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## INTERPRETIVE SUMMARY

## TYPEABILITY OF MALDI-TOF FOR NON-*AUREUS* STAPHYLOCOCCI IDENTIFICATION

**MAHMMOD**

MALDI-TOF has never been investigated for NAS from non-milk samples. We evaluated the typeability of MALDI-TOF for identification of NAS associated with bovine IMI and teat apex colonization. Proportion of NAS isolates correctly identified from milk (91%) was higher than proportion of isolates correctly identified from teat skin (68%). In total, 93% of isolates were successfully identified as NAS, while the remaining (7%) were shown to be other bacterial species using MALDI-TOF. MALDI-TOF is efficient in NAS identification from milk but it may not always be acceptable for routine identification of NAS from non-milk samples. Nucleic-acid based tools is vital for accurate species identification of some NAS species.

# TYPEABILITY OF MALDI-TOF FOR NON-*AUREUS* STAPHYLOCOCCI IDENTIFICATION

## Typeability of MALDI-TOF Assay for Identification of Non-*Aureus* Staphylococci associated with Bovine Intramammary Infections and Teat Apex Colonization

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47 **ABSTRACT**

48 Matrix-assisted laser desorption/ionization time of flight (**MALDI-TOF**), a culture-dependent assay,
49 has recently been implemented for routine identification of non-*aureus* Staphylococcus (**NAS**)
50 species from milk, but the assay has never been investigated for NAS from non-milk or
51 environmental samples. The objective of this study was to evaluate the typeability of MALDI-TOF
52 assay for the identification and differentiation of bovine-associated NAS species on aseptically
53 collected quarter-milk and teat skin in dairy herds. In eight herds, 14 to 20 cows with elevated
54 somatic cell count were randomly selected for teat skin swabs and foremilk samples from right hind
55 and left front quarters. Teat skin swabs and milk samples were collected aseptically for preliminary
56 identification using bacterial culture on chromogenic and calf blood agars. Colonies from milk and
57 teat skin samples with suspicion of having NAS were identified to species-level by MALDI-TOF
58 assay. Out of 511 isolates from 284 quarters (142 cows), 78% (n = 399) were identified by MALDI-
59 TOF. The percentage of correctly identified NAS from milk (91% 105/115) using MALDI-TOF was
60 higher than the percentage from teat skin (68%, 268/396). Out of the identified isolates, 93% (n =
61 373) were successfully identified as NAS, while the remaining 26 (7%) were shown to be other
62 bacterial species. Out of 26 non-NAS isolates, one originated from milk (*Corynebacterium stationis*),
63 while 25 originated from teat skin representing *Aerococcus viridans* (n = 7), *Bacillus pumilis* (n =
64 13), *Enterococcus saccharolyticus* (n = 1), *Clostridum septicum* (n = 1), *Corynebacterium stationis*
65 (n = 2), and *Corynebacterium casei* (n = 1). MALDI-TOF identified 85% (98/115) and 62%
66 (245/396) of the isolates in the first test. Isolates that were not identified to species-level at first test
67 were subjected to a second test, and here 47% (8/17) and 32% (48/151) from milk and teat skin,
68 respectively, were identified. After two rounds of MALDI-TOF, 22% (n = 112) of the isolates were
69 not identified representing 103 from teat skin and nine from milk. Eighteen isolates without
70 identification by MALDI-TOF were successfully identified to species-level using sequencing, where
71 16 were correctly identified as NAS, while the other two were *Corynebacterium stationis.*

In conclusion, MALDI-TOF is a reliable assay for identification and typeability of NAS species from aseptically collected quarter milk samples. The assay may be used for identification of NAS species from teat skin swabs. However, confirmation using nucleic-acid based tools is vital for accurate species identification of some species and strains.

**KEYWORDS:** non-aureus staphylococci; bovine mastitis; teat skin colonization; phenotypic identification

## INTRODUCTION

Non-*aureus* Staphylococci (**NAS**) are a heterogeneous group of bacterial species (Schukken et al., 2009), which regarded as a common cause of IMI in dairy herds (Zadoks and Watts, 2009). Moreover, NAS are abundantly colonizing the teat skin, teat apex and teat canal and hence, many studies have shown that teat colonization with NAS could have a significant role in initiation or development of IMI with NAS in dairy cows (Leroy et al., 2015; De Visscher et al., 2016). Recent studies documented that some species are more important than others in relation to udder health (Supré et al., 2011; De Visscher et al., 2016). Furthermore, De Visscher et al. (2014) provided evidence that the group of NAS species is comprised of environmental, opportunistic and host-adapted species, which differ in ecology. Additionally, the authors concluded that some of the extramammary niches, such as the teat apex, might act as infection sources for IMI-causing NAS. Except for *Staphylococcus xylosus*, an association was observed between teat canal colonization and IMI by all NAS species, in which the majority of IMI were preceded by teat canal colonization (Quirk et al., 2012).

NAS are known to be a common teat apex colonizer (Braem et al., 2013; Falentin et al., 2016), and are among the mastitis-causing bacteria most likely to enter the mammary gland through the teat orifice (Fox and Norell, 1994) resulting in the establishment of IMI (Pyörälä and Taponen, 2009; Piepers et al., 2010). Braem et al. (2012) identified staphylococci among the bacterial genera with the highest percentage (31%) of colonized teat apices and they were detected with equal prevalence from teat apices of non-infected, subclinically infected, and clinically infected quarters. Therefore, it is crucial to identify and differentiate the NAS species colonizing teat skin or inhabiting milk causing IMI to understand their epidemiology and to evaluate the clinical relevance and feasibility of species-specific infection control measures (Zadoks and Watts, 2009). Furthermore, it is important for the routine microbiological laboratories, because rapid and correct identification of mastitis causing

pathogens will influence the choice of antibiotic before the final determination of antibiotic resistance of the isolate (Nagy et al., 2014). For many years, the species identification of NAS relied on phenotypic characteristics, which is difficult, time consuming, laborious, and often inaccurate (Watts et al., 1991; Vanderhaeghen et al., 2014). Although biochemical assays such as API systems are widely used for identification of NAS, the accuracy and speed is not optimal (Taponen et al., 2006; Capurro et al., 2009; Sampimon et al., 2009; Park et al., 2011).

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (**MALDI-TOF**) is a rapid method, which is able to identify a great variety of the isolated bacteria based on the composition of conserved ribosomal proteins (Kliem and Sauer, 2012). This technique is based on the acquisition of protein (ribosomal proteins) "fingerprints" directly from intact microorganisms, since such profiles vary considerably among microorganisms (Singhal et al., 2015). The assay provides a new diagnostic platform, which overcomes the limitations of traditional diagnostics for NAS being time consuming and laborious, or the need of sugar fermentation or test kits (Watts et al., 1991; Capurro et al., 2009; Vanderhaeghen et al., 2014; Taponen et al., 2016). The technique is increasingly used in human medicine and, recently, it has been expanded as a routine diagnostic tool in veterinary medicine (Randall et al., 2015; Pizauro et al., 2017). In the recent decade, research studies showed that MALDI-TOF is a powerful and reliable diagnostic tool for identification and discrimination of mastitis causing pathogens including NAS from bovine mastitis samples (Tomazi et al., 2014; Gonçalves et al., 2014; Cameron et al., 2017a, b), and spiked milk samples (Barreiro et al., 2017). MALDI-TOF assay was validated against other routine diagnostics for NAS such as Vitek 2 compact system (Elbehiry et al., 2016) and PCR (Pizauro et al., 2017) where it showed a better performance in identification and discrimination of NAS species from bovine mastitis. To the best of our knowledge there is no available literature describing the performance of MALDI-TOF assay for identification of NAS from non-milk cow samples or environmental samples in dairy herds. The

objective of this study was to evaluate the typeability of MALDI-TOF assay for the identification and differentiation of bovine-associated NAS species on quarter level from aseptically collected milk (IMI) and teat skin (teat apex colonization) habitats in dairy herds.

## MATERIAL AND METHODS

### *Study population*

Eight dairy herds with Danish Holstein cows were selected for participating in a project on *Streptococcus agalactiae* and *Staphylococcus aureus* IMI. To be eligible for inclusion in the present study, herds had to have automatic milking systems **(AMS)** with ≥ 3 milking robots and bulk tank milk PCR cycle threshold value ≤ 32 for *Streptococcus agalactiae.* About 30 to 40 lactating dairy cows were selected randomly from each herd on the basis of the criteria of having no clinical mastitis, SCC ≥ 200,000 cells/mL at the preceding milk recording, and not subjected to antibiotic therapy during the four weeks prior to sample collection. From each cow with an odd laboratory running number, teat skin swab and aseptic milk samples were taken from right hind and left front quarters (Mahmmod et al., 2018).

### *Sampling Procedures*

Each herd was visited once to collect teat swab samples and aseptically collected quarter foremilk samples for bacterial culture. The farmers were asked to separate the selected cows for sampling. Cows were fixed in head lockers or tied. Teat swab samples were collected according to the modified wet-dry method (Paduch et al., 2013). Briefly, the teat skin was sampled after cleaning with dry tissue paper. The first rayon swab (DaklaPack, Glostrup, Denmark) was moistened with ¼ Ringer's solution (Merck, Darmstadt, Germany) and rotated 360° around the teat about one cm from the teat canal orifice. The same procedure was carried out with a dry swab (second). Immediately after

153 sampling, the tips of both swabs were transferred into one tube with 2 mL of sterile ¼ Ringer's
154 solution.
155 Quarter milk samples were collected directly after harvesting the teat swab samples according to
156 National Mastitis Council (1999) guidelines. Briefly, the teat end was thoroughly disinfected with
157 cotton swabs drenched with ethanol (70%). Individual quarter foremilk samples were then
158 aseptically collected in sterile screw-cap plastic tubes. New latex gloves were worn at each sampling
159 procedure and were changed after each cow. Tubes containing the teat swabs and aseptically
160 collected milk samples were stored at 5°C in ice boxes until the samples were delivered to the
161 microbiological laboratory within 24h. All study activities including farm visits, collection of
162 samples and laboratory examination were carried out during the period from February to May 2017.
163
164 ***Laboratory Procedures***
165 ***Bacteriological examination***
166 Bacterial culture for milk samples was conducted in accordance with National Mastitis Council
167 recommendations (1999). After vortexing, 0.01 mL of the milk sample from each quarter was
168 streaked using disposable, calibrated bacterial loops on a calf blood agar plate and simultaneously
169 another 0.01 mL of the same sample was streaked on chromogenic agar plates selective for
170 staphylococci species (SA*SELECT*, Bio-Rad, Marnes-la-Coquette, France). Bacterial culture of teat
171 swab samples was performed according to the procedures of Paduch et al. (2013). Briefly, the teat
172 swab sample was vortexed before removing the swab tips from the tubes. The agar plates were
173 inoculated with 0.1 mL of a swab solution prepared with ¼ Ringer's solution. The inoculum was
174 spread with a sterile Drigalski spatula onto the agar surface of a calf blood agar plate and
175 SA*SELECT* media simultaneously for each quarter.
176 All the inoculated plates were marked by the laboratory running number and sequence of the quarter
177 and were incubated aerobically at 37°C for 48h and examined for growth of NAS colonies after 24

and 48h. *Staphylococcus* species were presumptively identified on the basis of the phenotypic characteristics of their colonies including shape (round, glossy) and color on the selective media. Up to three different NAS species per sample, according to the colony color on the SA*SELECT* media, were considered for further identification at species-level. Cut-off ≥ five CFU on the plate was regarded as an acceptable cut-off point for definition the positivity of NAS according to Thorberg et al. (2009) from milk and teat skin samples based on bacterial culture.

***MALDI-TOF Assay and Species Identification***

Isolates of NAS species that were identified based on bacterial culture were subcultured individually on new calf blood agar plates and incubated for 24 h at 37ºC to be submitted freshly to MALDI-TOF Bruker Biotyper software system (Microflex LT, Bruker Daltonics GmbH, Bremen, Germany) using an Autoflex Speed for identification of NAS at species-level. The bacteria were prepared for mass spectrometry analysis according to a standard extraction protocol using formic acid (Bizzini et al., 2010), as recommended by the manufacturer. A sterile wooden applicator was used to pick the material from a single bacterial colony followed by smearing a thin film of colony material onto a MTP 384 ground steel target plate (Bruker Daltonics). Subsequently, each spot was overlaid with 1.0 µL of a saturated α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, and 2.5% trifluoroacetic acid solution and air-dried at room temperature. Mass spectra were obtained on an Autoflex Speed calibrated with the recommended Bruker *Escherichia coli* Bacterial Test Standard for Mass Spectrometry.

Spectra of the analyzed bacteria were obtained in positive linear mode, within the mass range of 2 to 20 kDa. Spectra were acquired using the flexControl 3.4 program, and subsequently, analyzed using MALDI Bruker Real-Time Classification 3.1 software equipped with the BDAL database (Bruker Daltonics) comprised with 6903 reference spectra combined with verified local spectra from National Veterinary Institute, Denmark. The software compares the 10 closest spectra in the database

to the entry and since expansion of the Bruker BDAL database is an ongoing process; local spectra were generated from isolates obtained by the Diagnostic laboratory at DTU and identified by biochemical methods and/or 16S rDNA sequencing to meet the requirements for both animal and human reference spectra in the database. Improvement of the local database was conducted and isolates submitted as reference strains for the extended database were implemented. These stains were identified either by 16S rDNA sequencing or by biochemical methods as described by Andresen et al. (2005). The 47 custom Main SPectra (MSPs) were created according to Nonnemann et al. (2013) (Supplementary material 1).

The analysis for each isolate was run in triplicate. If the isolate was not identified to species-level in the first run, it was subcultured and resubmitted for MALDI-TOF to exclude the reason of "no identification" due to handling of the samples and to confirm that the non-identified isolates were either due to new species or to limitation of the database. After two rounds of MALDI-TOF, the unidentified isolates were considered as "no possible identification". A cut-off score $\geq$ 1.7 was regarded as a reliable threshold for the bacterial identification of NAS at species-level according to Cameron et al. (2017a, b).

***Identification by 16S rDNA sequencing***

A set of NAS isolates without possible identification by MALDI-TOF were subjected to 16S rDNA sequencing for identification. Those NAS isolates were selected on the basis of having a cut-off threshold < 1.7 in the triplicate runs of the two rounds of MALDI-TOF. One bacterial colony was suspended in 1 mL PBS and centrifuged for 5 min at 10,000 *g*. The supernatant was removed and the pellet resuspended in 100 µL water. The sample was boiled for 10 min and immediately placed on ice. The lysate was centrifuged for 2 min at 20,000*g*. The concentration of the DNA was quantified on a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and then was adjusted to 50 ng/µL.

The 16S rRNA gene was amplified by PCR in two separate products with following primers; 8f – 804r (5`- AGA GTT TGA TC (AC) TGG CTC AG -3´ and 5'- GTA TTA CCG CGG CTG CTG G-3') and (5'- CCA GCA GCC GCG GTA ATA C – 3' and 5´- GTT ACC TTG TTA CGA CTT CAC -3´) The amplification program ran as follows: denaturation at 94°C for 6 min, 33 cycles 94°C for 45 sec, 56 °C for 45 sec, and 72°C for 90 sec, final extension 72°C for 10 min in a reaction volume of 50 µL. Amplified PCR products, 796 bp and 990 bp expected length, respectively, were verified by E-Gel (Invitrogen). Fourty µL PCR product was purified with MinElute PCR purification kit (Qiagen) and eluted in 20 µL EB buffer. The amplified 16s rRNA gene was sequenced on an ABI 3130 Genetic analyzer using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem) according to manufacturer protocol. The resulting sequences were compared to the described sequences by blasting with the GenBank database (http://www.ncbi.nlm.nih.gov) as previously described (Strube et al. 2015).

***Statistical analysis***

A logistic mixed regression model with herd and cow treated as random intercept was performed to investigate if teat apex colonization with a specific NAS species increased the odds of IMI with the given species in the corresponding quarter. Different models were therefore performed for each of the NAS IMI species recovered from the quarter milk samples. Statistical analysis was carried out in R version 3.3.3 (The R Foundation for Statistical Computing). Results were considered significant if $P \leq 0.05$.

## RESULTS

Out of 150 eligible cows, 16 quarters were excluded for being dry or sampled twice. Out of 284 quarters of 142 cows, 228 quarters harbored at least one NAS species ≥ five CFU/0.01 mL identified by culture. Table 1 shows the number and distribution of NAS isolates from milk and teat skin samples submitted to MALDI-TOF assay. Out of total 511 culture isolates, 399 (78%) were

identified to species-level by MALDI-TOF, while the remaining 112 (22%) were not identified after two submissions to MALDI-TOF and were labeled as "no possible identification". Out of the 399 isolates identified by MALDI-TOF, 373 (93%) were successfully identified as NAS, while the remaining 26 isolates (7%) were diagnosed to be other bacterial species such as *Aerococcus viridans, Bacillus pumilus, Enterococcus saccharolyticus, Clostridum septicum* and *Corynebacterium casei*, Table 1. Out of the 26 isolates identified as other bacteria, one originated from milk and 25 from teat skin. Out of the 373 isolates, 28% were isolated from milk (n = 105) and 72% from teat skin (n = 268). In the first round of MALDI-TOF, 85% (98/115) of the submitted isolates were identified from milk, while the corresponding percentage was much lower for isolates from teat skin (62%, 245/396). In the second round, 47% (8/17) were identified from milk while it was 32% (48/151) for teat skin isolates. The number of isolates without possible identification after two rounds of MALDI-TOF from teat skin (n = 103) was about 10 times higher than that the number of isolates from milk (n = 9).

In total, MALDI-TOF identified 16 different NAS species, 15 species from teat skin and 10 species from milk, Table 2. In milk samples, *S. epidermidis* (49.5%, 52/105) was the most frequently isolated NAS species, while *S. arlettae* and *S. warneri* (1/105) were the least frequently isolated species. In teat skin swabs, *S. equorum* (43.3%, 116/268) was the most common species, while *S. vitulinus* and *S. warneri* (1/268) were the least frequently isolated species. *S. equorum* was the most frequently isolated NAS species from both left front (35.5%, 63/183) and right hind (30%, 57/190) quarters. Teat apex colonization with *S. chromogenes, S. equorum S. cohnii, S. epidermidis, S. haemolyticus* and *S. xylosus* was not found to significantly increase the odds of IMI with these NAS species, Table 2. Eighteen isolates without identification by MALDI-TOF were successfully identified using sequencing analysis, where 89% (n = 16) were correctly identified as NAS, while the other 11% (n = 2) were *Corynebacterium stationis*, Table 3.

## DISCUSSION

To the best of our knowledge, this is the first study to evaluate the application of MALDI-TOF for identification and differentiation of NAS species on quarter level from milk and teat skin in dairy cows milked with AMS. Our findings showed that MALDI-TOF was able to identify 92% (106/115) of the submitted culture isolates originating from milk samples indicating that it is a reliable assay for the rapid and accurate identification of NAS species from milk samples. This finding is similar to previous reports (Banach et al., 2016; Cameron et al., 2017a, b; Goetz et al., 2017; Savage et al., 2017). For instance, Elbehiry et al. (2016) showed that MALDI-TOF correctly identified 100% (44/44) of NAS species isolated from milk, while Tomazi et al. (2014) demonstrated that the sensitivity of MALDI-TOF for the identification of NAS isolated from milk was 95.4% in comparison to PCR-RFLP. Similarly, MALDI-TOF showed a better performance (93.2%) for identification of 234 NAS representing 20 different species than Phoenix (75.6%) and Vitek-2 (75.2%) (Dupont et al., 2010). Loonen et al., (2012) reported a good performance of MALDI-TOF for identification of NAS with a correct-identification rate of 99.3%. A lower identification percentage (78%) was reported by Ayeni et al. (2017) based on 171 isolates (13 species) using MALDI-TOF. However, the authors found that a drawback in identifying NAS with MALDI-TOF was the inability to identify *S. gallinarum* in their study because it was absent from the MALDI-TOF database at the time of study.

After two rounds of triplicate MALDI-TOF the overall number of isolates remaining without possible identification was 22% (112/511) representing 103 from teat skin and nine from milk. This is similar to Cameron et al. (2017b), who demonstrated that 40 isolates were not identified to species-level after the first round of duplicate MALDI-TOF, but after being subjected to a second round MALDI-TOF resulted in the identification of all except seven isolates. In line with that finding

Pizauro et al. (2017) reported that not all NAS found in buffalo milk could be identified by MALDI-TOF. Additionally, Banach et al. (2016) reported that six isolates of NAS were not identified to species-level by MALDI-TOF but were classified by means of routine bacteriological testing, and comprised of *S. sciuri* (three strains), *S. xylosus* (two strains), and *S. equorum* (one strain). This finding is in agreement with previous reports about the limitations of MALDI-TOF and could have been caused by limited detection capacity of the bacteria due to a limited database of different bacteria (Moussaoui et al., 2010; Barreiro et al., 2017; Cameron et al., 2017a). Furthermore, some studies have shown that the conditions of bacterial growth, preparation of samples, number of reference strains, and version of the software Biotyper may be a reason for the variability of NAS identification (Benagli et al., 2011; Tomazi et al., 2014).

Our findings indicate that MALDI-TOF provides a valuable tool for identification and typeability of NAS species from milk samples, while for isolates from non-milk samples, the assay showed a limited performance. Furthermore, in both the first and second submissions, the performance of MALDI-TOF for identification of isolates originating from milk (85% and 47%) was higher than the assay performance in identification of isolates from teat skin (62% and 32%, respectively). One possible explanation is that these unidentified bacteria from teat skin come from a natural teat skin microbiota (commensal bacteria), which have not been previously included in the BDAL database. For that reason, they may be out of interest of the microbiological diagnosticians and, therefore were not considered in the database of MALDI-TOF. Moreover, our findings showed that the vast majority of the unidentifiable NAS isolates were originated from teat skin. A reasonable explanation could be (a) those isolates are new NAS species. Supré et al. (2010) have classified 10 non-motile, Gram-stain-positive, coagulase-negative staphylococci isolated from bovine milk and teat apices as *S. devriesei* sp. nov using 16S rRNA gene and four housekeeping genes (rpoB, hsp60, tuf and dnaJ) in combination with tRNA-intergenic spacer length analysis. Another reason (b) these isolates could

328 be known NAS species but are not included in our BDAL database. It may be worth to mention that
329 the database of MALDI-TOF we used in this study, is not based only on the commercial version of
330 BDAL database. Our database was updated regularly because the assay is being used on research and
331 routine diagnostic service purposes for identification and differentiation of bacterial pathogens in
332 different samples types from both human and animals. We think that adding additional microbial
333 spectra, MSPs for some staphylococci species will improve the identification capacity of MALDI-
334 TOF assay both from milk and teat skin. In line with that statement, Cameron et al. (2017b) showed
335 that using a custom reference spectra expanded database, which included an additional 13 in-house
336 created reference spectra, isolates were identified by MALDI-TOF mass spectrometry with 99.2%
337 (854/861) typeability and 99.4% (849/854) accuracy.
338
339 Moreover, the BDAL database is mainly bacterial isolates originated from humans and then it
340 gradually extended to cover veterinary isolates (Tomazi et al., 2014). In support to that, Randall et al.
341 (2015) stated that the majority of reference spectra included in commercial MALDI-TOF databases
342 are derived from human isolates and, consequently, small differences between human and animal
343 isolates of the same bacterial species may influence the results obtained when testing some isolates
344 from animals. Remarkably, 89% of 18 isolates without possible identification by MALDI-TOF were
345 correctly identified as NAS using PCR indicating that confirmation using nucleic-acid tools is
346 essential for those suspected NAS isolates of environmental origin. By examination of the genetic
347 diversity among NAS species using molecular typing methods, Piessens et al. (2012) identified five
348 genotypes among *S. chromogenes* in six dairy farms. Cameron et al. (2017a) added that these
349 genotypes would have different spectra and would require their own entry within the database. We
350 updated the MALDI-TOF database on the basis of the findings in this study. Adding the spectra for
351 eight NAS species based on both prior knowledge of the species and results of the assessment of the
352 database, the authors were able to eliminate the unidentified risk from 8% unidentified to 0%

353 unidentified. Therefore, in future studies, we will expect better agreement in the performance of
354 MALDI-TOF between teat skin and milk samples.
355 Consequently, MALDI-TOF is a valuable routine diagnostic tool for identification and
356 differentiation of mastitis pathogens but it has some limitations with regard to different detection
357 capacity of the pathogens depending on the bacterial species, and limited database of pathogens
358 (Moussaoui et al., 2010; Barreiro et al., 2017; Cameron et al., 2017a). In line with that statement, our
359 findings showed that the isolates without possible identification by MALDI-TOF were successfully
360 identified by PCR, and sequencing analysis confirming the limited database of MALDI-TOF.
361 However, the database can continuously be expanded to accommodate new species and spectra.
362 Furthermore, the MALDI-TOF assay depends on initial microbiological culture, which is time-
363 consuming and laborious (i.e., plate preparation, sterilization of materials, time of incubation,
364 biochemistry tests) (Barreiro et al., 2010; Cameron et al., 2017a). Additionally, the reading is not
365 constant and differs by changing the cut-off (identification score) for species-level identification
366 (Barreiro et al., 2017; Cameron et al., 2017a) and extra rounds of identification may be necessary for
367 achieving accurate identification as shown in this study. Moreover, some previous studies have
368 shown that the conditions of bacterial growth, preparation of samples, number of reference strains or
369 spectra, database and version of the Biotyper may be a reason for the variability of NAS
370 identification (Benagli et al., 2011; Tomazi et al., 2014). Out of the initially unidentifiable isolates on
371 first round of MALDI-TOF, our findings showed that the assay identified 8/17 (47%; milk) and
372 48/151 (32%; teat skin) on the second round of identification. A plausable argument could be related
373 to the variation in the conditions of bacterial growth, extraction procedure and handling and
374 preparation of samples between the first and second rounds of MALDI-TOF. MALDI-TOF may be
375 sensitive to the sample preparations and handling due to individual variations. This may also explain
376 some of the deviations between different rounds, supporting previous reports (Benagli et al., 2011;
377 Tomazi et al., 2014).

Remarkably, we noticed that NAS isolates originating from teat skin may require extra rounds for identification by MALDI-TOF in comparison to NAS isolates originating from milk. That phenomenon is supported by the marked high number of isolates from teat skin remaining "unidentifiable" after the first and second rounds of identfication. A possible explanation could be that bacterial isolates from the enviroment may have developed an extra layer or capsule of protein material as a mean of protection against unfavorable enviromental conditions.

MALDI-TOF as culture-dependent assay relies on direct analysis of proteins from bacterial cell extracts of the reference strains included in the database (Liang et al., 1996). So, when a given NAS strain is tested, the species of the reference strain with the closest match is retained for identification of the tested strain (Ayeni et al., 2017). Therefore, an up-to-date database is essential for bacterial identification and more spectra of appropriate reference strains of NAS should be added to the database for accurate identification (van Veen et al., 2010; Cameron et al., 2017a). The same conclusion was supported by Murugaiyan et al. (2014) and Randall et al. (2015), who reported that the ongoing supplementation of the Bruker database library should further improve the utility of MALDI-TOF in routine veterinary diagnostic laboratories. Murugaiyan et al. (2014) demonstrated that 17 isolates initially diagnosed as *S. intermedius* with the current content of the BDAL database were identified as *S. pseudintermedius* by applying the in-house reference spectra extended version indicating that updating the reference spectra library allowed species identification of NAS.

## CONCLUSION

MALDI-TOF provides a valuable and efficient platform for identification and typeability of NAS species from aseptically collected quarter milk samples. MALDI-TOF may be used for identification

of NAS species from teat skin swabs. However, confirmation using nucleic-acid based tools is essential for accurate identification of some NAS species and strains.

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588

589 **Table 1.** Number and distribution of NAS isolated from milk and teat skin of dairy cows in eight

590 dairy herds submitted to MALDI-TOF assay for species identification of NAS.

| Sample type | MALDI-TOF identification | | | | No possible identification | Bacterial species (n) wrongly identified as NAS on culture |
|---|---|---|---|---|---|---|
| | First round | | Second round | | | |
| | Submitted | Identified | Submitted | Identified | | |
| Milk | 115 | 98 | 17 | 8 | 9 | *Corynebacterium stationis (1)* |
| Teat skin | 396 | 245 | 151 | 48 | 103 | *Aerococcus viridans (7); Corynebacterium casei (1); Bacillus pumilus (13); Clostridum septicum (1); Enterococcus saccharolyticus (1); Corynebacterium stationis (2)* |
| Overall | 511 | 343 | 168 | 56 | 112 | 26 |

591

**Table 2.** Description of 16 species of NAS analyzed by MALDI-TOF and their association from aseptic quarter milk and teat skin habitats collected from 142 cows (284 quarters) in eight dairy herds with automatic milking systems.

| NAS species [a] | Number (%) | Sample type (%) | | OR [c] (95% CI) | P-value [*] |
|---|---|---|---|---|---|
| | | Milk | Teat skin | | |
| *S* [b]*. arlettae* | 12 (3.2) | 1 (0.9) | 11 (4.1) | --- | --- |
| *S. capitis* | 3 (0.8) | --- | 3 (1.1) | --- | --- |
| *S. cohnii* | 43 (11.5) | 5 (4.8) | 38 (14.2) | 2.23 (0.11 - 15.6) | 0.48 |
| *S. epidermidis* | 60 (16.1) | 52 (49.5) | 8 (3.0) | 0.88 (0.05 - 5.07) | 0.90 |
| *S. haemolyticus* | 58 (15.6) | 16 (15.2) | 42 (15.7) | 1.13 (0.17 - 4.24) | 0.55 |
| *S. hominis* | 17 (4.6) | 3 (2.9) | 14 (5.2) | --- | --- |
| *S. piscifermentans* | 2 (0.5) | --- | 2 (0.8) | --- | --- |
| *S. saprophyticus* | 5 (1.3) | --- | 5 (1.9) | --- | --- |
| *S. sciuri* | 9 (2.4) | --- | 9 (3.4) | --- | --- |
| *S. simulans* | 2 (0.5) | 2 (1.9) | --- | --- | --- |
| *S. succinus* | 2 (0.5) | --- | 2 (0.8) | --- | --- |
| *S. vitulinus* | 1 (0.3) | --- | 1 (0.4) | --- | --- |
| *S. warneri* | 2 (0.5) | 1 (0.9) | 1 (0.4) | --- | --- |
| *S. chromogenes* | 16 (4.3) | 11 (10.5) | 5 (1.9) | NA [d] | NA |
| *S. equorum* | 122 (32.7) | 6 (5.7) | 116 (43.3) | NA | NA |
| *S. xylosus* | 19 (5.1) | 8 (7.6) | 11 (4.1) | NA | NA |
| *Other NAS (S. chromogenes, S. equorum, S. xylosus)* [e] | 157 (42.1) | 25 (23.8) | 132 (49.3) | 0.40 (0.09 - 1.69) | 0.21 |
| Total | 373 (100) | 105 (100) | 268 (100) | --- | --- |

[a] *Staphylococcus arlettae*, *S. warneri*, and *S. hominis* were not considered in the statistical analysis because of the few number of observations (< 5), while *S. capitis*, *S. piscifermentans*, *S. saprophyticus*, *S. sciuri*, *S. simulans*, S. *succinus*, and *S. vitulinus* were not isolated from milk and/or teat skin.

[b] S= *Staphylococcus*, [c] OR= Odds ratio; , [d] NA= not applicable; [*] significance at $P < 0.05$

[e] *Other NAS (S. chromogenes, S. equorum, S. xylosus)*: they are grouped together for a valid statistical analysis because the mixed model does not work for each species separately.

602 **Table 3.** Collection of bacterial isolates (n=18) from bovine teat skin suspected to be NAS on culture
603 and with no possible identification by MALDI-TOF after two rounds of submissions and were
604 correctly identified by 16S sequencing.

| NAS species identified by sequencing | Number (%) |
|---|---|
| *Jeotgalicoccus psychrophilus* | 1 (5.6) |
| *Corynebacterium stationis* | 2 (11.1) |
| *Staphylococcus cohnii* | 7 (38.9) |
| *Staphylococcus devriesei* | 1 (5.6) |
| *Staphylococcus equorum* | 3 (16.7) |
| *Staphylococcus arlettae* | 2 (11.1) |
| *Staphylococcus vitulinus* | 2 (11.1) |
| Total | 18 (100) |

605