Species identification by MALDI-TOF MS and gap PCR–RFLP of non-aureus Staphylococcus, Mammaliicoccus, and Streptococcus spp. associated with sheep and goat mastitis

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Abstract
Staphylococci and streptococci are common causes of intramammary infection in small ruminants, and reliable species identification is crucial for understanding epidemiology and impact on animal health and welfare. We applied MALDI-TOF MS and gap PCR–RFLP to 204 non-aureus staphylococci (NAS) and mammaliicocci (NASM) and to 57 streptococci isolated from the milk of sheep and goats with mastitis. The top identified NAS was Staphylococcus epidermidis (28.9%) followed by Staph. chromogenes (27.9%), haemolyticus (15.7%), caprae, and simulans (6.4% each), according to both methods (agreement rate, AR, 100%). By MALDI-TOF MS, 13.2% were Staph. microti (2.9%), xylosus (2.0%), equorum, petrassi and warneri (1.5% each), Staph. sciu (now Mammaliicoccus sciu, 1.0%), arlettae, capitis, cohni, lentus (now M. lentus), pseudintermedius, succinus (0.5% each), and 3 isolates (1.5%) were not identified. PCR–RFLP showed 100% AR for Staph. equorum, warneri, arlettae, capitis, and pseudintermedius, 50% for Staph. xylosus, and 0% for the remaining NASM. The top identified streptococcus was Streptococcus uberis (89.5%), followed by Strept. dysgalactiae and parauberis (3.5% each) and by Strept. galolyticus (1.8%) according to both methods (AR 100%). Only one isolate was identified as a different species by MALDI-TOF MS and PCR–RFLP. In conclusion, MALDI-TOF MS and PCR–RFLP showed a high level of agreement in the identification of the most prevalent NAS and streptococci causing small ruminant mastitis. Therefore, gap PCR–RFLP can represent a good identification alternative when MALDI-TOF MS is not available. Nevertheless, some issues remain for Staph. haemolyticus, minor NAS species including Staph. microti, and species of the novel genus Mammaliicoccus.

Keywords: Small ruminant, milk, coagulase-negative staphylococci, streptococci, mammaliicocci, NAS, NASM, species identification, Gap gene

Introduction
Mastitis is one of the most common and costly diseases affecting dairy sheep and goats. Therefore, monitoring and maintaining udder health is critical for small ruminant welfare and dairy production yield and quality [1, 2]. Infectious mastitis outbreaks can be caused by a wide variety of bacterial species, including the genera Staphylococcus (Staph.) and Streptococcus (Strep.) [2,
Among *Staphylococcus* spp., non-*aureus* staphylococci (NAS) are the most prevalent and cause mainly subclinical intramammary infection (IMI) in both ewes and goats, leading to significant economic losses and reduced animal welfare [4, 5]. Recently, based on 16S rRNA sequences, five NAS species were reclassified and assigned to the novel genus *Mammi licoccus* with *Mammi licoccus sciuri* as the type species [6]. These species are collectively indicated by the acronym NASM. The large NASM bacterial group includes numerous species with different prevalence and epidemiology [2, 3, 7]. From studies on bovine subclinical mastitis, it is clear that some species are more associated with IMI, subclinical mastitis and somatic cell count (SCC) increase than others, which are instead more related to the farm environment or the mammary gland microbiota [6, 8–10]. Obtaining precise information on the species of NASM causing IMI and mastitis is therefore crucial for understanding the epidemiology and the respective roles in mammary gland health and disease in order to make more appropriate management decisions when these bacteria are isolated from the milk of small ruminants [7, 11].

In the microbiological laboratory, staphylococci and streptococci isolated from milk samples are mainly identified at the species level by means of biochemical tests or commercial biochemical galleries. However, these methods often fail in correctly identifying bacterial species of veterinary interest, also because they have been optimized for the strains associated with human infections [12–15]. In the last decade, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a fast and accurate microbial identification approach [16] and is being successfully applied to bacteria isolated from bovine milk [17–19]. However, there are scarce reports of its performance in the identification of bacteria isolated from small ruminant milk, especially concerning staphylococci and sheep. Identification methods based on molecular rather than phenotypic characteristics can also provide a reliable alternative. Accordingly, the Istituto Zooprofilar tico Sperimentale della Sardegna (IZSSA) has developed, comparatively assessed, and implemented a PCR-restriction fragment length polymorphism (PCR–RFLP) method based on PCR amplification of the *gap* gene followed by *AluI* enzyme digestion for the identification of staphylococci and streptococci isolated from routine diagnosis [12, 13, 20].

In this study, we applied MALDI-TOF MS and *gap* PCR–RFLP for the species identification of staphylococci and streptococci isolated from the milk of sheep and goats with mastitis. Then, we compared their respective results to identify potential issues and solutions. Bacterial isolates were collected in Sardinia, the region with the largest small ruminant population in Italy.

**Materials and methods**

**Bacterial isolates**

From January 2021 to May 2022, 204 NASM and 57 *Streptococcus* spp. were isolated from sheep and goat milk samples that routinely arrive at the IZSSA laboratories for identification of the IMI agent. Milk samples are accompanied by a form with a checkbox indicating the presence of clinical mastitis/visible milk alterations to be selected by the farm veterinarian. Only isolates derived from samples with these characteristics were included in the study. Milk samples were cultured following the standard procedures provided by the IZSSA. Briefly, 10 µL of milk were seeded in 5% sheep blood agar, incubated at 37 °C, and evaluated at 24 and 48 h. Upon growth of more than one morphologically different bacterial colony, identifications were not performed, and the sample was classified as “mixed bacterial flora”. Colonies were re-isolated in blood agar and examined with Gram stain, catalase, and coagulase tests to discriminate between staphylococci and streptococci. All isolates were stored at −20 °C in Brain Heart Infusion broth (BHI, Beckton-Dickinson, Sparks, MD, USA) containing 20% glycerol, until further investigation. Only one NASM or streptococcus isolate was selected from each farm for identification by MALDI-TOF MS and PCR–RFLP, for a total of 261 non-duplicate isolates. A geographic map indicating the site of collection of each isolate in the different provinces of Sardinia was created with the software Microsoft Power BI using the farm code of each flock. The geo-referenced coordinates were extracted from the Banca Dati Nazionale (BDN) of the Italian Health Ministry. Additional file 1 illustrates the animal species originating the isolates and their geographical distribution.

**MALDI-TOF MS for bacterial identification**

At the IZSSA, all bacterial isolates were retrieved from the frozen archives, seeded in 5% sheep blood agar, and incubated at 37 °C. All isolates were passaged twice in the solid medium before identification at 24 h of growth. For MALDI-TOF MS analysis, the direct colony transfer protocol was applied. A small amount of an isolated colony was deposited in duplicate wells of disposable target plates using a toothpick, overlaid with 1 µL of α-cyano-4-hydroxycinnamic acid (HCCA) solution in 50% acetonitrile, 47.5% water, 2.5% trifluoroacetic acid (Bruker Daltonik GmbH, Bremen, Germany), and left to dry. The target plates were prepared at the IZZSA in the afternoon, placed in an empty disposable target container, once dry, and transported at room temperature to the animal infectious diseases laboratory at the University
of Milan (MILab). In the morning of the following day, i.e. within 24 h as recommended by the MALDI Biotyper™ (MBT) Bruker user manual, the mass spectra were acquired with the MBT Microflex LT/SH MALDI-TOF mass spectrometer (Bruker Daltonik GmbH) in the positive mode. Each target plate included two spots of bacteria. Two sets of 3893 species/entries were obtained during the course of this study. The obtained spectra were interpreted against the MBT Compass® Library Revision H (2021), covering 3893 species/entries. The Staphylococcus and Streptococcus species included in this library revision are listed in Additional file 2. The two Mammallicoccus species M. sciuri and M. lentus were still reported as Staph. sciuri and Staph. lentus in the library revision available at the time of this study. The following similarity log score thresholds were considered [16]: a log score ≥ 2.0 indicated a reliable species level identification, while a log score between 1.7 and 2.0 indicated a presumptive species level identification. Identifications with log scores below 1.7 were considered unreliable. All samples producing scores below 1.7 were processed again with the direct transfer, extended direct transfer, and protein extraction procedures. Specifically, for the extended direct transfer procedure, after depositing a small amount of the isolated colony in duplicate wells of the disposable target plate using a toothpick, the sample was overlaid with 1 µL of 70% formic acid and left to dry before adding the HCCA matrix solution for the protein extraction procedure, bacteria from 4 isolated colonies were deposited into 300 µL of HPLC-grade water and vortexed. Then, 900 µL of ethanol absolute were added, the tube was vortexed again, and centrifuged for 2 min at maximum speed in a microcentrifuge. The supernatant was removed carefully, and the pellet was left to dry for 10 min at room temperature. Once dried, the pellet was thoroughly resuspended in 25 µL of 70% formic acid, 25 µL of acetonitrile were added, and the suspension was mixed by pipetting. The sample was centrifuged as above, and 1 µL of supernatant was deposited on the MALDI target and left to dry before overlaying with the HCCA matrix.

**DNA extraction and PCR amplification**

At the IZSSA, genomic DNA was extracted from all 261 isolates and Type/Reference Strains (T/RS) as described previously [12]. Species identification was based on PCR amplification of the glyceraldehyde-3-phosphate dehydrogenase gene (gap) gene [20, 21]. The primers GF-1 (5'-ATGGTTTTTGGTAGAATTTGCTGTTTA-3') and GR-2 (5'-GACATTTCGTTATCATACCAAGCTG-3') were used for staphylococci whereas the primers Strept-gap-F (5'-ACTCAAAGTGTAGCAACAGAT-3') and Strept-gap-R (5'-GTCTTGCATTCCGTCGTAT-3') were used for streptococci. PCR was performed in a reaction mixture containing 2.5 µl 10× reaction buffer, 1.5 µl dNTPs 1.25 mM, 1 µl of each primer (25 pmol each), 1 µl DNA template, 0.5 µl Fast Taq (Roche, Basel, Switzerland), and distilled water up to 25 µl. Reactions were carried out in an automated DNA thermal cycler (GeneAmp 9700, Applied Biosystems, CA, USA). Amplification conditions: initial denaturation for 5 min at 95 °C followed by 30 cycles of 1 min at 95 °C, 1 min at 50 °C (for staphylococci)/1 min at 54 °C (for streptococci), and 1 min at 72 °C with a final extension step of 10 min at 72 °C. The 933-bp (staphylococci) and 945-bp (streptococci) amplicons were examined by electrophoresis in 1% agarose gels, stained with Sybr® Safe DNA gel stain (Invitrogen, CA, USA), and visualized under a UV transilluminator.

**Restriction fragment length polymorphism (RFLP) analysis**

Fifteen microliters of both PCR amplifications were digested in a 30 µl volume containing 10× FastDigest Green buffer, 0.25 µl of 20 mg/ml acetylated BSA, and 1 µl of FastDigest AluI enzyme (Thermo Scientific, CA, USA). Reaction mixtures were incubated at 37 °C for 15 min and directly loaded on the precast gels. Twenty microliters of digested gap amplifications were loaded in 12% Bis–Tris NuPAGE™ gels (Invitrogen) and then electrophoresed in a vertical gel apparatus. For staphylococci, the following 14 T/RS were used as reference strains for restriction pattern comparison: *Staph. epidermidis* ATCC 35983, *Staph. xylosus* ATCC 29971 T, *Staph. saprophyticus* ATCC 15305 T, *Staph. capitis* ATCC 27840 T, *Staph. haemolyticus* ATCC 29970 T, *Staph. simulans* ATCC 27848 T, *Staph. warneri* ATCC 27836 T, *Staph. arlettae* ATCC 43957 T, *Staph. chromogenes* ATCC 43764 T, *Staph. equorum* ATCC 43958 T, *Staph. caprae* ATCC 35538 T, *Staph. sciuri* ATCC 29062 T, *Staph. hycus* ATCC 11249 T, and *Staph. intermedius* ATCC 29663 T. The gap PCR–RFLP patterns used for *Staphylococcus* species assignment are illustrated in Additional file 3. For streptococci, the following 7 T/RS were used as reference strains for restriction pattern comparison: *Strep. uberis* ATCC 700407, *Strep. dysgalactiae* subsp. *dysgalactiae* ATCC 43078 T, *Strep. dysgalactiae* subsp. *equisimilis* DSM 23147 T, *Strep. agalactiae* ATCC 13813 T, *Strep. gallolyticus* subsp. *gallolyticus* ATCC 49475, *Strep. equi* subsp. *zoepidermicus* NCTC 6180, and *Strep. suis* ATCC 43765. The gap PCR–RFLP patterns used for *Streptococcus* species assignment are illustrated in Additional file 4. All reference strain species identities were confirmed by MALDI-TOF MS.

**Amplicon sequencing**

The gap gene amplicons of all isolates with PCR–RFLP profiles different from those of *Staphylococcus* and *Streptococcus* T/RS were sequenced at BMR Genomics with...
the Sanger sequencing option. The nucleotide sequences were compared to sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST).

**Comparison of PCR–RFLP and MALDI-TOF MS results**

All the data related to MALDI-TOF MS results, log scores, PCR–RFLP identification, gap gene sequencing results, and animal species, were plotted with Microsoft Excel for calculation of agreement ratio (AR), score distribution, and relative percentage values. Plots were generated with Microsoft Excel.

**Results**

**Isolates and geographical distribution.**

From January 2021 to May 2022, 204 NASM and 57 streptococci, for a total of 261 isolates, were obtained from sheep and goat milk samples sent to the IZSSA for microbiological analysis with a diagnosis of clinical mastitis/visible milk alterations by the farm veterinarian. Ovine isolates were 246, of which 191 NASM and 55 streptococci. Caprine isolates were 15, of which 13 NASM and 2 streptococci (Additional file 5).

**MALDI-TOF MS of NASM isolates**

All 204 isolates typed as NASM by culture and primary biochemical tests were subjected to MALDI-TOF MS identification by processing two spots per colony with the direct transfer procedure after 24 h of bacterial growth. This enabled the successful species identification of 201 isolates (98.5%), with log scores $\geq 2.00$ in 165 (80.9%) and between 1.70–1.99 in 36 (17.6%). For the remaining 3 isolates (1.5%), log scores were $< 1.70$ with no identification possible even after a second round of identification with the direct transfer, extended direct transfer, and protein extraction procedures (Table 1).

The most frequently identified species were *Staph. epidermidis* (59, 28.9%), *Staph. chromogenes* (57, 27.9%), *Staph. haemolyticus* (32, 15.7%), *Staph. caprae* (13, 6.4%), and *Staph. simulans* (13, 6.4%), accounting for 85.3% of all NASM isolates. The remaining 13.2% was represented by *Staph. microti* (6, 2.9%), *Staph. xylosus* (4, 2.0%), *Staph. equorum, petrasii, and warneri* (3 each, 1.5%), *Staph. sciuiri* (now *M. sciuiri*) (2, 1.0%), and *Staph. arlettae, capitis, cohnnii, lentus* (now *M. lentus*), *pseudointermedius*, and *succinus* (1 each, 0.5%).

**MALDI-TOF MS of streptococcus isolates**

MALDI-TOF MS identification of the 57 streptococci isolates included in this study enabled the successful species identification of all isolates (100%) with log scores $\geq 2.00$ in 53 (93.0%) and between 1.7–1.99 in 4 (7.0%). The most frequently identified species was *Strep. uberis* (51, 89.5%), followed by *Strep. galgalactiae and parauberis* (2 each, 3.5%), and by *Strep. galgalyticus and suis* (1 each, 1.8%) (Table 1).

**PCR–RFLP of NASM isolates**

All 204 isolates typed as NASM by culture and primary biochemical tests were also subjected to species identification by *gap* PCR–RFLP. A total of 187 (91.7%) isolates showed restriction profiles identical to the reference strains, while 17 (8.3%) isolates showed a different PCR–RFLP pattern (Table 1). Upon *gap* gene sequencing (Additional file 6), 12 of them were identified as *Staph. chromogenes* (1), *devriesei* (1), *epidermidis* (1), *haemolyticus* (3), *hyicus* (1), *jettensis* (1), *muscae* (1), *pseudointermedius* (1), *pseudoxylosus* (1), and *simulans* (1), while 5 remaining isolates were classified as *Staph. muscae* (4) and *Staph. devriesei* (1) by matching with the isolate classified by *gap* gene sequencing (Table 2).

As a result, the top three identified species were *Staph. epidermidis* (61, 29.9%), *Staph. chromogenes* (58, 28.4%), and *Staph. haemolyticus* (37, 18.1%), followed by *Staph. simulans* (14, 6.9%) *Staph. caprae* (13, 6.4%), *Staph. muscae* (5, 2.5%), *Staph. equorum and warneri* (3, 1.5%), *Staph. devriesei* and *xylosus* (2 each, 1.0%), and *Staph. arlettae, capitis, hyicus, jettensis, pseudointermedius, and pseudoxylosus* (1 each, 0.5%).

**PCR–RFLP of streptococcus isolates**

Out of the 57 isolates typed as *Streptococcus* sp. by culture and primary biochemical tests, 53 (93.0%) showed *gap* PCR–RFLP profiles identical to the reference strains, while 4 (7.0%) did not. Upon *gap* gene sequencing (Additional file 7), these were identified as *Strep. parauberis* (2), *Strep. uberis* (1), and *Strep. ruminantium* (1) (Table 1 and Table 2). As a result, the top identified species was *Strep. uberis* (51, 89.5%), followed by *Strep. dysgalactiae* and *parauberis* (2 each, 3.5%), and by *Strep. galgalyticus and ruminantium* (1 each, 1.8%).

**Comparison of gap PCR–RFLP and MALDI-TOF MS results**

The species identification results obtained by MALDI-TOF MS and *gap* PCR–RFLP were in general agreement as detailed in Table 1 and illustrated in Figure 1.

For the 5 most frequently identified NAS (*Staph. epidermidis, chromogenes, haemolyticus, caprae, and simulans*) the agreement rate (AR) between MALDI-TOF MS and *gap* PCR–RFLP was 100%. Log scores were mostly $\geq 2.00$, although for *Staph. haemolyticus* only 56.3% of the identifications had log scores $\geq 2.00$. The AR between the two identification methods was 100% also for *Staph. equorum, warneri, arlettae, capitis, and pseudointermedius*. On the other hand, AR was 50% for *Staph. xylosus* and 0% for *Staph. microti, Staph. petrasii, Staph.
The discordant species identifications between the two approaches are reported in Table 2. Of note was the case of 6 isolates identified as *Staphylococcus microti* by MALDI-TOF MS and as *Staphylococcus muscae* (5 isolates) and *Staphylococcus simulans* (1 isolate) by gap sequencing. Among less frequently identified NASM species, only gap sequencing provided as identifications *Staphylococcus muscae*, *devriesei*, *hyicus*, *jettensis*, and *pseudoxylosus*, while only MALDI-TOF MS identifications were made for *Staphylococcus cohnii*, *Staphylococcus lentus* (now *Mammaliicoccus lentus* [6]), and *Staphylococcus succinus* (Table 1).

### Table 1 Summary of species identification results obtained by MALDI-TOF MS and gap PCR–RFLP on the 261 isolates evaluated in this study

| Species ID by MALDI-TOF MS | N. of isolates | Log score | AR with gap PCR–RFLP |
|----------------------------|---------------|-----------|----------------------|
|                            |               | ≥ 2.0 | 1.7–1.99 | < 1.7 (no ID) |
| **Staphylococci**          |               |       |          |                |
| *Staphylococcus epidermidis* | 59 (28.9%) | 55 (93.2%) | 4 (6.8%) | – | 59 (100%)² |
| *Staphylococcus chromogenes* | 57 (27.9%) | 53 (93.0%) | 4 (7.0%) | – | 57 (100%)² |
| *Staphylococcus haemolyticus* | 32 (15.7%) | 18 (56.3%) | 14 (43.7%) | – | 32 (100%)³ |
| *Staphylococcus caprae* | 13 (6.4%) | 10 (76.9%) | 3 (23.1%) | – | 13 (100%)³ |
| *Staphylococcus simulans* | 13 (6.4%) | 13 (100%) | – | – | 13 (100%)³ |
| *Staphylococcus microti* | 6 (2.9%) | 3 (50%) | 3 (50%) | – | 0 (0%)² |
| *Staphylococcus xylosus* | 4 (2.0%)⁸ | 2 (50%) | 2 (50%) | – | 2 (50%)³ |
| *Staphylococcus equorum* | 3 (1.5%) | 3 (100%) | – | – | 3 (100%)³ |
| *Staphylococcus petrasii* | 3 (1.5%) | 3 (100%) | – | – | 0 (0%)² |
| *Staphylococcus warneri* | 3 (1.5%) | 2 (66.7%) | 1 (33.3%) | – | 3 (100%)³ |
| *Staphylococcus sciuri*⁹ | 2 (1.0%) | 2 (100%) | – | – | 0 (0%)³ |
| *Staphylococcus arlettae* | 1 (0.5%) | – | 1 (100%) | – | 1 (100%)³ |
| *Staphylococcus capitis* | 1 (0.5%) | 1 (100%) | – | – | 1 (100%)³ |
| *Staphylococcus cohnii* | 1 (0.5%) | – | 1 (100%) | – | 0 (0%)² |
| *Staphylococcus lentus*⁹ | 1 (0.5%)⁹ | – | 1 (100%) | – | 0 (0%)² |
| *Staphylococcus pseudintermedius* | 1 (0.5%) | – | 1 (100%) | – | 1 (100%)³ |
| *Staphylococcus succinus* | 1 (0.5%) | – | 1 (100%) | – | 0 (0%)² |
| **Total staphylococci** | 204 | 165 (80.9%) | 36 (17.6%) | 3 (1.5%) | 185 (90.7%)³ |
| **Streptococci** | | | | | |
| *Streptococcus uberis* | 51 (89.5%) | 48 (94.1%) | 3 (5.9%) | – | 51 (100%)³ |
| *Streptococcus dysgalactiae*⁹¹⁰ | 2 (3.5%) | 2 (100%) | – | – | 2 (100%)³ |
| *Streptococcus parauberis* | 2 (3.5%) | 2 (100%) | – | – | 2 (100%)³ |
| *Streptococcus galilaeus* | 1 (1.8%) | 1 (100%) | – | – | 1 (100%)³ |
| *Streptococcus suis* | 1 (1.8%) | – | 1 (100%) | – | 0 (0%)³ |
| **Unidentified** | 3 (1.5%) | – | – | 3 (100%) | – |
| **Total streptococci** | 57 | 53 (93.0%) | 4 (7.0%) | 0 (0%) | 56 (98.2%)³ |

* Based on the Bruker MALDI Biotyper System Compass® Library Revision H (2021), covering 3893 species/entries.
* Percent data represent the proportion of a given species isolated among all staphylococci or streptococci (within column).
* Percent data represent the proportion of isolates identified with MALDI log scores ≥ 2.0 among all isolates of the same species.
* Percent data represent the proportion of isolates identified with MALDI log scores 1.7–1.99 among all isolates of the same species.
* Percent data represent the proportion of isolates with MALDI log scores < 1.7 among all isolates of the same species and no identification possible (no ID).
* Number of isolates identified as the same species by MALDI-TOF MS and gap PCR–RFLP. Percent data represent the proportion of isolates identified as the same species (Agreement Rate, AR).
* The reference strain for this species was included in the gap PCR–RFLP identification panel (please see the materials and methods section for the reference isolate list).
* These species have been reclassified in the *Mammaliicoccus* genus [6].
* For one isolate, the first round of MALDI-TOF MS identification was not successful and it was repeated.
* *Streptococcus dysgalactiae* and *Streptococcus canis* cannot be resolved by MALDI-TOF MS.
* Both assigned by gap gene amplicon sequencing and alignment with sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST). Details are reported in Additional file 7.

sciuri (now *M. sciuri*), *Staphylococcus cohnii*, *Staphylococcus lentus* (now *M. lentus*), and *Staphylococcus succinus* (Table 1).
providing Staph. microti, Staph. petrasii, Staph. sciuri (now M. sciuri), Staph. lentus (now M. lentus), and Staph. succinus.

For streptococci, the AR between MALDI-TOF MS and gap PCR–RFLP was 98.2% (56 of 57). The AR was 100% for the identification of Strept. iberis, with log scores ≥ 2.00 in 94.1% of cases. The AR was also 100% for Strept. dysgalactiae, parauberis, and gallolyticus. Only one species was identified as Strept. suis by MALDI-TOF MS (log score 1.78) and as Strept. ruminantium by gap sequencing (Table 2).

Discussion
NAS and streptococci are the most prevalent etiological agents of small ruminant mastitis [2, 3]. However, NAS, mammaliicocci, and streptococci can also be found in milk as contaminants, as well as components of the mammary gland microbiota [6, 9, 10, 22]. Obtaining a reliable species identification is therefore essential for understanding their epidemiology and roles in mammary gland health and disease in order to make more meaningful management decisions [7, 8, 23].

In the last decade, MALDI-TOF MS has emerged as a dependable method for microbial identification and is being increasingly applied to bacteria and fungi isolated from bovine milk. When MALDI-TOF MS instrumentation is available in the laboratory, this approach is rapid, cost-effective, high-throughput, reliable, and does not require specific knowledge of mass spectrometry or molecular biology. An isolated colony is sufficient for identification and results are available within minutes. Thanks to the short analytical times, a high number of targets can be processed during the day, making it possible to identify thousands of microbial isolates. A further

Table 2 Detail of the 20 discordant species identification results between MALDI-TOF MS and gap PCR–RFLP integrated with gap gene sequencing

| MALDI-TOF MSa | Log score | ID by gap PCR–RFLPb | ID by gap sequencingc (% identity)d |
|---------------|-----------|----------------------|-----------------------------------|
| Staphylococci |           |                      |                                   |
| Staph. microti | 1.99      | –                    | Staphylococcus muscae (92.79%)    |
| Staph. microti | 2.05      | –                    | Staphylococcus muscae (92.79%)    |
| Staph. microti | 2.06      | –                    | Staphylococcus muscae (92.79%)    |
| Staph. microti | 1.91      | –                    | Staphylococcus muscae (92.79%)    |
| Staph. microti | 1.76      | –                    | Staphylococcus muscae (92.79%)    |
| Staph. microti | 2.04      | –                    | Staphylococcus simulans (99.89%)  |
| Staph. petrasii | 2.1       | Staphylococcus haemolyticus | –                          |
| Staph. petrasii | 2.03      |                      | Staphylococcus haemolyticus | –                      |
| Staph. petrasii | 2.24      | –                    | Staphylococcus jettensis (99.29%)|
| Staph. sciuri | 2.23      | –                    | Staphylococcus chromogenes (98.75%)|
| Staph. sciuri | 2.16      | –                    | Staphylococcus hyicus (98.69%)    |
| Staph. xylosus | 1.88      | –                    | Staphylococcus devriesei (99.78%)|
| Staph. cohnii | 1.7h      | –                    | Staphylococcus haemolyticus (98.91%)|
| Staph. lentus | 1.8h      | –                    | Staphylococcus epidermidis (98.46%)|
| Staph. succinus | 1.93     | Staphylococcus epidermidis | –                      |
| Unidentified  | 1.6       | –                    | Staphylococcus haemolyticus (99.07%)|
| Unidentified  | 1.28      | –                    | Staphylococcus haemolyticus (98.26%)|
| Unidentified  | 1.44      | –                    | Staphylococcus devriesei (99.77%)|
| Streptococci  |           |                      |                                   |
| Strept. suis  | 1.78      | –                    | Streptococcus ruminantium (98.15%)|

a Based on the Bruker MALDI Biotyper System Compass® Library Revision H (2021), covering 3893 species/entries.
b Species identification was assigned by matching the enzyme digestion pattern of the gap gene amplicon with the reference strain.
c All amplicons producing an enzyme digestion pattern different than the reference strains were subjected to genomic sequencing for species identification.
d Percent identity of the gap gene amplicon with sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST). Details are reported in Additional files 6 and 7.
e Based on the restriction pattern of the Staphylococcus muscae isolate identified by gap sequencing.
f These species have been reclassified in the Mammaliicoccus genus [6].
g Based on the restriction pattern of the Staphylococcus devriesei isolate identified by gap sequencing.
h The first round of MALDI-TOF MS identification was not successful and it was repeated.
advantage of MALDI-TOF MS is the possibility of creating personalized spectrum libraries including isolates of specific interest, thereby improving bacterial identification reliability [24].

In the last decade, the IZSSA has developed and applied in its diagnostic routine a gap PCR–RFLP method enabling the identification of NAS and streptococci isolated from small ruminant milk [12, 13, 20]. This approach is reliable but moderately expensive in terms of materials and labor as it requires DNA extraction, PCR amplification, amplicon digestion, electrophoresis, and result interpretation by comparison with RFLP profiles from reference strains. The restricted number of reference strains is also a limitation, and it may eventually require gap gene amplicon sequencing for a tentative identification by homology. However, gap PCR–RFLP may represent a valuable alternative when MALDI-TOF instrumentation is not available. In fact, in spite of the low cost per test, the mass spectrometer acquisition costs are still too high for many veterinary diagnostic facilities, especially in low-resource contexts or in peripheral laboratories. As a further consideration, scarce data are available in the literature on the application of MALDI-TOF MS identification to the microorganisms isolated from small ruminant milk, especially concerning NAS and sheep.

In this work, we carried out the identification of NASM and streptococci isolated from the milk of small ruminants with mastitis by MALDI-TOF MS and gap PCR–RFLP integrated with amplicon sequencing. As a result, the species most frequently identified in our study were in line with those reported as causing small ruminant IMI worldwide [11, 25], that is *Staphylococcus epidermidis*, *Chromogenes haemolyticus*, *Caprae* and *Simulans* for NAS, and *Streptococcus uberis* for streptococci. Notably, the two identification approaches showed a very high level of agreement, even for MALDI-TOF MS identifications with log scores ≥ 1.7. A cutoff log score ≥ 1.7 for species identification has been validated by various authors [19, 26, 27] as highly appropriate and accurate for bovine NAS. Han et al. [27] found that the ≥ 1.7 log score threshold enabled to reach a significantly higher level of NAS species identification without sacrificing specificity. Cameron et al. [28] also found that the reduction of the species level cutoff improved method performance from 64 to 92% when classifying bovine-associated NAS isolates. In the more recent study by Conesa et al. [19] the ≥ 1.7 score made it possible to successfully identify 36 more strains, as validated by the comparison with genotypic methods. Based on our results, a log score threshold of ≥ 1.7 could be considered reliable also for the most prevalent small ruminant NAS.
For *Staph. haemolyticus*, however, the log scores were generally lower, as 43.7% of isolates had values \(< 2.0\). Notably, 2 isolates were identified as *Staph. haemolyticus* by PCR–RFLP and as *Staph. petrasii* by MALDI-TOF MS with log scores \(> 2.0\). Moreover, the gap gene of three isolates showed a very high sequence identity to *Staph. haemolyticus* but these were either identified as *Staph. cohnii* with a very low log score (1.7) or were not identified by MALDI-TOF MS. In our routine work, *Staph. haemolyticus* isolated from cow milk is also typically identified with lower scores among the most prevalent *Staphylococcus* spp. (M.F.A., personal communication). *Staph. haemolyticus* may be more problematic to identify because of a higher genomic variability and similarity of marker genes with other species. Wanecka et al. [14] found that 27 of 33 of their *Staph. haemolyticus* isolates had 99.5–100% similarity of the 16S rRNA gene with *Staph. petrasii* subsp. *jettensis* (now *Staph. petrasii* subsp. *petrasii*), *Staph. hominis*, *Staph. epidermidis*, or *Staph. devriesii*. Also recently, some *Staph. haemolyticus* have been reclassified as *Staph. borealis* [29]. Therefore, molecular techniques may have limitations including insufficient discriminatory power in the case of closely related species or the lack of quality of sequences deposited in the GenBank [30], as also observed in this work for gap gene sequencing. On the other hand, the absence of reference spectra for these highly similar minor species in the MALDI-TOF MS database might be the reason for their identification as *Staph. haemolyticus* with lower log scores.

All the 6 isolates identified as *Staph. microti* by MALDI-TOF MS could not be identified by gap PCR–RFLP. The gap gene sequence showed the highest identity with *Staph. muscae* (5 out of 6) and *Staph. simulans* (1 out of 6). *Staph. microti* has been described for the first time by Nováková et al. in 2010 [31] as an isolate from *Microtus arvalis*, the common vole, with *Staph. muscae* as the nearest relative. Its report as a staphyloccocal species associated with mastitis in bovine and bubaline cows is increasing in association with the implementation of MALDI-TOF MS for NAS identification [18, 32, 33]. However, in consideration of the high similarity between these species, as well as with other closely related species not included in the Bruker spectrum library such as *Staph. rostri*, this will require further evaluation, possibly followed by spectrum library integration.

An isolate identified as *Staph. petrasii* by MALDI-TOF MS with log score \(\geq 2.0\) was identified as *Staph. jettensis* upon gap gene sequencing. The species description of *Staph. petrasii* has been emended, and *Staph. jettensis* should be reclassified as a novel subspecies within *Staph. petrasii* for which the name *Staphylococcus petrasii* subsp. *jettensis* subsp. nov [34]. As discussed above, the identification of this NAS as *Staph. jettensis* by gap gene sequencing may have resulted from matching with GenBank sequences that were not updated following taxonomic reclassification.

In 2020, five NAS species were proposed to be reclassified to the novel *Mammaliicoccus* genus, including *Staph. sciuri* and *Staph. lentus*, with *M. sciuri* as the type species [6]. The same authors proposed the reclassification of *Staph. cohnii* subsp. *urealyticus* as the novel species *Staph. urealyticus*. The *Mammaliicoccus* genus is not included in the current release of the Bruker library. Therefore, this should be considered when these species are identified by MALDI-TOF MS with the MBT System and the commercial spectrum library release available at the time of this study.

Concerning the two isolates identified as *Staph. devriesii* by gap gene sequencing and PCR–RFLP, one was not identified by MALDI-TOF MS, while the other was identified as *Staph. xylosus* with a score \(< 2.00\). Moreover, one isolate identified as *Staph. hyicus* by gap gene sequencing was identified as *Staph. sciuri* by MALDI-TOF MS with score \(\geq 2.00\). *Staph. devriesii* falls in the *Staph. haemolyticus* group [11], and *Staph. sciuri* is also reported as belonging to a separate group than *Staph. hyicus*. Nevertheless, *Staph. hyicus* can be difficult to differentiate from *Staph. agnetis* [35] and the latter species was not present in the MALDI-TOF MS spectrum database.

An isolate was identified as *Staph. xylosus* by MALDI-TOF MS after a second round of identification with score 1.81. The same isolate was identified as *Staph. pseudoxys-losus* by gap gene sequencing; however, this species was not included in the spectrum library. Analogously, no *Strep. ruminantium* spectra were present. However, for this latter species a presumptive identification as *Strep. suis* was provided, with score \(\geq 1.7\). *Strep. ruminantium* is indeed a new species of the *suis* group. *Strep. suis* includes 35 serotypes, of which 6 have been re-classified to other bacterial species [36]. Among them, *Strep. suis* serotype 33 has been recently classified as a new species, *Streptococcus ruminantium* [37], of which the reference strain was originally isolated from a lamb. As the two species are biochemically very similar, differentiation is difficult and a PCR specific for *Strep. ruminantium* has recently been described [38]. All other streptococci showed a very high agreement rate; therefore, both MALDI-TOF MS and PCR–RFLP enable to overcome the known difficulties in the identification of Gram-positive, catalase-negative cocci by biochemical reactions [39, 40] also with streptococci isolated from small ruminant milk.

As a further consideration, in this work we carried out microbial cultivation and target preparation at the IZSSA in Sassari, Sardinia, and MALDI-TOF MS
identification at the University of Milan in Lodi, Lombardy, on the following day. According to the Bruker MBT User Manual, result reliability is maintained if spectra are generated within 24 h from target preparation. Therefore, this opens the possibility that these may be prepared in one laboratory and sent to a shared core facility for identification within the following day, provided that convenient logistic solutions are in place. This approach would enable to contain instrumental costs while centralizing the mass spectrometry instrumentation and the spectrum library, and it might also represent a reasonable alternative to the gap gene sequencing to integrate the PCR–RFLP identification approach. If planning such a setup, however, it would be advisable to thoroughly assess the impact of target transportation temperatures and conditions on reliability of the MALDI-TOF MS identification results.

In conclusion, our results concerning the species of NASM and streptococci associated with sheep and goat mastitis were in line with those reported as causing small ruminant IMI worldwide [11, 25]. For the most prevalent species, MALDI-TOF MS and gap PCR–RFLP provided comparable results. Therefore, gap PCR–RFLP can offer a reliable identification alternative when MALDI-TOF MS is not available, but restriction profiles differing from the validated reference isolates [12, 13, 20] may not be easily resolved by gap gene sequencing. Concerning MALDI-TOF MS, integrating the spectrum library with small ruminant strains of Staph. haemolyticus as well as of Staph. microti and their closely related species might further improve identification performances and it is advised.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13567-022-01102-4.

Additional file 1: Geographical distribution of all 261 isolates included in this study.

Additional file 2: Staphylococcus and Streptococcus species included in the MBT Compass® Library Revision H (2021).

Additional file 3: Restriction fragment length polymorphism (RFLP) pattern of PCR products of the gap gene obtained after digestion with AluI and used for Staphylococcus species assignment.

Additional file 4: Restriction fragment length polymorphism (RFLP) pattern of PCR products of the gap gene obtained after digestion with AluI and used for Streptococcus species assignment.

Additional file 5: Excel file detailing PCR–RFLP identification, gap gene sequencing information where obtained, MALDI-TOF MS identification, best log score, and animal species.

Additional file 6: Genomic sequence of the gap gene and sequence similarity data for staphylococci.

Additional file 7: Genomic sequence of the gap gene and sequence similarity data for streptococci.

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Authors’ contributions
NM, MP and SF-P performed the laboratory experiments. MFA and ST designed the study, analyzed the data, and wrote the manuscript. All authors read and approved the final manuscript.

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Declarations

Competing interests
The authors declare that they have no competing interests.

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