In vitro antimicrobial activity of non-*aureus Staphylococci* and *Mammaliicocci* against *Staphylococcus aureus* and *Streptococcus uberis* and their relationship with the presence of intramammary infections in organic dairy cows

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ABSTRACT

This study aimed to: (1) Assess the *in vitro* antimicrobial activity of non-*aureus Staphylococci* and *Mammaliicocci* (**NASM**) isolates from organic cows teat apices with and without intramammary infections (**IMI**) by *Staphylococcus aureus* (**SAU**) or *Streptococcus* spp. and *Streptococcus*-like organisms (**SSLO**); (2) Investigate the relationship between IMI status and NASM counts on teat apex; (3) Explore the relationship between NASM species assigned taxonomy and the inhibitory activity against SAU and *Streptococcus uberis* (**SUB**). A total of 114 cows from 2 farms were sampled and followed from 8 weeks before to 5 weeks after calving. Weekly milk samples were cultured and used to design a case control study. Cases were defined as cows that had an IMI by SAU or SSLO, and controls were randomly chosen from cows without an IMI by these microorganisms. One teat apex sample from the week prior to IMI diagnosis was aerobically cultured. Taxonomy of recovered isolates was confirmed using mass spectrometry and whole genome sequencing. The inhibitory activity against SAU and SUB was investigated using agar dilution methods. Isolates were ranked based on in vitro inhibitory activity against SAU and SUB and categorized as "top 10" with the lowest minimum inhibitory concentration or not. The presence of NASM isolates classified as “top 10” against SAU was highest in controls (19.4%) compared to cases (5.8%). No difference in NASM counts on teat apex was observed between cases and controls. The inhibitory activity varied across NASM, with *Staphylococcus succinus* showing the highest inhibitory activity against both SAU and SUB.

KEY WORDS

Coagulase negative *Staphylococci*. Organic heifers. Antimicrobial activity.

INTRODUCTION

Mastitis is the leading cause of morbidity (USDA, 2007) and milk production loss in dairy farms (Heikkila et al., 2018). Although preventive measures are important for the control of mastitis, antimicrobials are still a major component of on-farm contagious mastitis control (Ruegg, 2017). However, the availability of antimicrobials for dairy farmers and veterinarians is at-risk due to concerns about antimicrobial resistance to medically important antibiotics and policy restrictions on antimicrobial use in livestock (Rajala-Schultz et al., 2021; McCubbin et al., 2022; Ruegg, 2022). This limitation is especially remarkable on United States organic-certified dairy farms, where regulations for production of organic milk include a strict prohibition against the use of antimicrobials and other synthetic substances (NMC, 2019).

Prior data from our group showed that *Staphylococcus aureus* (**SAU**) and *Streptococcus* spp. had a high prevalence and persistence in the mammary gland of dairy cows in organic farms in the first 35 days of lactation (Peña-Mosca et al., 2023b). Moreover, this study showed that SAU and *Streptococcus* spp. in early lactation were associated with a higher risk of high somatic cell count (**SCC**) during the first 180 days of lactation in organic dairy cows (Peña-Mosca et al., 2023a). Non-*aureus Staphylococci* and *Mammaliicocci* (**NASM**) are also highly prevalent in milk samples (De Buck et al., 2021; Peña-Mosca et al., 2023b), but unlike SAU and *Streptococcus*, NASM have been associated with increased milk production (Piepers et al., 2010). Indeed, the infusion into the mammary gland of NASM has been associated with decreased risk for intramammary infection (**IMI**) with SAU and *Streptococcus* spp., suggesting a potential protective effect of NASM against these two pathogens (Reyher et al., 2012). *In vitro*

studies using isolates obtained from the mammary gland of dairy cows have shown that NASM are capable of inhibiting the growth of SAU (De Vliegher et al., 2004a; Carson et al., 2017; Toledo-Silva et al., 2022). Using the cross-streak method for inhibitory testing, these studies reported that between 9.1% and 14.3% of the isolates were able to inhibit the growth of SAU (Carson et al., 2017; Toledo-Silva et al., 2022). Recently, agar dilution methods have also been utilized to investigate the inhibitory capabilities of NASM species on mastitis pathogens (Toledo-Silva et al., 2022). Moreover, studies in humans have also reported that specific NASM strains were associated with a decreased risk of skin colonization by SAU (Nakatsuji et al., 2017).

These findings have raised the hypothesis that certain NASM species could be used for the development of products to prevent or treat mastitis in dairy farms (De Buck et al., 2021). However, despite the *in vitro* antimicrobial activity demonstrated by NASM, it remains uncertain whether any species or strains within the NASM group would yield *in vivo* protective effects against IMI by major mastitis pathogens. Lastly, the use of the agar dilution method has only been investigated using a few isolates, and a more comprehensive investigation needs to be performed to assess the inhibitory activity of different NASM species.

The first objective of this study was to investigate the phenotypic *in vitro* antimicrobial activity of NASM isolated from the teat apex of organic cows with and without an IMI caused by SAU or *Streptococcus* spp. and *Streptococcus*-like organisms (**SSLO**) on the week preceding IMI diagnosis. We hypothesized that NASM isolates from cows without an IMI caused by SAU or SSLO (i.e., controls) would exhibit higher *in vitro* inhibitory activity against SAU and *Streptococcus uberis* (**SUB**) compared to NASM isolates from cows with SAU or SSLO IMI (i.e., cases). Our second objective was to investigate the relationship between the presence of an

IMI caused by SAU or SSLO and NASM counts on teat-apex swab samples collected the week prior to the diagnosis of SAU or SSLO IMI. We hypothesized that the NASM counts from the teat apex samples would be higher in organic cows without an IMI compared to cows with an IMI. Lastly, our third objective was to explore the relationship between the assigned taxonomy of NASM species and the presence of inhibitory activity against SAU and SUB. We hypothesized that there will be differences in the *in vitro* inhibitory across different NASM species, with a few specific species showing high *in vitro* antimicrobial activity.

MATERIALS AND METHODS

The Strengthening the Reporting of Observational Studies in Epidemiology - Veterinary (STROBE-Vet) Statement guidelines were followed for the preparation of this manuscript (Sargeant et al., 2016). All study activities were approved by the University of Minnesota Institutional Animal Care and Use Committee (**IACUC**) (Protocol Number: 1807: 36109A), Colorado State University IACUC (Protocol number: 1442), and Texas Tech University IACUC (Protocol number: 18068-10). Laboratory activities were approved by the Institutional Biosafety Committee protocol: 2205-40001H.

Inclusion criteria

Only cows from USDA organic certified dairy farms were enrolled in this study. Farms, from different regions of the United States and with different herd sizes, were selected for the study. Enrollment was based on willingness to participate, and proximity to the Universities involved in the study. This study was part of a larger research initiative to investigate potential associations between the udder microbiome and udder health (Dean et al., 2021). For this purpose, cows from 5 organic dairy farms were enrolled 8 weeks before calving and followed up during the first 5 weeks of lactation (Peña-Mosca et al., 2023b). In this manuscript, data from

only two of these farms were included, as teat apex samples for bacteriological culture were only collected in these two farms due to limited research labor availability. Specifically, this manuscript used samples collected from a subset of 114 cows (Farm A [n =21] and Farm B [n =93]) out of the 596 cows enrolled in the main research project. Samples from Farm A were collected between August 2019 and January 2020, and included all first-lactation cows that calved during that period. Samples from Farm B were collected between February and July 2021 and included both first and second lactation cows that calved throughout that interval.

Milk sample collection

After calving, quarter milk samples were collected weekly during the first 5 postpartum weeks prior to the morning milking by members of the research team, following guidelines from the National Mastitis Council (NMC, 2017). Upon removal of the pre-dipping disinfectant, 3 to 4 streams of milk were discarded from each quarter, and the teat apex of each quarter was scrubbed with a gauze soaked in 70% ethanol. Milk samples were collected in a clockwise direction, beginning with the right rear quarter, and ending with the right front quarter. Approximately 10 mL of milk from each quarter were collected into separate 60 mL plastic vials (Capitol Vials, Thermo Fisher Scientific). Samples were placed on ice immediately after collection and stored in a freezer at -20 °C until further processing.

Milk pooling

Quarter milk samples were combined into composite samples using the following protocol (Peña-Mosca et al., 2023b). First, milk samples were thawed at 4 °C overnight. Next, quarter milk samples were homogenized by inverting them upside down twice. Then, 2 mL was extracted from each vial and dispensed into a single plastic vial inside a laminar hood. Lab-

pooled composite samples were then submitted to the Laboratory for Udder Health at the University of Minnesota (St. Paul, MN, USA) for milk culture.

Milk culture

Using a cotton swab, approximately 100 µL of milk were plated onto Columbia CNA agar with 5% sheep blood and MacConkey agar. Agar plates were incubated in aerobic conditions at 37 °C for a total of 42 to 48 h. Plates were examined by a trained technician to evaluate the presence of growth of distinct isolates at 18 to 24 hours and 42 to 48 hours. Samples were defined as contaminated if more than 3 different isolates were identified and omitted from further analysis (Dean et al., 2022). Taxonomic assignments of cultured isolates were made using a Matrix-Assisted Laser Desorption/Ionization-Time of Flight (**MALDI-TOF**) mass spectrometer (**MS**) (Microflex; BrukerDaltonics Inc.) (Jahan et al., 2021). Mass spectra profiles produced from each isolate were matched against the Biotyper reference library. Confidence scores were used to assign genus- and species-level classifications (≥1.8 and ≥ 2.0 for genus- and species- classification, respectively) (Jahan et al., 2021). An IMI was defined as a composite sample containing one or more than one colony forming units (10 CFU/mL) of any cultured isolate.

Implementation of case control study

Milk culture results were used for the implementation of a case control study. Cases were defined as cows that had an IMI caused by SAU or SSLO during the first 35 DIM. Controls, with 1 control chosen for each case, were randomly chosen from the list of sampled cows that did not have an IMI caused by these microorganisms during the first 35 DIM. Cases and controls were individually matched by farm, lactation number, and DIM of when SAU or SSLO IMI were diagnosed (±7 DIM). From each selected cow, one teat apex sample from the week prior to the

diagnosis of a SAU or SSLO IMI was processed using the protocol outlined below. Because 3 cows did not have a teat apex sample available in the week prior to first diagnosis of SAU or SSLO IMI, 2 samples were processed for these animals from the 2 closest available samples before IMI diagnosis, resulting in a total of 71 samples from 68 cows (34 cases and 34 controls).

Teat apex sample collection

Cows enrolled from two of the participating farms, where labor was available, were sampled weekly from 8 weeks before calving to 5 weeks after calving in the milking parlor prior to the morning milking. First, teats were visually inspected, and any visible debris was removed using a paper towel. Then, using a new pair of disposable gloves, a single sterile pre-moistened gauze was used to thoroughly swab the distal third of all four teats. After sampling, gauze was placed in individual tubes containing a pre-loaded solution of 50% glycerol. Gauze samples were transported in a cooler with ice and stored in -80 °C after arrival to research facilities. Postpartum samples were collected before pre-dipping disinfectant solutions were applied to the teat and before milk samples were collected.

Teat apex sample culture

The methods for culturing gauze samples collected from teat apex were adapted from protocols utilized in the Laboratory for Udder Health at the University of Minnesota to process towel samples (Rowe et al., 2019). Before sample processing and to reduce the possibility of batch effects, sample IDs for each gauze sample were randomly sorted using Excel (Microsoft Corporation). In accordance with this order, isolates were selected for each sampling processing date in batches of 10 isolates. Gauze samples were thawed at 4 °C overnight and then transferred into 50 mL round-bottom falcon tubes (Corning Inc.). Afterwards, 5 mL of Phosphate-Buffered Saline (**PBS**; Gibco, ThermoFisher Scientific) was added to reduce viscosity. Samples were

homogenized carefully by turning tubes upside down twice and vortexing for 10 seconds at low speed. Samples were allowed to stand for 10 minutes, and the homogenization process was repeated once. Following homogenization, a single 1 in 10 serial dilution was done using PBS. A 100 uL aliquot from each sample (i.e. undiluted and 10^{-1}) was inoculated onto Mannitol salt agar (Oxoid). Plates were then incubated in aerobic conditions for 24 hours at 37 °C. Following incubation, bacterial groups were visually assessed and counted from the dilution plate with the optimal number of colonies (25 to 250 per plate). Isolates of visually distinct colonies were then picked from the plate using a sterile calibrated loop (ThermoFisher Scientific), streaked onto blood agar (Hardy Diagnostics) and incubated for 24 hours at 37 °C.

Species-level identification of isolates

After reaching pure growth, the identity of isolates was investigated using MALDI-TOF MS, using similar protocols to those outlined above for isolates from milk samples. Non-*aureus Staphylococci* and *Mammaliicocci* isolates were then stored for further investigation in -80 °C using commercial cryovials (Hardy Diagnostics). Isolates identified as NASM using MALDI-TOF MS were further investigated using whole genome sequencing. Isolates were first grown on brain heart infusion for 24 hours, centrifuged at 10,000 rpm for 5 minutes, and the pellet was collected in 2 mL sterile polypropylene microcentrifuge tubes (Thermo Fisher Scientific). Following this procedure, DNA extraction was performed utilizing commercial kits and following manufacturer's protocol (Qiagen's DNeasy 96 Blood & Tissue Kit, Qiagen). The amount and quality of extracted DNA was evaluated using PicoGreen fluorometer and NanoDrop spectrophotometer (ThermoFisher Scientific). Subsequently, library preparation was performed using the DNA Prep Sample Preparation Kit (Illumina Inc.), and libraries were pooled for 2x300 paired-end sequencing on the Illumina MISEQ platform, with a targeted depth of 50x

coverage across the *Staphylococcus* spp. genome for each library. Quality control of raw sequencing data was performed using FastQC (Andrews, 2010) and MultiQC (Ewels et al., 2016). Trimmomatic was utilized to trim low quality bases and adapters from raw reads (Bolger et al., 2014). Genomes were assembled utilizing SPAdes version 3.15.5 (Bankevich et al., 2012), and assembly quality was evaluated using Quast version 5.2.0 (Gurevich et al., 2013). Species identification of isolates was performed utilizing GTDB-Tk version 1.7.0 (Chaumeil et al., 2020) and GTDB release 202 (Parks et al., 2022) as implemented in KBase (Arkin et al., 2018).

Determination of in vitro antimicrobial activity of NASM isolates using agar dilution methods

The methods utilized in this study were based on procedures previously described by others (Toledo-Silva et al., 2022). In each batch of samples, on day 1, isolates that were previously identified using MALDI-TOF MS as NASM were randomly sorted using Excel (Microsoft Corporation). Following this procedure, isolates were grown for 24 hours in blood agar. If evidence of mixed growth (more than 1 colony type) was present, samples were replated. On day 2, one colony was picked with a 10 uL disposable loop and was grown on brain heart infusion (**BHI**; Research Products International Corporation) media for 18 to 20 hours. The next day, 1 in 10 serial dilutions solutions were created using sterile PBS in a 96 well plate (ThermoFisher Scientific). Bacterial counts (CFU/mL) were estimated by plating 100 uL of the serial dilutions on blood agar plates that were then aerobically incubated for 18 to 20 h at 37 °C. One hundred microliters of bacterial suspensions containing NASM isolates at different dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}) were added to 10 mL of 2% Tryptic soy agar (**TSA**; Oxoid) inside Falcon round-bottom polypropylene tubes (Corning Inc.). The temperature was examined using a laboratory digital thermometer (Thermco) before the inclusion of the 2% TSA. The media were not used until the temperature reached 50 °C (Toledo-Silva et al., 2022). Tubes were

then homogenized and poured into pre-heated (37 °C) TSA plates. Following aerobic incubation for 18 to 20 h, 10 uL of the bacterial suspensions containing approximately 10,000 CFU of SAU (American Type Culture Collection (**ATCC**) strain Seattle 1945; *Staphylococcus aureus* subsp. aureus Rosenbach 25923 ™) and SUB (ATCC strain 01040 J; *Streptococcus uberis* Diernhofer BAA-854 ™) were inoculated onto the top of plates containing different concentrations of NASM isolates and incubated for 18 to 20 h. For each batch of samples, negative controls were included by incubating a 2% TSA agar plate with the inclusion of 100 uL of BHI media only (instead of NASM isolates). Additionally, a 10 µL droplet of PBS was added on top of the media on each plate to serve as an additional negative control, ensuring the absence of PBS contamination. *Staphylococcus aureus* and SUB were incorporated into the media as positive controls, and their self-inhibition was tested to explore the determination of MIC driven by nutrient competition rather than *in vitro* inhibitory activity. Growth of SAU and SUB (i.e., yes vs no) was evaluated by visual examination of each of the plates and comparison with negative control plates within each batch. Minimum inhibitory concentration (**MIC**) was defined as the minimum concentration of NASM (CFU/mL) on the 2% TSA top layer able to inhibit the growth of SAU or SUB.

Statistical analysis

All analyses were performed in R 4.3.2 (https://www.r-project.org/). Code and output can be found online: https://fepenamosca.github.io/NASM_in_vitro_inhibitory_activity.io/ . No priori sample size calculation was performed for this exploratory study. Before modeling, descriptive characteristics for numerical variables and categorical outcomes were evaluated using the “table 1” package in R (Rich et al., 2023). Data distribution was evaluated using density plots as implemented in the “car” package in R (Fox et al., 2022). The relationship between IMI status

(i.e., cases versus controls) and $\log_{10}$ -transformed bacterial counts from teat apex samples was investigated using linear regression models. The relationship between IMI status and presence of NASM on the teat apex samples (i.e., yes vs no) was investigated using a generalized linear model with a "binomial" family and "logit" link (logistic regression). For analysis, NASM taxonomy was determined based on the assigned taxonomy using MALDI-TOF MS and whole genome sequencing. It was represented as a six-level multi-level variable (*Staphylococcus chromogenes*, *Staphylococcus haemolyticus*, *Staphylococcus sciuri*, Staphylococcus *succinus*, *Staphylococcus xylosus/pseudoxylosus*, and other rare NASM [*Staphylococcus cohnii*, *Staphylococcus devrieseii*, *Staphylococcus equorum*]). Cox proportional hazards regression was used to assess the association between presence of IMI, NASM taxonomy and the concentration to NASM *in vitro* growth inhibition against SAU and SUB. Observations were right censored when inhibition was still not present at maximum NASM concentration analyzed (i.e., 10^{-7} dilution). Proportional hazards assumption was tested by evaluating the Schoenfeld residuals. Since multiple isolates were harbored from each cow, the non-independence of observations within each enrolled animal was accounted for using the robust sandwich estimator. The NASM MIC ($\log_{10}$, CFU/mL) against SAU and SUB was further explored and compared across cases and controls and different NASM species using mixed linear regression. These models incorporated cow-ID as a random effect to address the non-independence of observations for isolates harbored from the same cow. Subsequently, isolates were ranked according to their MIC (e.g., 1 to 79) and classified as being in the top 10 isolates with lowest MIC or not ("top 10"). The relationship between IMI status and presence of a "top 10" NASM on the teat apex was investigated using a generalized linear model (logistic regression) with "binomial" family and "logit" link. When performing this analysis by NASM species, we used the Fisher exact test due

to the absence of “top 10” isolates for some NASM species. The intraclass correlation coefficient was estimated using the “performance” package (Lüdecke et al., 2021). Estimated marginal means were estimated using the “emmeans” package in R (Lenth et al., 2024) . Normality assumption was investigated by evaluation of model residuals and the use of quantile-quantile plots. Multiple comparisons were accounted for with Tukey adjustment as implemented in the “emmeans" package (Lenth et al., 2024). Farm-ID was forced into all models that investigated the relationship between IMI status and NASM inhibitory activity against SAU and SUB. Because farm ID was strongly associated with the prevalence of different NASM species (i.e., each of the NASM species were in most cases highly prevalent in one farm but rarely found in the other one), this variable was not included in the models comparing *in vitro* inhibitory activity across different NASM species.

For all models, processing batch (i.e., the date at which gauze samples were processed or antimicrobial activity of NASM was evaluated) and parity of the cow (i.e., first vs second lactation) were offered as potential confounders. Presence of confounding was evaluated by examining the change in the estimates after adjustment by each confounder ([Crude estimate – Adjusted estimate] / Adjusted estimate). Confounders that changed the estimates by 10% or more were kept in the models.

RESULTS

Descriptive characteristics

Farms enrolled in this study were located in Minnesota (farm A) and Colorado (farm B) with herd sizes of 100 and 1,700 milking cows, respectively. Both farms utilized a free-stall housing system and cows consumed at least 30% of dry matter intake from pasture, when possible, following organic regulations. All enrolled cows from farm A (21/21) were starting

their first lactation, while in farm B 46.2% (43/93) were starting their first lactation and 53.8% (50/93) were transitioning to their second lactation. A total of 68 animals (34 cases and 34 controls) were included in this case control study (**Table 1**). Fifteen of these cases had a SAU-IMI, 15 had a SSLO-IMI and 4 had an IMI caused by both microorganisms. In the first postpartum sample, 64.7% (22/34) of the cases had the presence of a SAU or SSLO IMI. Teat apex samples processed from cases and controls showed similar days relative to calving (mean [SD]; Controls: -2.8 [15.2] vs Cases: -1.2 [16.5]). A flowchart describing the *in vitro* study as well as the number of observations lost at each step is presented in **Figure 1**.

In vitro inhibitory activity of* NASM *isolated from case and control cows against Staphylococcus aureus and Streptococcus uberis

A total of 275 isolates were submitted for MALDI-TOF MS as shown in **Table 2**. Surprisingly, *Bacillus* spp. were the most frequently identified genus in controls (61.9% [83/134]) and cases (61.7% [87/141]). Non-*aureus Staphylococci Mammaliicocci* were identified in 32.8% (44/134) and 27.7% (39/141) of the isolates obtained from the teat apex samples from controls and cases, respectively. The most frequent species identified within this group included *Staphylococcus chromogenes* (8.0% [22/275]) and *Staphylococcus haemolyticus* (7.6% [21/275]). The overall inhibitory activity of NASM on SAU was not different between isolates obtained from cases and controls (HR (95%CI): 0.92 (0.56, 1.49), P = 0.72; **Figure 2**). The adjusted MIC ($\log_{10}$, CFU/mL) for SAU was 3.26 and 3.37 for isolates recovered from controls and cases, respectively (Estimate [95%CI]: 0.11 [-0.21, 0.44]; P = 0.49**)**. Similarly, we did not find evidence of a difference in the inhibitory activity of NASM on SUB between isolates obtained from cases and controls (HR (95%CI): 1.23 (0.74, 2.08), P=0.45; **Figure 2**). The

adjusted MIC (log_{10}, CFU/ml) of NASM against SUB was 3.17 in isolates from controls and 3.08 in isolates from cases (Estimate [95%CI]: -0.10 [-0.45, 0.25]; *P* = 0.58).

During the study, we evaluated the inhibitory activity of 79 NASM isolates. We were not able to determine the MIC against SAU and SUB for 5 and 14 NASM isolates, respectively (**Figure 3**). Among these isolates, the average (SD) highest concentration with an undetermined MIC was 3.98 (0.45) CFU/mL for SAU and 4.30 (0.52) CFU/mL for SUB.

When SAU was included in the media as a positive control, the MIC against SAU was 3.09. Among the studied NASM isolates, 34.2% (27/52) displayed an MIC below this value (38.1% [16/42] and 30.0% [11/37] for isolates recovered from controls and cases, respectively). Upon the inclusion of SUB in the media as a positive control, its MIC against SUB was 3.61. Twenty percent of the isolates (16/79) displayed a MIC below this value (21.4% [9/42] for isolates from controls and 18.9% [7/37] for those from cases).

To explore isolates with high inhibitory activity, we arranged the isolates based on their MIC values against SAU and SUB (**Figure 3A and 3B**). The 10 isolates with lowest MICs displayed a mean (SD) MIC (log_{10}, CFU/ml) of 2.41 (0.26) and 2.16 (0.32) for SAU and SUB, respectively. These isolates showed MICs that ranged between 1.92 to 2.66 log_{10} CFU/mL against SAU growth and 1.65 to 2.56 log_{10} CFU/mL against SUB growth. Top 10 isolates with lowest MIC against SAU were more prevalent in controls (19.4% (7/36)) than in cases (5.8% (2/35)) (OR (95%CI): 0.25 (0.04, 1.13), *P* = 0.07). However, no evidence of an association was found between IMI status and the likelihood of harboring a 10 top isolate for SUB (controls: 11.1% (4/35) vs cases: 14.7% (5/36); (OR (95%CI): 1.33 (0.32, 5.83), *P* = 0.69).

Relationship between case control status and bacterial counts on teat apex

The association between IMI status and bacterial count in the teat apex samples is presented in **Table 3**. No evidence of a difference in NASM counts was found between cases and controls (Estimate [95%CI]: -0.09 [-0.51, 0.36], $P = 0.72$). The likelihood of harboring NASM on the teat apex was not different between controls (66.7% [22/36]) and cases (62.9% [24/35]) (OR (95%CI): 0.85 (0.32, 2.25), $P = 0.74$) (**Table 4**). The distribution of NASM species differed by farm. *Staphylococcus chromogenes, Staphylococcus haemolyticus* and *Staphylococcus succinus* showed a high prevalence in farm A (28.1% [16/57], 28.1% [16/57] and 14.1% [8/57], respectively), but were rarely found on farm B (7.1% [1/14], 7.1% [1/14] and 0% [0/14], respectively). On the other hand, *Staphylococcus xylosus* or *Staphylococcus pseudoxylosus* were found in half of the samples from farm B (50.0% [7/14]) but showed a low prevalence in farm A (1.8% [1/57]).

Relationship between NASM taxonomy and inhibitory activity against Staphylococcus aureus and Streptococcus uberis

The inhibitory activity of NASM on SAU and SUB differed across different NASM species ($P < 0.001$; **Figure 3, Figure 4**). *Staphylococcus haemolyticus* and other rare NASM species (i.e., *Staphylococcus cohni*, *Staphylococcus devrieseii*, *Staphylococcus equorum*) showed lower inhibitory activity against SAU compared to *Staphylococcus chromogenes* (HR [95%CI]: 0.28 [0.09, 0.90], P=0.02; and HR [95%CI]: 0.15 [0.03, 0.71], P=0.006, respectively), *Staphylococcus sciuri* (HR [95%CI]: 0.21 [0.08, 0.58]; $P = 0.001$, and HR [95%CI]: 0.11 [0.02, 0.50], P<0.001, *respectively*), *Staphylococcus succinus* (HR [95%CI]: 0.15 [0.05, 0.42], P<0.001; and HR [95%CI]: 0.08 [0.02, 0.36], P<0.001, respectively) and *Staphylococcus xylosus*/*pseudoxylosus* (HR [95%CI]: 0.14 [0.05, 0.38], P<0.001; and HR [95%CI]: 0.07 [0.02,

0.28], $P<0.001$, respectively). The adjusted MIC ($\log_{10}$ CFU/ml) of NASM on SAU for different NASM species ranged between 2.92 and 3.99 (**Table 5)**. Similarly, when inhibition of SUB was the outcome of interest, *Staphylococcus haemolyticus* displayed a reduced ability to inhibit SUB compared to *Staphylococcus chromogenes* (HR [95%CI]: 0.11 [0.02, 0.49]), *Staphylococcus sciuri* (HR (95%CI): 0.17 (0.06, 0.48)), *Staphylococcus succinus* (HR [95%CI]: 0.13 [0.03, 0.50]) and *Staphylococcus xylosus*/*pseudoxylosus* (HR [95%CI]: 0.11 [0.03, 0.43]), all $P<0.001$). However, the inhibitory activity of *Staphylococcus haemolyticus* against SUB was not different from other rare NASM species (e.g., *Staphylococcus cohnii*, *Staphylococcus devriesei*, *Staphylococcus equorum*) (HR [95%CI]: 0.62 [0.06, 6.31], $P=0.99$). The adjusted MIC ($\log_{10}$ CFU/ml) of NASM against SUB in different NASM species ranged between 2.92 and 3.63 (**Table 5)**. The distribution of isolates ranked by their MIC on SAU and SUB growth is shown in **Figure 3C and 3D**. The proportion of isolates classified as "top 10" with respect to SAU and SUB was different between the NASM species ($P = 0.04$ and 0.06, respectively). Only 4.8% (1/21) and 0% (0/5) of the isolates identified as *Staphylococcus haemolyticus* and other rare NASM species, respectively, were classified as being "top 10" against both SAU and SUB. By comparison, 45.5% (5/11) of *Staphylococcus succinus* isolates were classified as "top 10" against both SAU and SUB (**Table 6**).

DISCUSSION

This study aimed to explore the association of naturally occurring NASM isolates and their ability to inhibit the growth of SAU and SUB, two major mastitis pathogens that are difficult to control and eradicate on dairy farms (Barkema et al., 2006; Peña-Mosca et al., 2023b; Woudstra et al., 2023). In our study, we observed an association between the presence of isolates with high *in vitro* antimicrobial activity against SAU and absence of IMI by SAU or SSLO. In

addition, the inhibitory activity of NASM showed substantial variation across different NASM species.

Relationship between in vitro antimicrobial activity of non-aureus Staphylococcus and Mammaliicocci isolates and presence of intramammary infections

Our primary objective was to investigate the *in vitro* antimicrobial activity of NASM isolates and their association with the presence of IMI. We discovered a wide variation in the inhibitory activity across NASM isolates. A considerable number of the NASM isolates did not show inhibitory activity at the maximum concentration studied suggesting the absence of inhibitory activity. Moreover, we did not find evidence of a difference in the average inhibitory activity of NASM on SAU or SUB across cases and controls. One possible explanation for these findings is that average antimicrobial activity may not be an important predictor of the risk of IMI, and only high antimicrobial activity against a specific pathogen may be effective to prevent the risk of IMI (Nakatsuji et al., 2017). In this study, we discovered that control cows had the majority of NASM isolates that were within the "top 10" with the lowest MIC against SAU. In addition, cows in the control group had a higher chance of having these highly inhibitory isolates on their teat apices than cows in the case group. Although our results need to be interpreted with caution, given the small sample size and imprecision of estimates, these results suggest the presence of a protective effect of NASM with high *in vitro* antimicrobial activity against SAU on the presence of IMI by SAU or SSLO. These findings are in line with studies performed in humans in which isolates with high phenotypic antimicrobial activity showed a high prevalence on the skin on controls, but were rarely identified on people with SAU skin infections (Nakatsuji et al., 2017). As whole, our results suggest that commensal NASM may provide defense against mammary gland pathogen invasion, albeit these research discoveries require more study.

Relationship between teat apex colonization by non-aureus Staphylococcus and Mammaliicocci and presence of intramammary infections

The prevalence of NASM on teat apex (64.8%) was similar to that reported by other studies in conventional dairy farms in which 70.1% (Mahmmod et al., 2018) and 71.8% (De Visscher et al., 2016) of the teat-apex samples showed presence of NASM. Consistently, we found a high prevalence of *Staphylococcus chromogenes* and differences in the prevalence of the different NASM species across the enrolled farms, which concurs with these previous reports (De Visscher et al., 2016; Mahmmod et al., 2018). These differences have been previously attributed to differences in management across the enrolled farms (De Buck et al., 2021) and are not surprising, given the differences in the prevalence and distribution of different microorganisms that cause IMI on organic dairy farms (Peña-Mosca et al., 2023b).

Contrary to our hypothesis, the counts or the presence of NASM on teat apex were not associated with the presence of SAU or SSLO IMI. These results are inconsistent to those reported by prior studies, in which prepartum colonization of teat apices by NASM was related to a lower risk of postpartum IMI (Piepers et al., 2011) and subclinical mastitis (De Vliegher et al., 2003). These findings suggest that, in our study population, the mere presence of NASM on the teat apex was not a significant factor driving the risk of IMI in early lactation cows. Other factors, which could potentially account for NASM's ability to prevent major pathogens IMI, as has been proposed in previous research (Reyher et al., 2012), including the presence of *in vitro* antimicrobial activity (Braem et al., 2014; Carson et al., 2017; Toledo-Silva et al., 2022).

Relationship between non-aureus Staphylococci and Mammaliicocci taxonomy and presence of in vitro antimicrobial activity

In our study, nine different NASM species isolated were identified from teat apices of organic dairy cows. This findings align with previous research (Braem et al., 2014; Carson et al., 2017), highlighting important differences among various NASM species. In these studies, the assessment of NASM's antimicrobial activity relied on the cross-streak method. This methodology revealed that between 9.1% and 13% of the isolates showed signs of antimicrobial activity (Braem et al., 2014; Carson et al., 2017). In our study, agar dilution methods were used (Wiegand et al., 2008). This methodology not only aligns with the cross-streak technique but also provides a quantitative measurement of the antimicrobial activity of bacterial isolates (Toledo-Silva et al., 2022).

To the best of our knowledge, the quantitative method has only been investigated in 3 different NASM species isolates from the udder of dairy cows, which included *Staphylococcus chromogenes*, *Staphylococcus epidermidis* and *Staphylococcus simulans*. The MIC of these species against *Staphylococcus aureus* ranged between 5.10 and 8.24 CFU/mL ($\log_{10}$) (Toledo-Silva et al., 2022). In our study, variations were observed in the inhibitory activity of NASM on SAU or SUB. Our results suggested that *Staphylococcus haemolyticus* possesses little to no inhibitory activity on SAU or SUB. This finding is consistent with earlier research that showed that SAU was not inhibited by *Staphylococcus haemolyticus* (Carson et al., 2017). In contrast, *Staphylococcus succinus* exhibited the lowest MIC values, with almost fifty percent of these isolates falling within the “top 10” isolates possessing the lowest MIC. This differs from other species, where this proportion did not exceed 20%. In addition, this contradicts prior research showing no inhibitory activity of *Staphylococcus succinus* against SAU (Carson et al., 2017).

These results may be explained by the observation that antibacterial activity in earlier research in humans appeared to be strain-specific (Nakatsuji et al., 2017). Hence, it is plausible that antimicrobial activity of NASM could be attributed more to specific strains rather than distinct species. Investigating the antimicrobial activity across different strains within each of the NASM species was beyond the scope of this project and should be further investigated in the future.

Study validity and limitations

This study investigated the quantitative antimicrobial activity of NASM isolates against SAU and SUB. While this method represents a novel approach that has only recently been described for its use on mastitis pathogens (Toledo-Silva et al., 2022), it also has certain limitations. For instance, the novelty of these methods in mastitis research, coupled with the absence of predetermined MIC cut-off points, complicates the interpretation of results. This makes it challenging to discern whether the absence or presence of inhibition is due to antimicrobial activity or merely a competition for nutrients that creates unfavorable conditions for the growth of target microorganisms in the media (De Vliegher et al., 2004b; Toledo-Silva et al., 2022). To overcome this limitation, we tested the self-inhibition of SAU and SUB, and we found that a considerable number of NASM isolates were able to inhibit the growth of these mastitis pathogens at lower concentrations than those necessary for self-inhibition of SAU and SUB, suggesting the presence of inhibitory activity.

In this investigation, steps to control for potential technical and biological confounders were taken. This included the randomization of samples for processing, matching by important confounders, and assessing the magnitude of confounding by including these variables in the models used in this study. Nonetheless, it is vital to understand that some of our results could be still biased by other unmeasured confounders. For instance, cow-level factors that increased the

likelihood of presence of IMI such as abnormally high or low body condition score, milk leakage (Fernandes et al., 2022), dysfunctional immune response (Sordillo, 2018), or dirty udders (Schreiner and Ruegg, 2003; Compton et al., 2007) could have influenced our results. Moreover, the small sample size and resulting imprecision of estimates could hinder our ability to make inference from the results in this exploratory study. The use of composite milk samples represents a potential limitation of this observational study, considering the lower sensitivity of milk culture to detect IMI (Reyher and Dohoo, 2011; Toft et al., 2019). However, repeated sampling within the first 35 DIM addressed this limitation, improving milk culture sensitivity for IMI detection (Buelow et al., 1996).

Lastly, one major limitation of our study was the fact that almost two thirds of SAU and SSLO IMI were detected in the first postpartum milk sample. This suggests that IMI may have been acquired before calving. This finding is not surprising given the challenges to control mastitis without antibiotics (NMC, 2019) and the increased prevalence of SAU on organic dairy farms (Pol and Ruegg, 2007; Cicconi-Hogan et al., 2013). All the above limits our ability to determine if colonization of NASM with antimicrobial activity preceded the acquisition of SAU or SSLO IMI. Therefore, it is conceivable that other factors not explored in this study, such as the presence of a close-up pen with poor bedding management, high stocking density, high fly presence, and presence of IMI carried across lactations for multiparous cows, may be influencing the results and explaining why some of these animals calved with IMI (Green et al., 2008; De Vliegher et al., 2012; Fernandes et al., 2022).

External validity

This study was conducted in two organic dairy farms located in Minnesota and Colorado. Considering the substantial differences in the prevalence NASM species across different dairy

farms in both this and previous studies (De Visscher et al., 2016; Mahmmod et al., 2018), it is prudent to restrict the generalization of results to dairy farms managed under similar conditions. In addition, because antimicrobial activity differed across different NASM species and even within the species, results should be confirmed including a larger number of isolates representative of different species that can be found on the mammary gland of dairy cows. Finally, to address the wide heterogeneity of NASM antimicrobial activity, strain-level identification is required to identify clades of bacteria and antimicrobial peptides connected to the presence of *in vitro* antimicrobial activity.

CONCLUSIONS

The presence of NASM isolates classified as "top 10" with lowest MIC against SAU was higher in cows without SAU or SSLO IMI (controls), compared to cows that had an IMI by these microorganisms (cases) in early lactation. However, no evidence of an association was found between presence of isolates with "top 10" lowest MIC against SUB and IMI status. In this study, no evidence of an association was found between NASM counts on teat apex and presence of IMI by SAU or SSLO. The inhibitory activity of NASM was different across different NASM species. This study provides initial information about the inhibitory capabilities of naturally occurring NASM isolated against SAU and SUB and contributes to our understanding of the relationship between NASM and udder health in dairy cows.

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DATA AVAILABILITY

The raw sequence data generated in this study has been submitted to the Sequence Read Archive on NCBI (Accession: PRJNA1070794).

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APPENDIX

Tables

Table 1. Descriptive characteristics of enrolled cases (n=34) and controls (n=34)

Item	Controls	Cases
Farm		
A	20.6% (7/34)	20.6% (7/34)
B	79.4% (27/34)	79.4% (27/34)
Parity		
First lactation	47.1% (16/34)	47.1% (16/34)
Second lactation	52.9% (18/34)	52.9% (18/34)
Case type		
Staph aureus only	-	44.1% (15/34)
SSLO only	-	44.1% (15/34)
SSLO and *Staph aureus*	-	11.8% (4/34)
Cows positive at calving	-	64.7% (22/34)
Days in milk new IMI	-	13.2 (12.0)
Days relative to calving teat apex sample †	-2.8 (15.2)	-1.2 (16.5)

Cows positive at calving: Cows with an IMI by *Staphylococcus aureus* or *Streptococcus* spp. and *Streptococcus*-like organisms (SSLO) on the first postpartum sample. † Teat apex sample: gauze sample extracted on the week prior to the first time a cow tested positive for an IMI.

Table 2. Bacterial counts on teat-apex (log10 CFU/square inch of gauze) of controls (referent) and cases (n=71 teat apex samples).

Outcome	Estimated marginal means (SE)		Estimate (95%CI)	*P-value*
	Controls	Cases		
Total bacterial count	1.81 (0.12)	1.91 (0.12)	0.10 (-0.19, 0.40)	0.50
NASM	1.12 (0.18)	1.02 (0.18)	-0.09 (-0.54, 0.35)	0.67
Staph chromogenes	0.26 (0.12)	0.20 (0.12)	-0.05 (-0.34, 0.23)	0.71
Staph haemolyticus	0.21 (0.13)	0.21 (0.13)	-0.005 (-0.33, 0.32)	0.98
Staph sciuri	0.19 (0.10)	0.21 (0.10)	0.02 (-0.23, 0.27)	0.86
Staph succinus [a]	0.17 (0.09)	0.17 (0.09)	-0.004 (-0.24, 0.24)	1.00
Staph xylosus/pseudoxylosus	0.46 (0.09)	0.53 (0.10)	0.06 (-0.17, 0.30)	0.60
Other	0.20 (0.07)	0.12 (0.07)	-0.08 (-0.26, 0.09)	0.35

[a] *Staph succinus*: Model did not account for farm, because it was not present in farm B. [b] Other: *Staph cohnii, Staph devreseii* and *Staph equorum.* NASM: non-*aureus Staphylococci* and *Mammaliicocci*.

Table 3. Presence of non-*aureus Staphylococci* and *Mammaliicocci* (NASM) isolates on teat-apex of controls (referent) and cases (n=71 teat apex samples).

Outcome	Presence on teat apex		OR (95%CI)	*P-value*
	Controls	Cases		
NASM	66.7% (24/36)	62.9% (22/35)	0.85 (0.32, 2.25)	0.74
Staph chromogenes	25.0% (9/36)	22.9% (8/35)	0.89 (0.29, 2.73)	0.84
Staph haemolyticus	25.0% (9/36)	22.9% (8/35)	0.89 (0.29, 2.73)	0.84
Staph sciuri	8.3% (3/36)	5.7% (2/35)	0.66 (0.08, 4.28)	0.66
Staph succinus [a]	11.1% (4/36)	11.4% (4/35)	1.03 (0.23, 4.72)	0.97
Staph xylosus/pseudoxylosus	11.1% (4/36)	17.1% (6/35)	2.17 (0.39, 14.47)	0.38
Other [b]	8.3% (3/36)	2.9% (1/35)	0.31 (0.01, 2.63)	0.29

[a] *Staph succinus*: Model did not account for farm, because it was not present in farm B. [b] Other: *Staph cohnii, Staph devreseii* and *Staph equorum.*

Table 4. Taxonomic identification of isolates harbored from teat apex samples and submitted for taxonomic identification using Matrix-Assisted Laser Desorption/Ionization-Time of Flight mass spectrometer (n=275 isolates) and confirmation for non-*aureus Staphylococci* and *Mammaliicocci* (NASM) using whole genome sequencing (n=83 isolates).

Taxonomy classification	Controls	Cases
Staph aureus	0% (0/134)	1.4% (2/141)
NASM	32.8% (44/134)	27.7% (39/141)
Staph chromogenes	9.0% (12/134)	7.1% (10/141)
Staph cohnii	0% (0/134)	0.7% (1/141)
Staph devreseii	1.5% (2/134)	0% (0/141)
Staph equorum	0.7% (1/134)	0% (0/141)
Staph haemolyticus	9.0% (12/134)	6.4% (9/141)
Staph pseudoxylosus	1.5% (2/134)	2.1% (3/141)
Staph sciuri	2.1% (3/134)	2.1% (3/141)
Staph succinus	4.5% (6/134)	4.3% (6/141)
Staph xylosus	4.5% (6/134)	5.0% (7/141)
Aerococcus viridans	0% (0/134)	1.4% (2/141)
Aerococcus sp.	0.8% (1/134)	2.8% (4/141)
Bacillus sp.	61.9% (83/134)	61.7% (87/141)
Gram-positive rod	4.5% (6/134)	5.0% (7/141)

Table 5. Relationship between species identification and minimum inhibitory concentration (MIC) of non-*aureus Staphylococci* and *Mammaliicocci* (NASM) against *Staphylococcus aureus* and *Streptococcus uberis* (n=79 isolates).

NASM taxonomy	*Staphylococcus aureus*		*Streptococcus uberis*	
	MIC (SE)	*P-value*	MIC (SE)	*P-value*
Staph chromogenes	3.32 (0.13) [a]	0.001	3.06 (0.16)	0.23
Staph haemolyticus	3.99 (0.14) [b]		3.63 (0.26)	
Staph sciuri	3.32 (0.24) [a]		3.49 (0.30)	
Staph succinus	2.92 (0.17) [a]		2.92 (0.22)	
Staph xylosus/pseudoxylosus	3.05 (0.14) [a]		3.05 (0.17)	
Other	4.39 (0.32) [b]		3.29 (0.49)	

Other NASM: *Staph cohnii, Staph devreseii, Staph equorum.* Different super indexes within the same column indicate statistical differences. Intraclass correlation coefficient cow-ID *Staph aureus*: Not available, *Strep uberis*: Not available.Other: *Staph cohnii, Staph devreseii* and *Staph equorum.* Only isolates in which MIC was determined were included for analysis; this included a total of 75 isolates for *Staph aureus*: *Staph chromogenes:* 100% (19/19), *Staph haemolyticus*: 81.0% (17/21), *Staph sciuri*: 100% (6/6), *Staph succinus*: 100% (11/11), *Staph xylosus/pseudoxylosus*: 100% (18/18), other NASM: 75.0% (3/4). For *Streptococcus uberis* MIC was determined in 63 isolates: *Staph chromogenes:* 100% (19/19), *Staph haemolyticus*: 33.0% (7/21), *Staph sciuri*: 100% (6/6), *Staph succinus*: 100.0% (11/11), *Staph xylosus/pseudoxylosus*: 100% (18/18), other NASM: 50.0% (2/4).

Table 5.6. Relationship between non-*aureus Staphylococci* and *Mammaliicocci* (NASM) identified species and isolates classification as the top 10 with the lowest minimum inhibitory concentration (MIC) against *Staphylococcus aureus* and *Streptococcus uberis* (n=79 isolates)

Outcome	NASM taxonomy	Presence of isolates with top 10 lowest MIC	*P-value*
Inhibition *Staph aureus*	*Staph chromogenes*	10.5% (2/19)	0.04
	Staph haemolyticus	4.8% (1/21)	
	Staph sciuri	16.9% (1/6)	
	Staph succinus	45.5% (5/11)	
	Staph xylosus/pseudoxylosus	5.6% (1/18)	
	Other	0.0% (0/4)	
Inhibition *Strep uberis*	*Staph chromogenes*	10.5% (2/19)	0.06
	Staph haemolyticus	4.8% (1/21)	
	Staph sciuri	0% (0/6)	
	Staph succinus	45.5% (5/11)	
	Staph xylosus/pseudoxylosus	11.1% (2/18)	
	Other	0.0% (0/4)	

Other: *Staph cohnii*, *Staph devreseii* and *Staph equorum*.

Figures

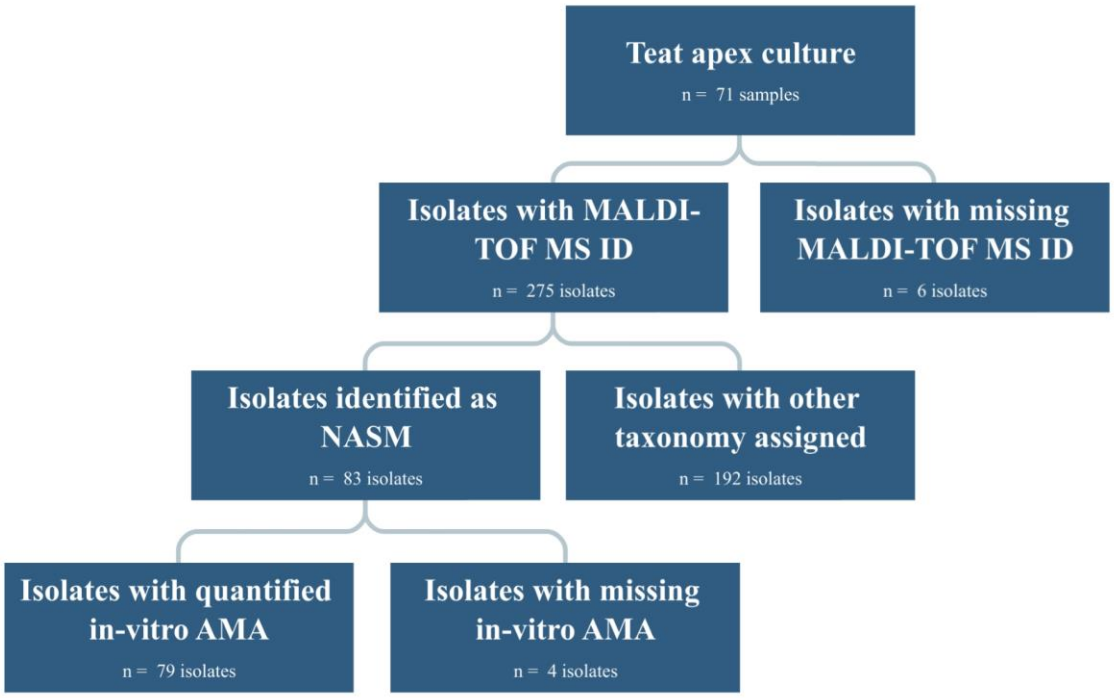

Figure 1. Outline of the study with number of observations lost at each step. NASM: non-*aureus Staphylococci* and *Mammaliicocci*. AMA: antimicrobial activity.

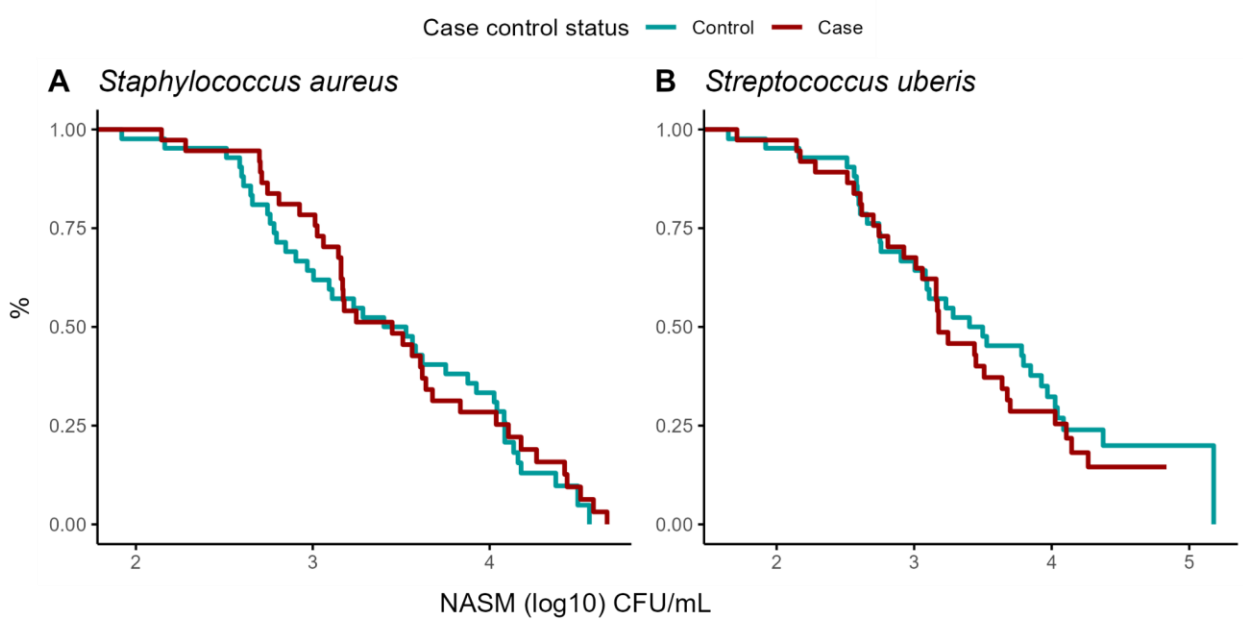

Figure 2. Kaplan meier plots showing presence of growth of *Staphylococcus aureus* and *Streptococcus uberis* at different concentrations of non-*aureus Staphylococci* and *Mammaliicocci* (NASM) on cases and controls (Referent group) (n=79 isolates). *Staphylococcus aureus*: HR (95%CI): 0.92 (0.56, 1.49), *P* =0.72. *Streptococcus uberis*: HR (95%CI): 1.23 (0.74, 2.08), *P*=0.45.

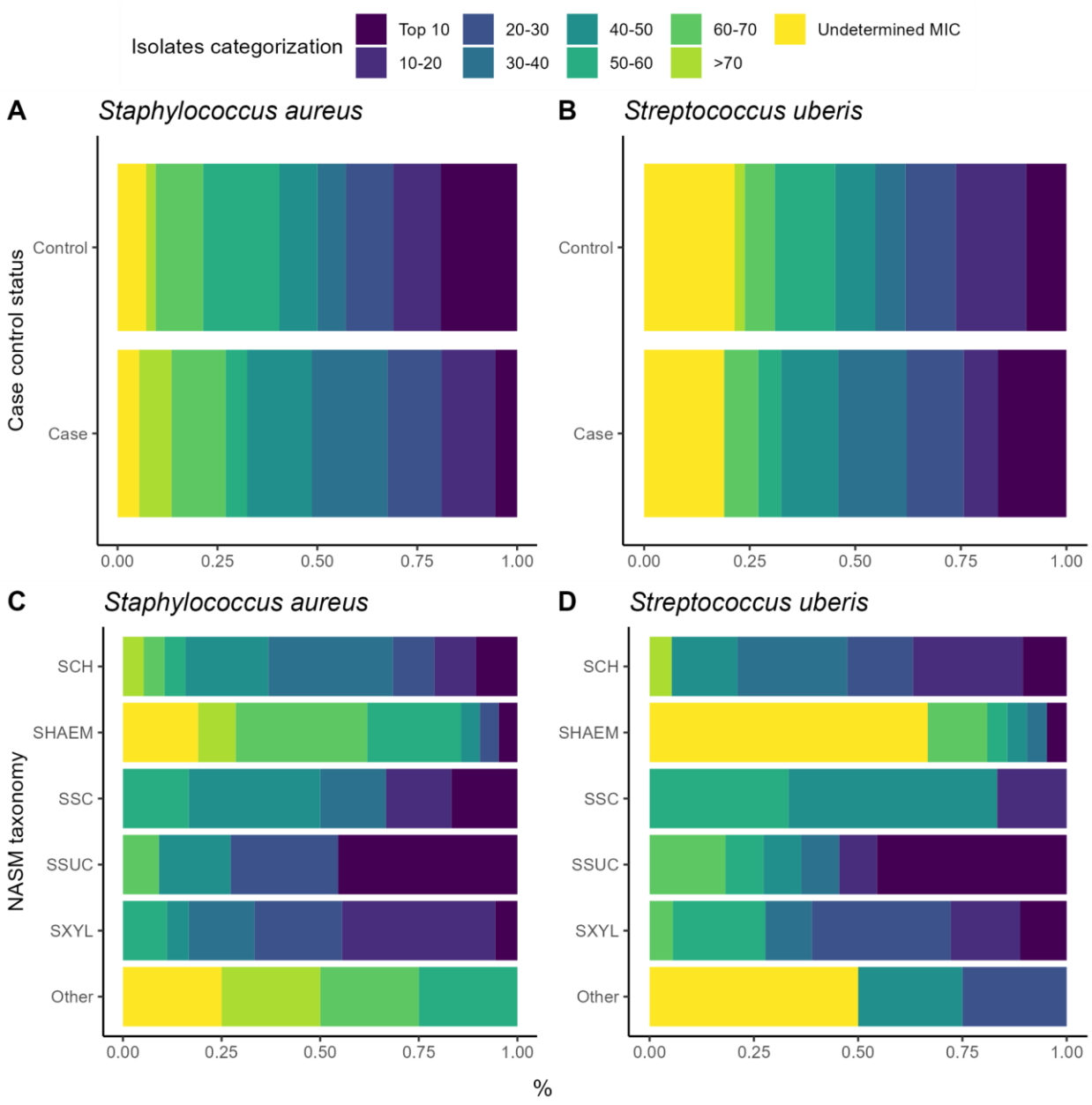

Figure 3. Ranking of 79 non-*aureus Staphylococci* and *Mammaliicocci* (NASM) isolates by minimum inhibitory concentration (MIC) against *Staphylococcus aureus* and *Streptococcus uberis* harbored from teat apex stratified by case control status (panel A/B) and assigned species (panel C/D) (n=79 isolates). Undetermined MIC: NASM isolates that showed growth of *Staphylococcus aureus* or *Streptococcus uberis* at the maximum investigated concentration on media. SCH: *Staphylococcus chromogenes*, SHAEM: *Staphylococcus haemolyticus*, SSC: *Staphylococcus sciuri*, SSUC: *Staphylococcus succinus*, SXYL: *Staphylococcus xylosus/pseudoxylosus*, Other: *Staphylococcus cohnii*, *Staphylococcus devreseii* and *Staphylococcus equorum*.

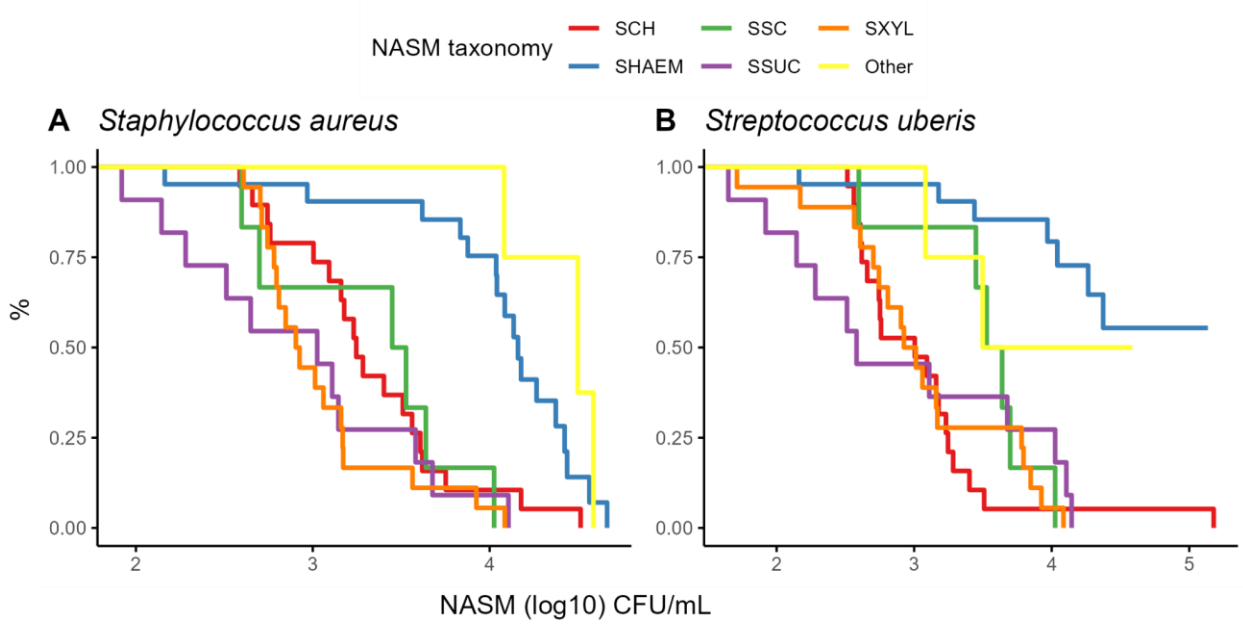

Figure 4. Kaplan meier plots showing presence of growth of *Staphylococcus aureus* and *Streptococcus uberis* at different concentrations of non-*aureus Staphylococci* and *Mammaliicocci* (NASM) for the different species isolated in this study (n=79 isolates). SCH: *Staphylococcus chromogenes*, SHAEM: *Staphylococcus haemolyticus*, SSC: *Staph sciuri*, SSUC: *Staphylococcus succinus*, SXYL: *Staphylococcus xylosus/pseudoxylosus*, Other: *Staphylococcus cohnii*, *Staphylococcus devreseii* and *Staphylococcus equorum.* Type III *P-value Staphylococcus aureus* < 0.001. Type III *P-value Streptococcus uberis* < 0.001.