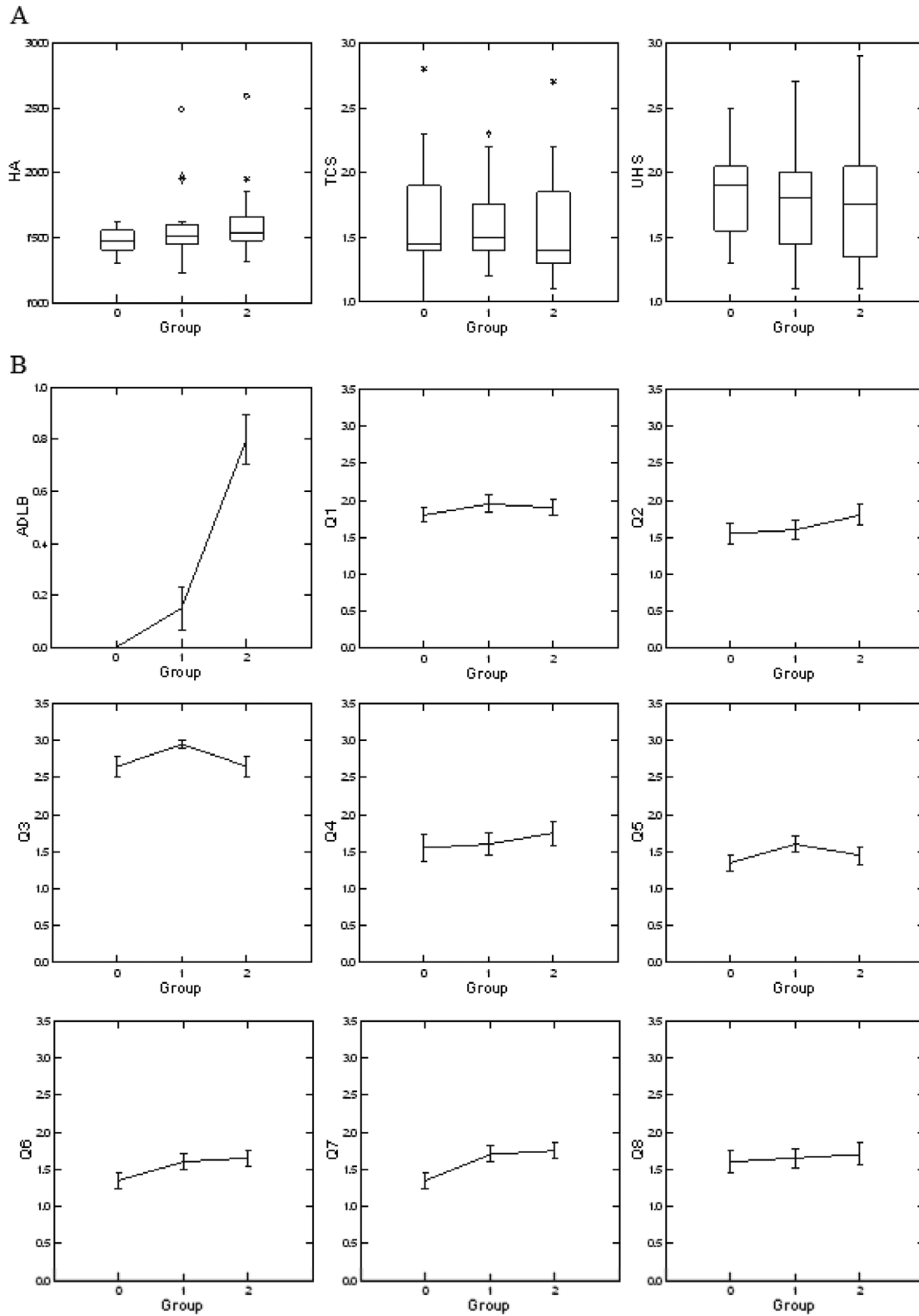
Figure 4. Graphical representation of the mean and SEM describing the relationships between different groups of prevalence (0 = low, 1 = intermediate, 2 = high) and the studied variables. (A) Continuous variables: herd age (HA), teat condition score (TCS), and udder hygiene score (UHS); (B) categorical variables: *adlb* status (ADLB) and questions (Q) 1 to 8 (Q1–Q8). A: The box is the area between the 25th and 75th percentiles, the line is the median, the whiskers are the limits (minimum and maximum), and the asterisks are outliers. The circles are the extreme outliers.

Table 4. Forward stepwise model selection for within-herd prevalence (Herd Prev) obtained from quasi-binomial regression[1]

Response variable	v-variable best model[2]	ϕ	Residual deviance	k	P-value
Herd Prev	~ 1[3]	14.8	961.6	1	—
	$\sim$pCC	6.9	401.2	6	9.5×10^{-10}
	$\sim$pCC + ADLB	5.4	287.1	7	2.8×10^{-5}
	$\sim$pCC + ADLB + HA	5.4	276.4	8	0.16

[1]Models were compared using drop-in-deviance tests. The best models for v explanatory variables are shown, with the dispersion parameter (ϕ), the residual deviance, the number of parameters (k), and the P-value of the comparison with the $v - 1$ variable best model (P-tests: <0.05 as the inclusion and exclusion criteria).

[2]pCC = predominant clonal complex; ADLB = *adlb*-positive strain; HA = herd age. Other explanatory variables included in the full model but not selected in the v-variable best models were udder hygiene score, teat condition score, and milk routine score.

[3]Null model.

the selection process) predicted a significantly higher *Staph. aureus* prevalence in farms where the pCC was CC126 (OR = 3.85, 95% CI: 2.26–6.54), and a significantly lower *Staph. aureus* prevalence in farms where the pCC was CC705 (OR = 0.21, 95% CI: 0.07–0.66) with respect to the benchmark. Instead, it did not predict significant differences in *Staph. aureus* prevalence with respect to the benchmark where the pCC was CC8 or CC97. Additionally, the 2-variable best model predicted a significantly higher *Staph. aureus* prevalence in farms where the circulating strains harbored the *adlb* gene compared with farms in which *Staph. aureus* strains were *adlb*-negative (OR = 4.06, 95% CI: 2.16–7.63).

DISCUSSION

Because *Staph. aureus* IMI is mainly chronic and subclinical, the contagiousness of this pathogen is of utmost importance in determining economic losses for the affected dairy farms. We investigated *Staph. aureus* IMI prevalence in 60 dairy farms located in northern Italy, with known *Staph. aureus* IMI and without other contagious microorganisms. Our study showed very variable prevalence of *Staph. aureus* IMI in the different herds, ranging from 0.7 to 73%. Of these, about one-third had a prevalence <8% and one-third >28%, and in 15% of the herds, prevalence was >50%. This is in line with previous Italian data (Luini et al., 2015; Magro et al., 2017) and confirms that very different situations can be found depending on the single farm considered. Indeed, in certain herds, *Staph. aureus* IMI are reported to remain confined to a few cows, whereas in many others, the infection appears to be widespread with up to 70 to 80% of cows infected, leading to serious economic losses and management problems (Cremonesi et al., 2015; Luini et al., 2015; Cosandey et al., 2016; Gazzola et al., 2020).

Our results are consistent with previous studies about the circulation of one predominant genotype within a farm; indeed, in most cases, when we isolated different genotypes within the same herd, only one of them was predominant (Joo et al., 2001; Capurro et al., 2010; Leuenberger et al., 2019). Interestingly, in herds with high prevalence of *Staph. aureus* IMI, we observed few genotypes, mostly CC8, whereas most of the different genotypes were isolated in the remaining herds.

To date, no study has clearly identified a single marker or combination of markers capable of predicting *Staph. aureus* contagiousness within a herd and that is universally valid in all geographical and farming conditions. In our study, we investigated the relationship between the prevalence of *Staph. aureus* IMI and environmental and management factors generally considered predisposing to IMI from contagious pathogens, such as the age of animals and the average number of milking cows (Cicconi-Hogan et al., 2013). In addition, we considered other factors related to hygiene and quality of milking, including UHS and TCS, as well as those strictly related to the milking routine, such as the hygienic level of the milking parlor and the milkers, udder preparation, the quality of pre- and postdipping, the use of back-flushing, the routine of milking procedure, and the cleaning and maintenance of milking equipment. Previously, Dufour et al. (2012) investigated manageable risk factors for *Staph. aureus* IMI incidence and prevalence, reporting that they seemed to be mostly related to milking procedures in herds where postmilking teat disinfection and blanket dry-cow therapy had already been implemented. In particular, wearing gloves during milking, adequate teat-end condition, and use of premilking teat disinfection were associated with lower IMI incidence and prevalence, highlighting the importance of good milking practices (Dufour et al., 2012). The association between TCS and mastitis in dairy cows has been the subject of a systematic review, which showed that only

severe teat condition was associated with the incidence or prevalence of *Staph. aureus* IMI (Pantoja et al., 2020). To avoid possible bias, we enrolled herds that did not practice segregation or culling of infected cows and that did not implement a specific dry-cow therapy, even if these remain best practices for the control and eradication of *Staph. aureus* (NMC, 2016).

Our descriptive analyses did not identify a significant association between most of the considered variables and the prevalence of *Staph. aureus* IMI, except for the udder and teat preparation for milking (Q7). In contrast, at least for Italian farms, some of the Q variables could not be individually used as predictors, because they were highly associated with each other as they reflect the farmer's attitude. For example, we noted that if the farmers performed a good milking procedure (Q5), they also wanted to maintain a clean milking parlor (Q1); if the personal hygienic level of the farmers was high (Q7), udder and teat preparation for milking (Q7) was also good, resulting in a general cleanliness; if the farmers cared for cleaning the milking equipment (Q6), they also maintained it in good condition (Q8) and performed good postmilking teat disinfection (Q4). Considering the global milking variable, that is, the combination of all Q variables, it had no or only a minimal impact on *Staph. aureus* cow prevalence within a herd. The HA had an effect, as reported by Barkema et al. (2006), but this small effect was not significant in the best model analysis. Our study showed a strong association between the prevalence of *Staph. aureus* IMI and the presence of the *adlb* gene ($P < 0.001$) in both the univariable and multivariable models. In the multivariable analysis, the model providing the best prediction included pCC and ADLB as the only significant predictors. Interestingly, the difference in OR obtained in the 1-variable and 2-variable models for CC8 and CC97 (the only CCs where *adlb* was detected) suggests that it is the presence of the *adlb* gene, not the circulation of these CCs in a herd per se, that leads to higher *Staph. aureus* within-herd prevalence. The within-herd prevalence of *Staph. aureus* IMI was always higher than the population average in farms in which CC8 or CC97 were the predominant CCs when they carried the *adlb* gene, whereas it was lower than the population average when the predominant CC8 and CC97 did not carry the gene.

These results demonstrate that the genetic properties of the *Staph. aureus* circulating within a herd may affect IMI prevalence and play a crucial role in the resultant herd problem. In particular, our findings show that the presence of a strain harboring *adlb* may be associated with the within-herd prevalence of IMI. As for herds with intermediate or high prevalence of IMI caused by *adlb*-negative *Staph. aureus*, such as CC126, on top of the standard factors HA, UHS, TCS, and MRS, other genetic factors may explain the increased cow prevalence. Other genotypes, such as CC705, seem to be associated with low within-herd prevalence of IMI and to behave similarly to environmental mastitis pathogens (Leuenberger et al., 2019). Interestingly, our results also suggest that, in spite of different environmental influences, intermediately contagious subtypes may occur as well, as in the case of CC97. They may have specific genetic properties that differ from both noncontagious and highly contagious types. However, as this was a cross-sectional study, we cannot exclude the possibility that cow prevalence in group 1 would have increased over time, reaching prevalence typically observed in group 2. Additional genomic and field studies are required to support the hypothesis of intermediate contagiousness.

These findings, together with our previous observations (Fournier et al., 2008; Graber et al., 2009; Cremonesi et al., 2015; Luini et al., 2015), indicate that bovine *Staph. aureus* per se is not contagious, but that it acquires this property most likely by horizontal transfer of appropriate genetic elements. In fact, our bioinformatics studies demonstrate that the *adlb* gene is part of a staphylococcal cassette chromosome that is known to be transferable (Malachowa and DeLeo, 2010). Based on these observations, the transfer of the *adlb* gene among different CCs is theoretically probable. Indeed, the present study showed that such a transfer can happen because the *adlb* gene was observed in both CC8 and CC97. However, further analyses are required to confirm and fully describe this gene transfer.

This study has potential limitations. Because we conducted a cross-sectional study, we cannot rigorously define the contagiousness of the strains circulating in the farms, which would require longitudinal studies that measure the incidence of infection over time. Considering the available resources and the need for farmers' consent, we preferred to conduct a cross-sectional study enrolling a greater number of herds (i.e., 60), rather than a longitudinal one on a restricted number of herds. Above all, this allowed us to collect a greater number of *Staph. aureus* isolates from different farms for the molecular analyses, with a cost-benefit ratio favorable to the informativeness of the study.

The second limitation concerns the checklist of 8 questions: although it is only internally validated, it is based on the experience of the Italian National Reference Center for milk quality to specifically address Italian milking practices, and it is largely inspired by the National Mastitis Council's Recommended Mastitis Control Program.

CONCLUSIONS

Our study showed the crucial role of the genetic properties of *Staph. aureus*, especially the *adlb* gene, in determining the prevalence of IMI within a herd. Environmental and management factors, which have long been considered predisposing to the spread of contagious mastitis (i.e., caused by *Strep. agalactiae*, *Mycoplasma bovis*, or *Staph. aureus*), may be less relevant if the disease is caused by a *Staph. aureus adlb*-positive strain. For these reasons, use of a molecular test such as *adlb*-targeted PCR in the diagnostic routine is of paramount importance. However, without a specific molecular characterization of the circulating *Staph. aureus*, hygienic and management measures for prevention of contagious mastitis should not be neglected, because they play a fundamental role in *Staph. aureus* mastitis control and eradication programs. Longitudinal studies may be useful to confirm the role of *adlb* in the mechanisms of contagiousness; further analyses using whole-genome sequencing could highlight other genes involved in the high prevalence of *Staph. aureus* IMI caused by *adlb*-negative strains, such as CC126.

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Bovine *Staphylococcus aureus*: a European study of contagiousness and antimicrobial resistance

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In dairy herds managements, mastitis is the leading cause of economic losses. One of the most important pathogens responsible for intra-mammary infections is *Staphylococcus aureus*. The genetic properties of *S. aureus* have a strong influence on its pathogenicity and contagiousness. In this study, we aimed to obtain a comprehensive overview of the key bovine *S. aureus* clinical properties, such as contagiousness and antimicrobial resistance, present in European strains. For this, 211 bovine *S. aureus* strains from ten European countries that were used in a previous study were used in this study. Contagiousness was assessed using qPCR for the detection of the marker gene *adlb*. Antimicrobial resistance was evaluated using a broth microdilution assay and mPCR for the detection of genes involved in penicillin resistance (*blaI*, *blaR1*, and *blaZ*). It was found that *adlb* was present in CC8/CLB strains; however, in Germany, it was found in CC97/CLI and in an unknown CC/CLR strains. CC705/CLC strains from all countries were found to be susceptible to all tested antibiotics. Major resistance to penicillin/ampicillin, chloramphenicol, clindamycin and tetracycline was detected. Resistance to oxacillin, trimethoprim/sulfamethoxazole and cephalosporins was rarely observed. In addition, contagiousness and antibiotic resistance seem to correlate with different CCs and genotypic clusters. Hence, it is recommended that multilocus sequence typing or genotyping be utilized as a clinical instrument to identify the most appropriate antibiotic to use in mastitis treatment. Actualization of the breakpoints of veterinary strains is necessary to address the existing antibiotic resistance of the bacteria involved in veterinary mastitis.

KEYWORDS

Staphylococcus aureus, *adlb*, antimicrobial resistance, minimum inhibitory concentration, multidrug resistant

1. Introduction

In veterinary medicine, mastitis is the leading cause of economic losses in dairy herds management. It contributes to reductions in milk quality and production, there are costs associated with its treatment, and animal culling can be a consequence of treatment failures (1, 2). In Switzerland, the total cost of mastitis is ∼$131 million annually, according to Heiniger et al. (3). One of the most important pathogens responsible for intramammary infections (IMIs) is *Staphylococcus aureus* (4). *S. aureus* may infect only some individual animals or may be contagious and infect the entire herd; infections usually resulting in subclinical chronic mastitis (5, 6). As shown previously (5, 7–12), the genetic properties of

S. aureus have a strong influence on its pathogenicity and contagiousness, making subtyping necessary to improve treatment success and dairy herd management. Using ribosomal spacer PCR (RS-PCR), it has been shown that the rate of infected cows in a herd is highly dependent on the bacterial genotype (GT) (7–10), and, *S. aureus* genotype B (GTB) and its variants may infect up to 100% of cows in the same herd (7–9, 11) due to its high contagiousness (13, 14). In contrast, other genotypes and their variants (e.g., GTC, GTF, GTS) are restricted to one or a few cows in a herd (7–10, 15, 16). In the electrophoresis of the RS-PCR product, variants differ in 1 electrophoretic band and as consequence, are named by superscripted roman numerals (e.g., GTR[I]). For further simplification, genotypes and their variants are combined into genotypic clusters (CL). For example, GTB and its variants form a cluster named CLB. Multilocus sequence typing (MLST) (17) results have shown that CLB is almost exclusively associated with clonal complex 8 (CC8), whereas CLC corresponds largely to CC705, and CLR to CC97 and CC133 (9, 18). In Europe, CLB, CLC, CLF, CLI, and CLR account for 76.6% of all *S. aureus* isolates obtained from clinical milk samples (19).

RS-PCR is particularly suitable for clinical applications as it is a low-cost, high-throughput method that provides analytical resolution at least as good as *spa* typing in bovine strains (9, 18). However, it is more appropriate to use MLST for subtyping at the biological level because a *S. aureus* clone can be used (17) and, consequently, evolutionary identity established (20, 21). To sanitize Swiss dairy herds infected with the contagious *S. aureus* CLB, Sartori et al., developed a real-time quantitative PCR (qPCR) assay to identify this pathogen in milk samples and achieved diagnostic sensitivity and specificity at the cow level of 99 and 100%, respectively (22). This new assay has been used to detect, with high specificity, the gene *adlb* which encodes the bovine adhesion-like protein located in the GTB-specific staphylococcal cassette chromosome SCCgtb (16, 22). It is a marker for contagiousness and high prevalence of intra-mammary infection (IMI) in dairy herds (11, 16).

Antibiotic (AB) treatment is still one of the most important measures for controlling bovine mastitis (23). However, the frequently unsatisfactory cure rates remain a serious concern, particularly for IMI caused by *S. aureus* (6, 24–27). One major reason for this drawback is the improper use of ABs (28, 29). Additionally, AB treatments applied at the herd level are usually not reported, even though various mastitis control plans strongly recommend performing these analyses and collecting the resultant data (30). Since 2019, it has been required for Swiss's farms to declare the AB treatments used at the herd level (31). In terms of the ABs used to treatment bovine IMIs caused by *S. aureus,* various classes of AB are used: typically, ß-lactams (penicillins and cephalosporins), aminoglycosides, lincosamides, and macrolides (32, 33). Penicillin G is the most commonly used AB for treating IMI in cows caused by *S. aureus* and other Gram-positive mastitis pathogens. In *S. aureus*, the *bla* operon mediates AB resistance against penicillin G and other β-lactamase-sensitive penicillins. The *bla* operon can be located on plasmids (as transposon) or on the chromosome (34, 35) and contains three genes: (1) *blaZ,* which encodes the βlactamase that hydrolyzes the β-lactam ring of AB, rending them inactive; (2) *blaI*, which encodes the repressor; (3)

blaR1 which encodes the sensor and antirepressor (35, 36). Ivanovic et al., recently showed that the *bla* operon plays a key role in phenotypic resistance to penicillin. Furthermore, for *S. aureus,* they highlighted the importance of using the minimum inhibitory concentration (MIC) value as the gold standard when assessing resistance to penicillin and probably other ABs (33).

As contagiousness and antimicrobial resistance (AMR) are critical pathogenic factors of the *S. aureus* strains responsible for bovine mastitis, a comprehensive study was performed to assess the distribution of these key clinical properties in strains from across Europe. Contagiousness was assessed using qPCR to detect the *adlb* gene, which is a staphylococcal marker for contagiousness and for high prevalence of intra-mammary infection in dairy herds. Furthermore, AMR was evaluated using an MIC assay and melting curve PCR (mPCR) to detect genes involved in penicillin resistance (*blaI*, *blaR1*, and *blaZ*).

2. Materials and methods

2.1. Strain collection

A total of 211 bovine strains of *S. aureus* were used in this study that had been collected from 10 European countries; Austria, Belgium, France, Germany, Ireland, Italy, Macedonia, Norway, Slovenia, and Switzerland. These strains were originally collected during two previous studies by Boss et al. (18) and Cosandey et al. (19). As described by Cosandey et al., the strains were aseptically collected from milk samples from individual quarters (19). The strains had been stored in skim milk at −20°C. They were plated onto Columbia agar plates containing 5% sheep blood (Biomérieux Suisse s.a., Geneva, Switzerland) and incubated at 37°C for 24 h (18, 19). The genotypes (GT) and the clonal complexes (CCs) information was obtained from previous studies. The distribution of the different CCs and the GT across the 10 European countries is shown in Table 1 (19).

2.2. DNA extraction

DNA was extracted from single *S. aureus* colonies. One colony was picked and resuspended in 100 μL of 10 mM Tris-HCl and 10 mM EDTA (pH = 8.5), incubated at 95°C for 10 min, and immediately placed on ice. The lysates were diluted 1:100 in qPCR H_2O (SINTETICA S.A, Mendrisio, Switzerland) for use as templates. The samples were stored at −20°C and were analyzed within 2 weeks of extraction (18).

2.3. Quantitative PCR (qPCR) with *adlb* and internal control gene

Real-time qPCR was performed with *adlb* and the internal control gene (N gene of canine distemper virus [CDVN]) according to the protocol of Sartori et al. (22). The characteristics of the utilized primers are listed in Supplementary Table S1. DNA amplification was performed using a Magnetic Induction Cycler

TABLE 1 Distribution of *Staphylococcus aureus* genotypes and clonal complexes across 10 European countries.

	Clonal complex (CC)	Genotype (GT)
Austria	CC8 (9)	GTB (5), GTAM (2), GTI^{IV} (1), GTBE (1)
	CC97 (10)	GTR (2), GTR^{I} (1), GTR^{VI} (3), GTBC (1), GTBL (2), GTE (1)
	CC705 (10)	GTC (7), GTC^{I} (1), GTR^{VI} (1), GTZ (1)
	CC20 (1)	GTF (1)
	CC9 (5)	GTF^{III} (4), GTR^{VI} (1)
	Other CC (13)	
	CC5 (1)	GTE (1)
	CC25 (1)	GTAK (1)
	CC30 (1)	GTB (1)
	CC71 (3)	GTR (2), GTR^{X} (1)
	CC101 (3)	GTAH (2), GTR^{VII} (1)
	CC133 (2)	GTR^{I} (1), GTR^{VI} (1)
	CC479 (1)	GTC (1)
	Unknown (1)	GTAH (1)
Belgium	CC8 (1)	GTB^{II} (1)
	CC97 (4)	GTI^{I} (4)
	CC705 (7)	GTC (4), GTC^{I} (2), GTC^{II} (1)
	CC20 (1)	GTF (1)
	Other CC (5)	
	CC70 (1)	GTC^{I} (1)
	CC71 (1)	GTI (1)
	CC133 (2)	GTR (1), GTZ (1)
	CC479 (1)	GTBG (1)
France	CC8 (2)	GTB (2)
	CC705 (3)	GTC (1), GTC^{I} (2)
	CC20 (4)	GTF (4)
	Other CC (2)	
	CC15 (1)	GTJ^{I} (1)
	CC133 (1)	GTR^{I} (1)
Germany	CC8 (11)	GTB (8), GTB^{I} (3)
	CC97 (5)	GTI^{I} (3), GTR^{VI} (2)
	CC705 (3)	GTC (1), GTC^{II} (2)
	CC9 (2)	GTF^{III} (2)
	Other CC (18)	
	CC1 (3)	GTAN (1), GTBA (1), GTBJ (1)
	CC7 (2)	GTL (1), GTM (1)
	CC15 (1)	GTJ (1)
	CC50 (1)	GTAU (1)
	CC71 (1)	GTI (1)

(Continued)

TABLE 1 (Continued)

	Clonal complex (CC)	Genotype (GT)
	CC133 (2)	GTR^{I} (1), GTR^{II} (1)
	CC398 (3)	GTS (3)
	CC479 (4)	GTP (1), GTZ (3)
	Unknown (1)	GTR^{I} (1)
Ireland	CC97 (2)	GTR (1), GTR^{VI} (1)
	CC705 (2)	GTC^{I} (1), GTO^{I} (1)
	Other CC (7)	
	CC5 (1)	GTE (1)
	CC71 (6)	GTAN (1), GTR (2), GTR^{VI} (3)
Italy	CC8 (9)	GTB (9)
	CC97 (3)	GTBE^{I} (1), GTF (1), GTI^{I} (1)
	CC20 (1)	GTF (1)
	CC9 (1)	GTF^{III} (1)
	Other CC (6)	
	CC22 (1)	GTP (1)
	CC30 (1)	GTBE^{I} (1)
	CC71 (1)	GTI^{I} (1)
	CC126 (2)	GTS^{I} (2)
	CC398 (1)	GTS (1)
Macedonia	CC97 (1)	GTR^{VI} (1)
	Other CC (2)	
	CC7 (1)	GTM (1)
	Unknown (1)	GTR^{VI} (1)
Norway	CC97 (2)	GTR (2)
	Other CC (4)	
	CC133 (2)	GTZ (2)
	CC479 (1)	GTZ (1)
	Unknown (1)	GTC (1)
Slovenia	CC97 (6)	GTR (1), GTR^{II} (2), GTAA (1), GTO (1), GTZ (1)
	CC20(1)	GTAT (1)
	CC9 (1)	GTBB (1)
	Other CC (5)	
	CC22 (1)	GTI^{II} (1)
	CC49 (2)	GTAA (1), GTR^{I} (1)
	CC101 (1)	GTAA (1)
	CC71 (1)	GTR (1)
Switzerland	CC8 (18)	GTB (18)
	CC97 (1)	GTR (1)
	CC705 (19)	GTC (16), GTC^{I} (1), GTA (1), GTH (1)
	Other CC (4)	
	CC5 (1)	GTE (1)

(Continued)

TABLE 1 (Continued)

	Clonal complex (CC)	Genotype (GT)
	CC59 (1)	GTD (1)
	CC70 (1)	GTC (1)
	Unknown (1)	GTC (1)

qPCR real-time thermal cycler (Bio Molecular Systems, Australia) and the following cycling conditions: initial denaturation at 95°C for 3 min followed by 45 running cycles of denaturation at 95°C for 3 s and annealing/elongation at 60°C for 20 s. Two reference strains that were positive for both targets were included as positive controls.

2.4. PCR analysis of the *bla* operon genes

The mPCR was performed according to the protocol of Ivanovic et al. (33). Each of the 211 strains was analyzed for the presence of *blaI*, *blaR1*, and *blaZ;* each gene was detected separately. As per Ivanovic et al., amplicons with a single melting peak identical to the positive control for *blaI*, *blaR1*, or *blaZ* were considered positive. The characteristics of the utilized primers are listed in Supplementary Table S2.

2.5. Assessment of antimicrobial sensitivity

The sensitivity of each strain to 30 antimicrobial agents was tested by minimum inhibitory concentration (MIC) using a PM32 panel (Beckman Coulter, Inc., Brea, CA, USA) following the manufacturer's instructions. The tested ABs concentrations (μg/mL) were as follows: amoxicillin/K clavulanate (0.5/0.25–8/4), ampicillin (0.5–8), azithromycin (1–2), cefepime (4–8), cefotaxime (1–2), cefuroxime (4–8), chloramphenicol (8), ciprofloxacin (0.5–1), clindamycin (0.25–0.5, 2), daptomycin (0.5–4), ertapenem (0.5–1), erythromycin (1–2), fosfomycin (32), fusic acid (2), gentamycin (1–4), imipenem (2–8), levofloxacin (1–2), linezolid (0.5–4), meropenem (2–8), moxifloxacin (0.5–1), nitrofurantoin (64), oxacillin (0.25–2), penicillin (0.03–0.25, 2), rifampin (0.5–2), synercid (1–4), teicoplanin (1–8), tetracycline (1–2), tobramycin (1–4), trimethoprim/sulfamethoxazole (1/19–4/76), and vancomycin (0.25–8). Additionally, cefoxitin (4 μg/mL) screening was performed to determine the presence of methicillin resistant *Staphylococcus aureus* (MRSA) strains. When possible, the current clinical breakpoint of the EUCAST was used (37), otherwise the range specified by the CLSI was applied (38). All the ABs tested and their breakpoints are listed in Supplementary Table S3.

2.6. Statistical analysis

Data are expressed as absolute numbers or percentage. To assess the associations among different AB, the corresponding *phi* coefficients were computed and plotted using R 4.0.5 (39) together

TABLE 2 Detailed distribution of *adlb* across different genotypes and clonal complexes, listed by country.

	Genotype	Clonal complexes
Austria	GTB (6)	CC8 (5)
		Other CC (1)
Belgium	ND	ND
France	GTB (2)	CC8 (2)
Germany	GTB (10)	CC8 (10)
	GTI (1)	CC97 (1)
	Other GT (1)	Other CC (1)
Ireland	ND	ND
Italy	GTB (8)	CC8 (8)
Macedonia	ND	ND
Norway	ND	ND
Slovenia	ND	ND
Switzerland	GTB (18)	CC8 (18)

ND, Not detected.

with the corrplot package v. 0.84. *Phi* values range from −1 to 1 (40). Negative *phi* values indicate a negative, inverse association among both variables, whereas positive *phi* values indicate a positive association. The Kappa test was performed using R 4.0.5 (39) to evaluate the agreement between the MIC and the *bla* mPCR results. Kappa values range from 0 to 1, with values of 0 and 1 indicating no and perfect agreement, respectively (41). To assess penicillin resistance, a loglinear model was computed to analyze the relationships among the factors penicillin, CC, country, and their interactions. The analysis was performed using Systat 13 (Systat Software Inc., Richmond, CA).

3. Results

3.1. Presence of *adlb* in European *S. aureus* strains

The 211 *S. aureus* strains collected from 10 European countries were assessed using qPCR for the presence of *adlb* and its association with GTs and CCs. Among the 211 strains, 46 were positive for *adlb*. The distribution of *adlb* among the different GTs and CCs and among the 10 European countries is shown in Table 2.

An analysis of the GTs found to contain *adlb*, showed that 44 of 47 (94%) CLB strains were positive for *adlb* and that only two strains were positive for *adlb* in the remaining 164 strains (1.2%). Furthermore, the gene was also observed in a German GTI[I] and a GTR[I] strain. GTB was not detected in Ireland, Macedonia, Slovenia, or Norway. In Italy, Germany, and Belgium, three GTB strains were found that did not contain *adlb*.

3.2. AMR overview in European *S. aureus* strains

An analysis of the MIC data showed that 65% of the strains ($n = 137$) were inhibited by all the tested ABs. Table 3 shows the strains that demonstrated AMR, sorted by CC. Only the ABs to which resistance was exhibited are included.

Among all the ABs, the greatest number of AMR strains were found to be resistant to penicillin/ampicillin, chloramphenicol, clindamycin and tetracycline. There was no AMR observed against most of the tested antibiotics, including vancomycin, trimethoprim/sulfamethoxazole, rifampin, synercid, meropenem, linezolid, imipenem, daptomycin, and ertapenem. Interestingly, no MRSA strains were found.

A total of nine strains (4.3%) were multidrug resistant (MDR). The MDR strains were detected in only four countries: Belgium ($n = 4$, 1.8%), Austria ($n = 1$, 0.5%), Italy ($n = 3$, 1.4%) and Germany ($n = 1$, 0.5%). It is worth noting that the four Belgian strains showed the same pattern of resistance to β-lactams (ampicillin and penicillin), chloramphenicol, and clindamycin. The most resistant strain originated in Italy and showed resistance to β-lactams (ampicillin and penicillin), chloramphenicol, quinolones (ciprofloxacin, levofloxacin, and moxifloxacin), tetracycline, and trimethoprim/sulfamethoxazole.

Supplementary Figure S1 shows the AMR associations found among different ABs (ampicillin, chloramphenicol, clindamycin, penicillin and tetracycline). A strong association was found between the β-lactam ABs (ampicillin and penicillin, $phi = 1.0$; $P < 0.001$). Additionally, a strong association ($phi = 0.79$; $P < 0.001$) was found between clindamycin and chloramphenicol.

To analyze the observed penicillin resistance in more detail, a statistical model was computed to analyze the relationships among the following factors: resistance to penicillin, the most abundant CCs (CC8, CC97, and CC705), countries, and their interactions. For penicillin ($n = 54$), significant interactions ($P < 0.001$ in each case) were observed between penicillin resistance and CCs and between penicillin resistance and countries. Significant values ($P < 0.001$ in each case) were also obtained for the interaction between the CCs and countries, and for individual factors except the CCs ($P = 0.055$). Regarding the CCs, 50% and 14% of the CC97 and CC8 strains, respectively, showed resistance to penicillin. In contrast, CC705 was always sensitive to penicillin. Resistance to penicillin was particularly prominent in Austria, Belgium, Germany, and Ireland, and was absent in Slovenia and Switzerland. An identical loglinear model was also calculated for the genotypic clusters; the most observed CCs were replaced by the three most common CLs (CLB, CLC, and CLR). Significant interactions were found between penicillin resistance and CLs ($P = 0.014$) and between the penicillin resistance and countries ($P < 0.001$). CLC strains were always sensitive to penicillin, whereas 13% of CLB strains and 37% of CLR strains were resistant to penicillin. The distribution of penicillin resistance among the countries was identical to the found in the CCs model. Similar analyses for ABs other than penicillin were not performed due to a lack of sufficient data. In fact, for chloramphenicol and tetracycline, the next most common resistance targets after penicillin, only 20 (9.5%) and 12 (5.7%) of strains demonstrated resistance to these ABs, respectively.

CC705 was not only susceptible to penicillin but also to all other ABs except for one strain that was resistant to azithromycin and erythromycin (both macrolides) and another one that was resistant to chloramphenicol (Table 3). CC97 showed resistance to penicillin, chloramphenicol, and clindamycin. Increased AMR rates, in particular to penicillin/ampicillin and chloramphenicol, were also detected in CC9, CC20, and CC133 (Supplementary Table S4).

3.3. Association between MIC and *bla* operon genes

All 54 strains that exhibited phenotypic resistance to penicillin (26% of all strains) showed the simultaneous presence of all *bla* operon genes. In contrast, in 34 strains that were positive for all *bla* genes, the corresponding MIC value was always $< 0.12 \, \mu g/mL$. Interestingly, this discrepancy was observed exclusively in CC8/CLB strains with the exception of one strain CC20/GTAT. For 123 trains, the MIC assay and mPCR for *bla* operon genes gave negative results.

4. Discussion

4.1. Prevalence of *adlb* in European *S. aureus* strains

Previous studies demonstrated that *S. aureus* CC8/CLB is highly contagious (13, 14) and can be detected very specifically by the qPCR assay for *adlb* (22) as also used in the present study. Indeed, with an inclusivity of 97% and exclusivity of 98%, the specificity of this test is very high (22), a fact that was recently confirmed by Gazzola et al. (42). Because of the tight association between CC8/CLB (contagious) and *adlb*, the gene turned out to be a marker for contagiousness and for high prevalence of IMI in dairy herds as shown by Sartori et al. in Swiss and by Maisano et al. in Italian dairy herds (11, 16). Based on the present results we further suggest that high staphylococcal IMI prevalence is also present in Austrian, French, and German dairy herds as *adlb* was regularly observed in the corresponding strains. Indeed, a recent examination of an Austrian and German dairy herd with high IMI prevalence caused by *S. aureus* revealed again the presence of the *adlb* gene. Whether *adlb* is the only staphylococcal marker for contagiousness and high IMI prevalence remains to be elucidated. In fact, the study by Maisano et al. demonstrated that in a small percentage of herds *adlb* was not linked to high staphylococcal IMI prevalence (16).

Interestingly, we detected the *adlb* gene in a German GTI[I] and a GTR[I] strain, genotypes that are not part of CLB/CC8. From ongoing studies, we know that the *adlb* gene is located on the staphylococcal cassette chromosome (SCC). As reviewed by Malachowa et al., SCCs may be transmitted among *S. aureus* strains by horizontal gene transfer; hence, the presence of *adlb* gene in GTI[I] and GTR[I] strains may be the result of this mechanism, with an *S. aureus* CC8/CLB most likely being the SCC donor (43).

TABLE 3 Detailed description of the isolates (*n* = 211), their genotypes, and their phenotypic (and mPCR) resistance to the tested antibiotics.

CCs	Country	Genotype cluster (CL)	GEN[a]	TOB[a]	CIP[b]	LEV[b]	MOX[b]	TEI[c]	CLI[d]	AZI[e]	ERY[e]	AMP[f]	OXA[f]	PEN[f]	TET[g]	CHL[h]	FOS[h]	FUA[h]	MOX[h]	NIT[h]	T/S[h]	*bla*
CC8 (50)	Austria	CLB (5)																				Pos (4)
		CLI (1)												1								Pos (1)
		CLOG (3)										2		2	1			1				Pos (2)
	Belgium	CLB (1)										1		1								Pos (1)
	France	CLB (2)										2		2								Pos (2)
	Germany	CLB (11)												1								Pos (8)
	Italy	CLB (9)							1			2		2	1	2						Pos (6)
	Switzerland	CLB (18)																				Pos (17)
CC97 (34)	Austria	CLR (6)										3		3								Pos (3)
		CLOG (4)										3		3								Pos (3)
	Belgium	CLI (4)							4			4		4		4						Pos (4)
	Germany	CLI (3)										2		2								Pos (2)
		CLR (2)										1		1								Pos (1)
	Ireland	CLR (2)										2		2								Pos (2)
	Italy	CLF (1)																				Neg
		CLI (1)										1		1								Pos (1)
		CLOG (1)							1			1		1	1							Pos (1)
	Macedonia	CLR (1)																				Neg
	Norway	CLR (2)																				Neg
	Slovenia	CLR (3)													1							Neg
		CLOG (3)						1								1						Neg
	Switzerland	CLR (1)																				Neg
CC705 (44)	Austria	CLC (8)																				Neg
		CLR (1)										1		1								Pos (1)
		CLOG (1)																				Neg
	Belgium	CLC (7)																				Neg

(Continued)

TABLE 3 (Continued)

CCs	Country	Genotype cluster (CL)	GEN[a]	TOB[a]	CIP[b]	LEV[b]	MOX[b]	TEI[c]	CLI[d]	AZI[e]	ERY[e]	AMP[f]	OXA[f]	PEN[f]	TET[g]	CHL[h]	FOS[h]	FUA[h]	MOX[h]	NIT[h]	T/S[h]	mPCR *bla*
	France	CLC (3)																				Neg
	Germany	CLC (3)																				Neg
	Ireland	CLC (1)																				Neg
		CLOG (1)																				Neg
	Switzerland	CLC (17)								1	1					1						Neg
		CLOG (2)																				Neg
CC20 (8)	Austria	CLF (1)							1			1		1								Pos (1)
	Belgium	CLF (1)																				Neg
	France	CLF (4)															1					Neg
	Italy	CLF (1)												1								Pos (1)
	Slovenia	CLOG (1)																				Pos (1)
CC9 (9)	Austria	CLF (4)										2		2		2						Pos (2)
		CLR (1)										1		1								Pos (1)
	Germany	CLF (2)										1		1		1						Pos (1)
	Italy	CLF (1)																				Neg
	Slovenia	CLOG (1)													1							Neg
Other CC (66)	Austria	CLB (1)																				Pos (1)
		CLC (1)																				Neg
		CLR (6)							1	1	1	1		1	1	1						Pos (1)
		CLOG (5)	1	1								1				2						Pos (1)
	Belgium	CLC (1)																				Neg
		CLI (1)										1		1								Pos (1)
		CLR (1)														1						Neg
		CLOG (2)																				Neg
	France	CLR (1)																				Neg
		CLOG (1)										1		1								Pos (1)
	Germany	CLI (1)										1		1								Pos (1)

(Continued)

TABLE 3 (Continued)

CCs	Country	Genotype cluster (CL)	GEN[a]	TOB[a]	CIP[b]	LEV[b]	MOX[b]	TEI[c]	CLI[d]	AZI[e]	ERY[e]	AMP[f]	OXA[f]	PEN[f]	TET[g]	CHL[h]	FOS[h]	FUA[h]	MOX[h]	NIT[h]	T/S[h]	*bla*
												Phenotypic results (MIC)										mPCR
		CLR (3)																				Neg
		CLOG (14)			1	1	1					5	1	5	2	1			1		1	Pos (5)
	Ireland	CLR (5)										5		5		1				3		Pos (5)
		CLOG (2)										1		1	1							Pos (1)
	Italy	CLI (1)							1													Neg
		CLOG (5)			1	1	1		1			4		4	2	1			1		1	Pos (4)
	Macedonia	CLR (1)																				Neg
		CLOG (1)										1		1								Pos (1)
	Norway	CLC (1)																				Neg
		CLOG (3)														1						Neg
	Slovenia	CLI (1)																				Neg
		CLR (2)																				Neg
		CLOG (2)														1						Neg
	Switzerland	CLC (2)																				Neg
		CLOG (2)														1						Neg
Total No.			1	1	2	2	2	1	10	2	2	51	1	53	12	20	1	1	2	3	2	88

The abbreviation used in the table for the antibiotics are listed below, and the antibiotics are categorized according to class:

[a] Aminoglycosides: GEN, gentamycin; TOB, tobramycin.

[b] Fluoroquinolones: CIP, ciprofloxacin; LEV, levofloxacin; MOX, moxifloxacin.

[c] Glycopeptides: TEI, teicoplanin.

[d] Lincosamides: CLI, clindamycin.

[e] Macrolides: AZI, azithromycin; ERY, erythromycin.

[f] Penicillins: AMP, ampicillin; OXA, oxacillin; PEN, penicillin.

[g] Tetracyclines: TET, tetracycline.

[h] CH, chloramphenicol; FOS, fosfomycin; FUA, fusic acid; NIT, nitrofurantoin; T/S, trimethoprim/sulfamethoxazole.

The different color gradient is based on the number of positive samples. One is the lowest number of positive samples (light yellow) and the highest number of positive samples is in red.

4.2. Prevalence of AMR in 10 European countries

In recent years, a general increase in AMR has been reported, and this increase is thought to mainly be due to AB misuse and abuse in agriculture (44, 45). In the worst-case scenario, this AMR could be transmitted to humans, which would aggravate the existing AMR situation faced in human medicine (29). Nevertheless, ABs continue to be a key factor in the treatment of bovine mastitis caused by *S. aureus* (11, 23, 46). Hence, it is vital to use the AB to which an isolate is fully susceptible to guarantee the successful of the therapy. According to our research, despite the large amounts of ABs that have been used to treat bovine IMIs in the past, the AMR status of *S. aureus* isolates from European mastitis cases is promising (47). In fact, all strains were susceptible to most of the 31 ABs tested. AMR was only observed for penicillin (25.6%) ampicillin (24.2%), chloramphenicol (9.5%), clindamycin (4.7%), and tetracyclines (5.7%). Penicillin, chloramphenicol, and tetracycline are ABs that have been widely used in cattle medicine over the past 50 years (48–50). These findings demonstrate and confirm previous observations that the regular use of ABs against *S. aureus* increases the possibility of the emergence of AMR (51, 52). This is in line with our observations that AMR was absent for all ABs whose application, at least in Switzerland, has not been approved for treatment of cattle (50); this is true for all the ABs on the World Health Organization (WHO) reserve list (53, 54), such as daptomycin, linezolid, and fifth-generation cephalosporins. This also holds true for most of the ABs on the WHO watch list (54) including quinolones, carbapenems, fusidic acid (one strain resistant), rifampin, teicoplanin, tobramycin (one strain resistant), and vancomycin; the exceptions were the very limited macrolide (0.9%) and tetracycline (5.7%) resistance. Interestingly, all strains were susceptible to oxacillin and all (except two strains) were susceptible to gentamicin and to trimethoprim/sulfamethoxazole. Obviously, these ABs are still efficient despite their extensive use in cattle medicine. In Switzerland, trimethoprim/sulfamethoxazole is exclusively used as a systemic treatment and is not applied intramammarily (55), so IMI-associated *S. aureus* strains are not in direct contact with this AB, which explain their susceptibility. This contrasts with oxacillin and gentamicin, which have been widely used for the treatment of IMIs in the past 40 years. The minimal AMR prevalence for these AB in bovine *S. aureus* demonstrates that the occurrence of AMRs is not only a matter of frequent use (penicillin and tetracycline). But that it considerably depends on the AB class (aminoglycosides) and even on the properties of the individual compound (oxacillin and penicillin). Considering MRSA, the present study and the one by El Garch co-authors (47) show that MRSA are of no to little concern in the field of bovine mastitis. These observations are in clear contrast to the situation in Swiss human isolates, where the prevalence of MRSA is 6.6% (56). These findings largely suggest that bovine mastitis isolates are not the source of MRSA at the human level.

With a prevalence of 25.6%, penicillin resistance was the most frequently observed type of AMR in our study. This finding aligns with the results of another European study (25.5%) (47) and of an international study (19.4%) that included strains from South America (Argentina, Brazil, and Colombia), South Africa, and the USA (57). Penicillin was introduced for the treatment of bovine mastitis as early as 1945 (58) and is still considered the AB of choice to treat Gram-positive mastitis pathogens (29), which demonstrates its importance in modern medicine.

It is worth noting that resistance to penicillin in bovine *S. aureus* strains can be misreported, as recently shown by Ivanovic et al. (33). Using whole genome sequencing and bioinformatics, the authors showed that the MIC assay, which was also used in the present study, provided the correct results, while analyses conducted using disk diffusion and PCR methods were remarkably flawed (33). Depending on the protocol applied, either too many false negative or false positive results were generated, and false positive results were also generated when the mPCR method was used to assed the *bla* operon genes (*blaI*, *blaR1*, *blaZ*). In the case of mPCR, it turned out that the discrepant results were always associated with *S. aureus* CC8/CLB strains. Further genomic analyses of these strains showed that the promoter of the *bla* operon present in the plasmid of the *S. aureus* CC8/CLB strains was inactivated by a 31-bp deletion (33); consequently, the *bla* operon genes that mediate penicillin resistance, were no longer expressed but could be detected by mPCR. The same association, which was explicit for the CC8/CLB strain, between negative MIC values and positive mPCR results was confirmed in the present study. Compared to the previous study (33), however, considerably more strains were evaluated here.

The present study further revealed two more very relevant findings. First, for the three major CCs (CC8, CC97, and CC705) and CLs (CLB, CLC, and CLR), penicillin resistance was highly dependent on the CC and CL. In fact, the CC705 and CLC strains were always susceptible to penicillin whereas penicillin resistance in the CC97 and CLR strains was high, at 50 and 37%, respectively. Penicillin resistance in the CC8 and CLB strains was intermediate, at 14 and 13%, respectively. Importantly, the CC705 and CLC strains were not only susceptible to penicillin but, with two exceptions, also to all other ABs, a property that was not observed for strains in the other CCs and CLs. Second, the prevalence of penicillin resistance is country dependent. Indeed, resistance to penicillin was particularly observed in strains from Austria, Belgium, Germany, and Ireland; however, it was completely absent in strains from Slovenia and Switzerland. It is likely that resistance to other ABs (i.e., chloramphenicol and tetracycline) is also country dependent, although this could not be assessed in the present study because the rate of resistance of other ABs were low and the data set was too small for statistical analyses. Unfortunately, the reason for the difference in penicillin resistance among countries remains unknown and requires further clinical and epidemiological investigations. Nevertheless, our findings demonstrate at least for penicillin, that the prevalence of AMR is country dependent and that caution is required when interpreting results. However, from a statistical and interpretative perspective there are no concerns about analyzing data from multiple-countries as a single entity. In our case, this means that, except for penicillin resistance, the observed prevalence of AMR reflects that at the European level.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1154550/full#supplementary-material

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7. Curriculum vitae

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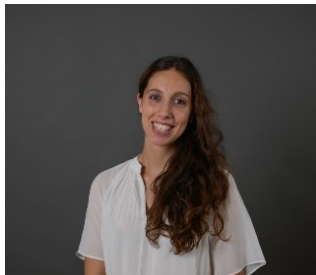
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<u>Other activities</u>

Student member of the **NMC**, National Mastitis Council; Award NMC Scholar 2023.
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Research Presentations

<u>Internal presentations of research:</u>

13.06.2018 Flash presentation: Analysis of the bovine intramammary resistome, the horizontal transfer of antibiotics resistance genes, and of the bacterial transmission during herd sanitation of Staphylococcus aureus GTB, Agroscope PhD and Post Doc Symposium 2018. Agroscope Reckenhölz.

12.10.2020 Presentation: Analysis of bovine intramammary resistome and of the bacterial transmission within dairy herds, Agroscope PhD Student & PostDoc Colloquium "evil & good microbiomes", Agroscope of Liebefeld.

28.01.2021 Poster: Analysis of bovine intramammary resistome and of the bacterial transmission within dairy herds, GCB Symposium 2021 (Online)

18.03.2021 Short presentation: Analysis of bovine intramammary resistome and of the bacterial transmission within dairy herds, Agroscope PhD and PostDoc Symposium 2021 (Online)

9.12.2021 Presentation: Analysis of bovine intramammary resistome and of the bacterial transmission within dairy herds, Riunione strategica progetto di risanamento SAGB, Bellinzona

27.01.2022 Flash presentation: Analysis of bovine intramammary resistome and of the bacterial transmission within dairy herds, GCB Symposium 2022 (Online).

02.06.2022 Presentation: Analysis of bovine intramammary bacteriome and resistome, Scientific Minisymposium "University Wageningen meets Agroscope", Agroscope of Liebefeld

13.10.2022 Presentation: Analysis of bovine intramammary bacteriome and resistome, Agroscope PhD and PostDoc symposium, Agroscope of Changins

01.12.2022 Presentation: Analysis of bovine intramammary bacteriome and resistome, Elucidation of the Bovine Intrammamary bacteriome and resistome from healthy cows of Swiss dairy farms in the Canton Tessin, TVL webinar, University of Zurich (Online)

27.02.2023 Presentation: Analysis of bovine intramammary resistome and of the bacterial transmission within dairy herds, Wissenstransfer, BLV, Liebefeld

16.05.2023 Presentation; Analysis of the intra-mammary bacteriome and resistome of healthy dairy cows, Agroscope Bioinformatics Symposium, Agroscope Reckenholz

Presentations at national meetings:

30.07.2021 Presentation: Analysis of the bovine intramammary resistome and of bacterial transmission within dairy herds, ASM Young Researcher's Symposium Switzerland (Online).

08.07.2022 Poster: Analysis of bovine intramammary bacteriome and resistome, 2nd ASM Swiss Young Microbiologist Symposium, Tiespital, University of Zurich.

Presentations at international meetings:

14-16th May 2019 Poster: The intramammary bacteriome of herds positive and negative for Staph. aureus genotype B IDF Mastitis Conference, Copenhagen, Denmark.

2-24th June 2021, Poster: Analysis of Bovine Intramammary Resistome and of Bacterial Transmission Within Dairy Herds, World Microbe Forum, ASM and FEMS Collaboration (Online Worldwide).

1-3rd February 2022, Poster: Analysis of Bovine Intramammary Resistome and of Bacterial Transmission Within Dairy Herds, National Mastitis Council Annual Meeting, San Diego, California, USA.

9-13th June 2022, Poster: Analysis of bovine intramammary bacteriome and resistome, ASM MICROBE, Washington, Columbia, USA.

9-13th June 2022, Poster: Staphylococcus aureus adlb gene is a major marker of contagiousness in bovine intramammary infections, ASM MICROBE, Washington, Columbia, USA.

30th of January-2nd February, Poster: Phenotypic: Genomic, and Phylogenetic Analyses of Mammaliicoccus sciuri Isolated from Bovine Milk and Environmental Samples of Swiss Dairy Herds, National Mastitis Council Annual Meeting, Atlanta, Georgia, USA.

8. List of Publications

Romanò A, Ivanovic I, Segessemann T, Vazquez Rojo L, Widmer J, Egger L, Dreier M, Sesso L, Vaccani M, Schuler M, Frei D, Frey J E, Ahrens C H, Steiner A, Graber H U (2023) Elucidation of the Bovine Intramammary Bacteriome and Resistome from healthy cows of Swiss dairy farms in the Canton Tessin, Frontiers in Microbiology, Front. Microbiol. 14:1183018. doi: 10.3389/fmicb.2023.1183018.

Nemati G*, **Romanó A***, Wahl F, Berger T, Rojo LV and Graber HU (2023) Short communication: Bovine *Staphylococcus aureus*: a European study of contagiousness and antimicrobial resistance. Front. Vet. Sci. 10:1154550. doi: 10.3389/fvets.2023.1154550. *= sharing co-first authorship.

Maisano AM, Luini M, Gazzola A, Sala L, Vezzoli F, Bertocchi L, Lorenzi V, Cremonesi P, Castiglioni B, Bergagna S, Romano A, Scaltriti E, Bolzoni L, Ivanovic I, **Romanò A**, Graber HU. (2023) Staphylococcus aureus adlb gene is associated with high prevalence of intramammary infection in dairy herds of northern Italy: A cross sectional study. J Dairy Sci. 2023 Mar 10:S0022-0302(23)00115-7. doi: 10.3168/jds.2022-22496.

Ivanovic I, Boss R, **Romanò A**, Guédon E, Le-Loir Y, Luini M, Graber HU. (2023) Penicillin resistance in bovine *Staphylococcus aureus*: Genomic evaluation of the discrepancy between phenotypic and molecular test methods. J Dairy Sci. Jan;106(1):462-475. doi:10.3168/jds.2022-22158.

Cremonesi P, Severgnini M, **Romanò A**, Sala L, Luini M, Castiglioni B. (2021) Bovine milk microbiota: comparison among three different DNA extraction protocols to identify a better approach for bacterial analysis. Microbiol Spectr 9:e00374-21. https://doi.org/10.1128/Spectrum.00374-21.

Romanò A, Gazzola A, Bianchini V, Cortimiglia C, Maisano AM, Cremonesi P, Graber HU, Vezzoli F and Luini M (2020) *Staphylococcus aureus* From Goats Are Genetically Heterogeneous and Distinct to Bovine Ones. Front. Vet. Sci. 7:628. doi: 10.3389/fvets.2020.00628.

Gazzola A, Maisano AM, Bianchini V, Vezzoli F, **Romanò A**, Graber HU, Cremonesi P, Zanardi G, Cappa V, Luini M. (2020) Short communication: Characterization of *Staphylococcus aureus* from bulk tank milk of dairy cattle in Lombardy (northern Italy). J Dairy Sci. Mar;103(3):2685-2692. doi: 10.3168/jds.2019-17181.

Maisano AM, Luini M, Lorenzi V, Bolzoni L, **Romanò A**, Spelta C, Angelucci A, Fusi F, Ferrara G, Vezzoli F, Bertocchi L. (2019) Diagnostic value of composite milk sample vs single

quarter milk sample for the detection of *Staphylococcus aureus* intra-mammary infections in cattle. Prev Vet Med. Jun 1;167:80-84. doi: 10.1016/j.prevetmed.2019.03.026.

Calonzi D, **Romanò A**, Monistero V, Moroni P, Luini MV, Biscarini F, Castiglioni B, Cremonesi P. (2020) Technical note: Development of multiplex PCR assays for the Molecular characterization of *Streptococcus uberis* strains isolated from bovine mastitis. J Dairy Sci. Jan;103(1):915-921. doi: 10.3168/jds.2019-16823.

Maisano, A.M., **Romano' A**., Maraschi F., Morandi S., Orlandelli C., Spelta C., Vezzoli F., Bertocchi L., Luini M. (2018) Indagini epidemiologiche, biomolecolari e di antibiotico-resistenza in aziende di bovine da latte con infezione da *Streptococcus agalactiae*, Large Animal Review 2018, 24, 11-18.

M. Ben Said, M.S. Abbassi, V. Bianchini, S. Sghaier, P. Cremonesi, **A. Romanò**, V. Gualdi, A. Hassen and M.V. Luini. (2016) Genetic characterization and antimicrobial resistance of *Staphylococcus aureus* isolated from bovine milk in Tunisia, Letters in Applied Microbiology 63, 473-481, 2016.

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Declaration of Originality

Last name, first name: Romano' Alicia

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I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.

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I am aware that in case of non-compliance, the Senate is entitled to withdraw the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 69, of 7 June 2011.

Place, date

Bern, 07.08.2023

Signature