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 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.

20 **IMPORTANCE**

21 Organic dairy farms need novel strategies to control mastitis. Non-*aureus Staphylococci*
22 and *Mammaliicocci* (**NASM**) have been proposed as potential targets for the development of
23 products to control mastitis, however, their protective activity against major mastitis pathogens
24 requires further investigation. This study provides initial information about the inhibitory
25 capabilities of naturally occurring NASM isolated against mastitis pathogens and its relationship
26 with udder health in organic dairy cows.

27 **INTRODUCTION**

28 Mastitis is the leading cause of morbidity (USDA, 2007) and milk production loss in
29 dairy farms (Heikkila et al., 2018). Although preventive measures are important for the control
30 of mastitis, antimicrobials are still a major component of on-farm contagious mastitis control
31 (Ruegg, 2017). However, the availability of antimicrobials for dairy farmers and veterinarians is
32 at-risk due to concerns about antimicrobial resistance to medically important antibiotics and
33 policy restrictions on antimicrobial use in livestock (Rajala-Schultz et al., 2021; McCubbin et al.,
34 2022; Ruegg, 2022). This limitation is especially remarkable on United States organic-certified
35 dairy farms, where regulations for production of organic milk include a strict prohibition against
36 the use of antimicrobials and other synthetic substances (NMC, 2019).

37 Prior data from our group showed that *Staphylococcus aureus* (**SAU**) and *Streptococcus*
38 spp. had a high prevalence and persistence in the mammary gland of dairy cows in organic farms
39 in the first 35 days of lactation (Peña-Mosca et al., 2023b). Moreover, this study showed that
40 SAU and *Streptococcus* spp. in early lactation were associated with a higher risk of high somatic
41 cell count (**SCC**) during the first 180 days of lactation in organic dairy cows (Peña-Mosca et al.,
42 2023a). Non-*aureus Staphylococci* and *Mammaliicocci* (**NASM**) are also highly prevalent in

43 milk samples (De Buck et al., 2021; Peña-Mosca et al., 2023b), but unlike SAU and
44 *Streptococcus*, NASM have been associated with increased milk production (Piepers et al.,
45 2010). Indeed, the presence of NASM has been associated with decreased risk for intramammary
46 infection (**IMI**) with SAU and *Streptococcus* spp., suggesting a potential protective effect of
47 NASM against these two pathogens (Reyher et al., 2012). *In vitro* studies using isolates obtained
48 from the mammary gland of dairy cows have shown that NASM are capable of inhibiting the
49 growth of SAU (De Vliegher et al., 2004a; Carson et al., 2017; Toledo-Silva et al., 2022). Using
50 the cross-streak method for inhibitory testing, these studies reported that between 9.1% and
51 14.3% of the isolates were able to inhibit the growth of SAU (Carson et al., 2017; Toledo-Silva
52 et al., 2022). Recently, agar dilution methods have also been utilized to investigate the inhibitory
53 capabilities of NASM species on mastitis pathogens (Toledo-Silva et al., 2022). Moreover,
54 studies in humans have also reported that specific NASM strains were associated with a
55 decreased risk of skin colonization by SAU (Nakatsuji et al., 2017).

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

63 The first objective of this study was to investigate the phenotypic *in vitro* antimicrobial
64 activity of NASM isolated from the teat apex of organic cows with and without an IMI caused by
65 SAU or *Streptococcus* spp. and *Streptococcus*-like organisms (**SSLO**) on the week preceding

66 IMI diagnosis. We hypothesized that NASM isolates from cows without an IMI caused by SAU
67 or SSLO (i.e., controls) would exhibit higher *in vitro* inhibitory activity against SAU and
68 *Streptococcus uberis* (**SUB**) compared to NASM isolates from cows with SAU or SSLO IMI
69 (i.e., cases). Our second objective was to investigate the relationship between the presence of an
70 IMI caused by SAU or SSLO and NASM counts on teat-apex swab samples collected the week
71 prior to the diagnosis of SAU or SSLO IMI. We hypothesized that the NASM counts from the
72 teat apex samples would be higher in organic cows without an IMI compared to cows with an
73 IMI. Lastly, our third objective was to explore the relationship between the assigned taxonomy
74 of NASM species and the presence of inhibitory activity against SAU and SUB. We
75 hypothesized that there will be differences in the *in vitro* inhibitory across different NASM
76 species, with a few specific species showing high *in vitro* antimicrobial activity.

77 **MATERIALS AND METHODS**

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

85 ***Inclusion criteria***

86 Only cows from USDA organic certified dairy farms were enrolled in this study. Farms,
87 from different regions of the United States and with different herd sizes, were selected for the
88 study. Enrollment was based on willingness to participate, and proximity to the Universities

89 involved in the study. This study was part of a larger research initiative to investigate potential
90 associations between the udder microbiome and udder health (Dean et al., 2021). For this
91 purpose, cows from 5 organic dairy farms were enrolled 8 weeks before calving and followed up
92 during the first 5 weeks of lactation (Peña-Mosca et al., 2023b). In this manuscript, data from
93 only two of these farms were included, as teat apex samples for bacteriological culture were only
94 collected in these two farms due to limited research labor availability. Specifically, this project
95 used samples collected from a subset of 114 cows (Farm A [n =21] and Farm B [n =93]) out of
96 the 596 cows enrolled in the main research project. Farm A samples were collected between
97 August 2019 and January of 2020 and Farm B samples were collected between February and
98 July of 2021.

99 ***Implementation of case control study***

100 Cases were defined as cows that had an IMI caused by SAU or SSLO during the first 35
101 DIM. Controls, with 1 control chosen for each case, were randomly chosen from the list of
102 sampled cows that did not have an IMI caused by these microorganisms during the first 35 DIM.
103 It should be emphasized that milk samples collected from both cases and controls might have
104 contained an IMI caused by other microorganisms (e.g., gram-negative bacteria, NASM, etc.).
105 However, these infections were not considered during the process of selecting cases and controls
106 or in the subsequent analysis. Cases and controls were individually matched by farm, lactation
107 number, and DIM of when SAU or SSLO IMI were diagnosed (±7 DIM). From each selected
108 cow, one teat apex sample from the week prior to the diagnosis of a SAU or SSLO IMI was
109 processed using the protocol outlined below. Because 3 cows did not have a teat apex sample
110 available in the week prior to first diagnosis of SAU or SSLO IMI, 2 samples were processed for

111 these animals from the 2 closest available samples surrounding IMI diagnosis, resulting in a total
112 of 71 samples from 68 cows (34 cases and 34 controls).

113 ***Teat apex sample collection***

114 All enrolled cows were sampled weekly from 8 weeks before calving to 5 weeks after
115 calving. First, teats were visually inspected, and any visible debris was removed using a paper
116 towel. Then, using a new pair of disposable gloves, a single sterile pre-moistened gauze was used
117 to thoroughly swab the distal third of all four teats. After sampling, gauze was placed in
118 individual tubes containing a pre-loaded solution of 50% glycerol. Gauze samples were
119 transported in a cooler with ice and stored in -80 °C after arrival to research facilities.
120 Postpartum samples were collected before pre-dipping disinfectant solutions were applied to the
121 teat and before milk samples were collected.

122 ***Teat apex culture and species identification***

123 The methods for culture and species-level identification were adapted from protocols
124 utilized in the Laboratory for Udder Health at the University of Minnesota to process towel
125 samples (Rowe et al., 2019). Before sample processing and to reduce the possibility of batch
126 effects, sample IDs were randomly sorted using Excel (Microsoft Corporation). In accordance
127 with this order, isolates were selected for each sampling processing date in batches of 10 isolates.
128 Gauze samples were thawed at 4 °C overnight and then transferred into 50 mL round-bottom
129 falcon tubes (Corning Inc.). Afterwards, 5 mL of Phosphate-Buffered Saline (**PBS**; Gibco,
130 ThermoFisher Scientific) was added to reduce viscosity. Samples were homogenized carefully
131 by turning tubes upside down twice and vortexing for 10 seconds at low speed. Samples were
132 allowed to stand for 10 minutes, and the homogenization process was repeated once. Following
133 homogenization, a single 1 in 10 serial dilution was done using PBS. A 100 uL aliquot from each

134 sample (i.e. undiluted and 10^{-1}) was inoculated onto Mannitol salt agar (Oxoid). Plates were then
135 incubated in aerobic conditions for 24 hours at 37 °C. Following incubation, bacterial groups
136 were visually assessed and counted from the dilution plate with the optimal number of colonies
137 (25 to 250 per plate). Isolates were then picked from the plate using a sterile calibrated loop
138 (ThermoFisher Scientific), streaked onto blood agar (Hardy Diagnostics) and incubated for 24
139 hours at 37 °C.

140 ***Species identification of isolates***

141 After reaching pure growth, the identity of representative colonies was further
142 investigated using a Matrix-Assisted Laser Desorption/Ionization-Time of Flight (**MALDI-**
143 **TOF**) mass spectrometer (**MS**) (Microflex; BrukerDaltonics Inc.) (Jahan et al., 2021). Mass
144 spectra profiles produced from each isolate were matched against the Biotyper reference library.
145 Confidence scores were used to assign genus- and species-level classifications (≥1.8 and ≥ 2.0
146 for genus- and species- classification, respectively) (Jahan et al., 2021). Non-*aureus*
147 *Staphylococci* and *Mammaliicocci* isolates were then stored for further investigation in -80 °C
148 using commercial cryovials (Hardy Diagnostics). Isolates identified as NASM using MALDI-
149 TOF MS were further investigated using whole genome sequencing. Isolates were first grown on
150 brain heart infusion for 24 hours, centrifuged at 10,000 rpm for 5 minutes, and the pellet was
151 collected in 2 mL sterile polypropylene microcentrifuge tubes (Thermo Fisher Scientific).
152 Following this procedure, DNA extraction was performed utilizing commercial kits and
153 following manufacturer's protocol (Qiagen's DNeasy 96 Blood & Tissue Kit, Qiagen). The
154 amount and quality of extracted DNA was evaluated using PicoGreen fluorometer and
155 NanoDrop spectrophotometer (ThermoFisher Scientific). Subsequently, library preparation was
156 performed using the DNA Prep Sample Preparation Kit (Illumina Inc.), and libraries were pooled

157 for 2x300 paired-end sequencing on the Illumina MISEQ platform, with a targeted depth of 50x
158 coverage across the *Staphylococcus* spp. genome for each library. Quality control of raw
159 sequencing data was performed using FastQC (Andrews, 2010) and MultiQC (Ewels et al.,
160 2016). Trimmomatic was utilized to trim low quality bases and adapters from raw reads (Bolger
161 et al., 2014). Genomes were assembled utilizing SPAdes version 3.15.5 (Bankevich et al., 2012),
162 and assembly quality was evaluated using Quast version 5.2.0 (Gurevich et al., 2013). Species
163 identification of isolates was performed utilizing GTDB-Tk version 1.7.0 (Chaumeil et al., 2020)
164 and GTDB release 202 (Parks et al., 2022) as implemented in KBase (Arkin et al., 2018).

165 ***Milk sample collection***

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

175 ***Milk pooling***

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

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

182 ***Milk culture***

183 Using a cotton swab, approximately 100 µL of milk were plated onto Columbia CNA
184 agar with 5% sheep blood and MacConkey agar. Agar plates were incubated in aerobic
185 conditions at 37 °C for a total of 42 to 48 h. Plates were examined by a trained technician to
186 evaluate the presence of growth of distinct isolates at 18 to 24 hours and 42 to 48 hours. Samples
187 were defined as contaminated if more than 3 different isolates were identified and omitted from
188 further analysis (Dean et al., 2022). Taxonomic assignments of cultured isolates were made using
189 a MALDI-TOF MS as previously described for teat-apex samples. An IMI was defined as a
190 composite sample containing one or more than one colony forming units (10 CFU/mL) of any
191 cultured isolates. Microorganisms were grouped according to their morphological and
192 epidemiological characteristics in different bacterial groups: SAU, NASM (members from
193 *Staphylococcus* genus that were not SAU), SSLO which include members from *Streptococcus*
194 genus, as well as *Streptococcus*-like organisms (e.g., *Enterococcus* spp., *Aerococcus* spp.,
195 *Lactococcus* spp. and *Micrococcus* spp.), gram negative microorganisms (e.g., *Escherichia* spp.,
196 *Klebsiella* spp., *Enterobacter* spp., *Pseudomonas* spp. and *Enterobacter* spp., *Pantoea* spp.) and
197 others (microorganisms that did not belong to any of the previous groups).

198 ***Determination of in vitro antimicrobial activity of NASM isolates using the agar dilution***
199 ***method***

200 The methods utilized in this study were based on procedures previously described by
201 others (Toledo-Silva et al., 2022). In each batch of samples, on day 1, isolates were randomly
202 sorted using Excel (Microsoft Corporation). Following this procedure, isolates that were

203 previously identified using MALDI-TOF MS as NASM were grown for 24 hours in blood agar.
204 If evidence of mixed growth (more than 1 colony type) was present, samples were replated. On
205 day 2, one colony was picked with a 10 uL disposable loop and was grown on brain heart
206 infusion (**BHI**; Research Products International Corporation) media for 18 to 20 hours. The next
207 day, 1 in 10 serial dilutions solutions were created using sterile PBS in a 96 well plate
208 (ThermoFisher Scientific). Bacterial counts (CFU/mL) were estimated by plating 100 uL of the
209 serial dilutions on blood agar plates that were then aerobically incubated for 18 to 20 h at 37 °C.
210 One hundred microliters of bacterial suspensions containing NASM isolates at different dilutions
211 (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}) were added to 10 mL of 2% Tryptic soy agar (**TSA**; Oxoid)
212 inside Falcon round-bottom polypropylene tubes (Corning Inc.). The temperature was examined
213 using a laboratory digital thermometer (Thermco) before the inclusion of the 2% TSA. The
214 media were not used until the temperature reached 50 °C (Toledo-Silva et al., 2022). Tubes were
215 then homogenized and poured into pre-heated (37 °C) TSA plates. Following aerobic incubation
216 for 18 to 20 h, 10 uL of the bacterial suspensions containing approximately 10,000 CFU of SAU
217 (American Type Culture Collection (**ATCC**) strain Seattle 1945; *Staphylococcus aureus* subsp.
218 aureus Rosenbach 25923 ™) and SUB (ATCC strain 01040 J; *Streptococcus uberis* Diernhofer
219 BAA-854 ™) were inoculated onto the top of plates containing different concentrations of NASM
220 isolates and incubated for 18 to 20 h. For each batch of samples, negative controls were included
221 by incubating a 2% TSA agar plate with the inclusion of 100 uL of BHI media only (instead of
222 NASM isolates). Additionally, a 10 µL droplet of PBS was added on top of the media on each
223 plate to serve as an additional negative control, ensuring the absence of PBS contamination.
224 *Staphylococcus aureus* and SUB were incorporated into the media as positive controls, and their
225 self-inhibition was tested to explore the determination of MIC driven by nutrient competition

226 rather than *in vitro* inhibitory activity. Growth of SAU and SUB (i.e., yes vs no) was evaluated
227 by visual examination of each of the plates and comparison with negative control plates within
228 each batch. Minimum inhibitory concentration (**MIC**) was defined as the minimum
229 concentration of NASM (CFU/mL) on the 2% TSA top layer able to inhibit the growth of SAU
230 or SUB.

231 ***Statistical analysis***

232 All analyses were performed in R 4.3.2 (https://www.r-project.org/). Code and output can
233 be found online: https://fepenamosca.github.io/NASM_in_vitro_inhibitory_activity.io/ . Before
234 modeling, descriptive characteristics for numerical variables and categorical outcomes were
235 evaluated using the "table 1" package in R (Rich et al., 2023). Data distribution was evaluated
236 using density plots as implemented in the "car" package in R (Fox et al., 2022). The relationship
237 between IMI status (i.e., cases versus controls) and $\log_{10}$ -transformed bacterial counts from teat
238 apex samples was investigated using linear regression models. The relationship between IMI
239 status and growth (yes or no) of NASM from the teat apex samples was investigated using a
240 generalized linear model with a "binomial" family and "logit" link (logistic regression). For
241 analysis, NASM taxonomy was determined based on the assigned taxonomy using MALDI-TOF
242 MS and whole genome sequencing. It was represented as a six-level multi-level variable
243 (*Staphylococcus chromogenes*, *Staphylococcus haemolyticus*, *Staphylococcus sciuri*,
244 Staphylococcus *succinus*, *Staphylococcus xylosus/pseudoxylosus*, other rare NASM
245 [*Staphylococcus cohnii*, *Staphylococcus devrieseii*, *Staphylococcus equorum*]). To assess the
246 association between NASM taxonomy and concentration to NASM *in vitro* growth inhibition on
247 SUB and SAU, Cox proportional hazards regression was utilized. Observations were right
248 censored when inhibition was still not present at maximum NASM concentration analyzed (i.e.,

249 10^{-7} dilution). Proportional hazards assumption was tested by evaluating the Schoenfeld
250 residuals. Since multiple isolates were harbored from each cow, the non-independence of
251 observations within each enrolled animal was accounted for using the robust sandwich estimator.
252 The NASM MIC ($\log_{10}$, CFU/mL) on SAU and SUB was further explored and compared across
253 cases and controls and different NASM species using mixed linear regression, incorporating
254 cow-ID as a random effect to address the non-independence of observations for isolates harbored
255 from the same cow. Subsequently, isolates were ranked according to their MIC (e.g., 1 to 79) and
256 classified as being in the top 10 isolates with lowest MIC or not ("top 10"). The relationship
257 between IMI status and presence of a "top 10" NASM on the teat apex was investigated using a
258 generalized linear model (logistic regression) with "binomial" family and "logit" link. When
259 performing this analysis by NASM species, we used the Fisher exact test due to the absence of
260 "top 10" isolates for some NASM species. The intraclass correlation coefficient was estimated
261 using the "performance" package (Lüdecke et al., 2021). Estimated marginal means were
262 estimated using the "emmeans" package in R (Lenth et al., 2022). Normality assumption was
263 investigated by evaluation of model residuals and the use of quantile-quantile plots. Multiple
264 comparisons were accounted for with Tukey adjustment as implemented in the "emmeans"
265 package (Lenth et al., 2022). Farm-ID was forced into all models that investigated the
266 relationship between IMI status and NASM inhibitory activity against SAU and SUB. Because
267 farm ID was strongly associated with the prevalence of different NASM species (i.e., each of the
268 NASM species were in most cases highly prevalent in one farm but rarely found in the other one)
269 , this variable was not included in the models comparing *in vitro* inhibitory activity across
270 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 3,000 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 cows in farm A (14/14) were starting their first lactation, while for farm B 46.2% (43/93) of the cows 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**.

291 ***In vitro inhibitory activity of* NASM *isolated from case and control cows against***

292 ***Staphylococcus aureus and Streptococcus uberis***

293 A total of 275 isolates were submitted for MALDI-TOF MS as shown in **Table 2**.
294 Surprisingly, *Bacillus* spp. were the most frequently identified genus in controls (61.9%
295 [83/134]) and cases (61.7% [87/141]). Non-*aureus Staphylococci Mammaliicocci* were identified
296 in 32.8% (44/134) and 27.7% (39/141) of the isolates obtained from the teat apex samples from
297 controls and cases, respectively. The most frequent species identified within this group included
298 *Staphylococcus chromogenes* (8.0% [22/275]) and *Staphylococcus haemolyticus* (7.6%
299 [21/275]). The overall inhibitory activity of NASM on SAU was not different between isolates
300 obtained from cases and controls (HR (95%CI): 0.92 (0.56, 1.49), $P = 0.72$; **Figure 2**). The
301 adjusted MIC ($\log_{10}$, CFU/mL) for SAU was 3.26 and 3.37 for isolates recovered from controls
302 and cases, respectively (Estimate [95%CI]: 0.11 [-0.21, 0.44]; $P = 0.49$**)**. Similarly, we did not
303 find evidence of a difference in the inhibitory activity of NASM on SUB between isolates
304 obtained from cases and controls (HR (95%CI): 1.23 (0.74, 2.08), P=0.45; **Figure 2**). The
305 adjusted MIC ($\log_{10}$, CFU/ml) of NASM against SUB was 3.17 in isolates from controls and
306 3.08 in isolates from cases (Estimate [95%CI]: -0.10 [-0.45, 0.25]; $P = 0.58$).

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

311 When SAU was included in the media as a positive control, the MIC against SAU was
312 3.09. Among the studied NASM isolates, 34.2% (27/52) displayed an MIC below this value
313 (38.1% [16/42] and 30.0% [11/37] for isolates recovered from controls and cases, respectively).

314 Upon the inclusion of SUB in the media as a positive control, its MIC against SUB was 3.61.
315 Twenty percent of the isolates (16/79) displayed a MIC below this value (21.4% [9/42] for
316 isolates from controls and 18.9% [7/37] for those from cases).

317 To explore isolates with high inhibitory activity, we arranged the isolates based on their
318 MIC values against SAU and SUB (**Figure 3A and 3B**). The 10 isolates with lowest MICs
319 displayed a mean (SD) MIC ($\log_{10}$, CFU/ml) of 2.41 (0.26) and 2.16 (0.32) for SAU and SUB,
320 respectively. These isolates showed MICs that ranged between 1.92 to 2.66 $\log_{10}$ CFU/mL
321 against SAU growth and 1.65 to 2.56 $\log_{10}$ CFU/mL against SUB growth. Top 10 isolates with
322 lowest MIC against SAU were more prevalent in controls (19.4% (7/36)) than in cases (5.8%
323 (2/35)) (OR (95%CI): 0.25 (0.04, 1.13), $P = 0.07$). However, no evidence of an association was
324 found between IMI status and the likelihood of harboring a 10 top isolate for SUB (controls:
325 11.1% (4/35) vs cases: 14.7% (5/36); (OR (95%CI): 1.33 (0.32, 5.83), $P = 0.69$).

326 ***Relationship between case control status and bacterial counts on teat apex***

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

336 found in half of the samples from farm B (50.0% [7/14]) but showed a low prevalence in farm A
337 (1.8% [1/57]).

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

340 The inhibitory activity of NASM on SAU and SUB differed across different NASM
341 species ($P < 0.001$; **Figure 3, Figure 4**). *Staphylococcus haemolyticus* and other rare NASM
342 species (i.e., *Staphylococcus cohni*, *Staphylococcus devrieseii*, *Staphylococcus equorum*) showed
343 lower inhibitory activity against SAU compared to *Staphylococcus chromogenes* (HR [95%CI:
344 0.28 [0.09, 0.90], P=0.02; and HR [95%CI]: 0.15 [0.03, 0.71], P=0.006, respectively),
345 *Staphylococcus sciuri* (HR [95%CI]: 0.21 [0.08, 0.58]; P = 0.001, and HR [95%CI]: 0.11 [0.02,
346 0.50], P<0.001, *respectively*), *Staphylococcus succinus* (HR [95%CI]: 0.15 [0.05, 0.42],
347 P<0.001; and HR [95%CI]: 0.08 [0.02, 0.36], P<0.001, respectively) and *Staphylococcus*
348 *xylosus*/*pseudoxylosus* (HR [95%CI]: 0.14 [0.05, 0.38], P<0.001; and HR [95%CI]: 0.07 [0.02,
349 0.28], P<0.001, respectively). The adjusted MIC ($\log_{10}$ CFU/ml) of NASM on SAU for different
350 NASM species ranged between 2.92 and 3.99 (**Table 5)**. Similarly, when inhibition of SUB was
351 the outcome of interest, *Staphylococcus haemolyticus* displayed a reduced ability to inhibit SUB
352 compared to *Staphylococcus chromogenes* (HR [95%CI]: 0.11 [0.02, 0.49]), *Staphylococcus*
353 *sciuri* (HR (95%CI): 0.17 (0.06, 0.48)), *Staphylococcus succinus* (HR [95%CI]: 0.13 [0.03,
354 0.50]) and *Staphylococcus xylosus*/*pseudoxylosus* (HR [95%CI]: 0.11 [0.03, 0.43]), all P<0.001).
355 However, the inhibitory activity of *Staphylococcus haemolyticus* against SUB was not different
356 from other rare NASM species (e.g., *Staphylococcus cohnii*, *Staphylococcus devriesei*,
357 *Staphylococcus equorum*) (HR [95%CI]: 0.62 [0.06, 6.31], P=0.99). The adjusted MIC ($\log_{10}$
358 CFU/ml) of NASM against SUB in different NASM species ranged between 2.92 and 3.63

359 (**Table 5)**. The distribution of isolates ranked by their MIC on SAU and SUB growth is shown in
360 **Figure 3C and 3D**. The proportion of isolates classified as "top 10" with respect to SAU and
361 SUB was different between the NASM species (P = 0.04 and 0.06, respectively). Only 4.8%
362 (1/21) and 0% (0/5) of the isolates identified as *Staphylococcus haemolyticus* and other rare
363 NASM species, respectively, were classified as being "top 10" against both SAU and SUB. By
364 comparison, 45.5% (5/11) of *Staphylococcus succinus* isolates were classified as "top 10" against
365 both SAU and SUB (**Table 6**).

366 **DISCUSSION**

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

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

376 Our primary objective was to investigate the *in vitro* antimicrobial activity of NASM
377 isolates and their association with the presence of IMI. We discovered a wide variation in the
378 inhibitory activity across NASM isolates. A considerable number of the NASM isolates did not
379 show inhibitory activity at the maximum concentration studied suggesting the absence of
380 important inhibitory activity for those microorganisms. Moreover, we did not find evidence of a
381 difference in the average inhibitory activity of NASM on SAU or SUB across cases and controls.

382 One possible explanation for these findings is that average antimicrobial activity may not be an
383 important predictor of the risk of IMI, and only high antimicrobial activity against a specific
384 pathogen may be effective to prevent the risk of IMI (Nakatsuji et al., 2017). In this study, we
385 discovered that control cows had the majority of NASM isolates that were within the "top 10"
386 with the lowest MIC against SAU. In addition, cows in the control group had a higher chance of
387 having these highly inhibitory isolates on their teat apices than cows in the case group. Although
388 our results need to be interpreted with caution, given the small sample size and imprecision of
389 estimates, these results suggest the presence of a protective effect of NASM with high *in vitro*
390 antimicrobial activity against SAU on the presence of IMI by SAU or SSLO. These findings are
391 in line with studies performed in humans in which isolates with high phenotypic antimicrobial
392 activity showed a high prevalence on the skin on controls, but were rarely identified on people
393 with SAU skin infections (Nakatsuji et al., 2017). As whole, our results suggest that commensal
394 NASM may provide defense against mammary gland pathogen invasion, albeit these research
395 discoveries require more study.

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

398 The prevalence of NASM on teat apex (64.8%) was similar to that reported by other
399 studies in conventional dairy farms in which 70.1% (Mahmmod et al., 2018) and 71.8% (De
400 Visscher et al., 2016) of the teat-apex samples showed presence of NASM. Consistently, we
401 found a high prevalence of *Staphylococcus chromogenes* and differences in the prevalence of the
402 different NASM species across the enrolled farms, which concurs with these previous reports
403 (De Visscher et al., 2016; Mahmmod et al., 2018). These differences have been previously
404 attributed to differences in management across the enrolled farms (De Buck et al., 2021) and are

405 not surprising, given the differences in the prevalence and distribution of different
406 microorganisms that cause IMI in organic dairy farms (Peña-Mosca et al., 2023b).

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

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

418 In our study, nine different NASM species isolated were identified from teat apices of
419 organic dairy cows. This findings align with previous research (Braem et al., 2014; Carson et al.,
420 2017), highlighting important differences among various NASM species. In these studies, the
421 assessment of NASM's antimicrobial activity relied on the cross-streak method. This
422 methodology revealed that between 9.1% and 13% of the isolates showed signs of antimicrobial
423 activity (Braem et al., 2014; Carson et al., 2017). In our study, agar dilution methods were used
424 (Wiegand et al., 2008). This methodology not only aligns with the cross-streak technique but
425 also provides a quantitative measurement of the antimicrobial activity of bacterial isolates
426 (Toledo-Silva et al., 2022).

427 To the best of our knowledge, the quantitative method has only been investigated in 3
428 different NASM species isolates from the udder of dairy cows, which included *Staphylococcus*
429 *chromogenes*, *Staphylococcus epidermidis* and *Staphylococcus simulans*. The MIC of these
430 species against *Staphylococcus aureus* ranged between 5.10 and 8.24 CFU/mL (log_{10}) (Toledo-
431 Silva et al., 2022). In our study, variations were observed in the inhibitory activity of NASM on
432 SAU or SUB. Our results suggested that *Staphylococcus haemolyticus* possesses little to no
433 inhibitory activity on SAU or SUB. This finding is consistent with earlier research that showed
434 that SAU was not inhibited by *Staphylococcus haemolyticus* (Carson et al., 2017). In contrast,
435 *Staphylococcus succinus* exhibited the lowest MIC values, with almost fifty percent of these
436 isolates falling within the "top 10" isolates possessing the lowest MIC. This differs from other
437 species, where this proportion did not exceed 20%. In addition, this contradicts prior research
438 showing no inhibitory activity of *Staphylococcus succinus* against SAU (Carson et al., 2017).
439 These results may be explained by the observation that antibacterial activity in earlier research in
440 humans appeared to be strain-specific (Nakatsuji et al., 2017). Hence, it is plausible that
441 antimicrobial activity of NASM could be attributed more to specific strains rather than distinct
442 species. Investigating the antimicrobial activity across different strains within each of the NASM
443 species was beyond the scope of this project and should be further investigated in the future.

444 ***Study validity and limitations***

445 This study investigated the quantitative antimicrobial activity of NASM isolates against
446 SAU and SUB. While this method represents a novel approach that has only recently been
447 described for its use on mastitis pathogens (Toledo-Silva et al., 2022), it also has certain
448 limitations. For instance, the novelty of these methods in mastitis research, coupled with the
449 absence of predetermined MIC cut-off points, complicates the interpretation of results. This

450 makes it challenging to discern whether the absence or presence of inhibition is due to
451 antimicrobial activity or merely a competition for nutrients that creates unfavorable conditions
452 for the growth of target microorganisms in the media (De Vliegher et al., 2004b; Toledo-Silva et
453 al., 2022). To overcome this limitation, we tested the self-inhibition of SAU and SUB, and we
454 found that a considerable number of NASM isolates were able to inhibit the growth of these
455 mastitis pathogens at lower concentrations than those necessary for self-inhibition of SAU and
456 SUB, suggesting the presence of inhibitory activity.

457 In this investigation, steps to control for potential technical and biological confounders
458 were taken. This included the randomization of samples for processing, matching by important
459 confounders, and assessing the magnitude of confounding by including these variables in the
460 models used in this study. Nonetheless, it is vital to understand that some of our results could be
461 still biased by other unmeasured confounders. For instance, cow-level factors that increased the
462 likelihood of presence of IMI such as abnormally high or low body condition score, milk leakage
463 (Fernandes et al., 2022), dysfunctional immune response (Sordillo, 2018), or dirty udders
464 (Schreiner and Ruegg, 2003; Compton et al., 2007) could have influenced our results. Moreover,
465 the small sample size and resulting imprecision of estimates could hinder our ability to make
466 inference from the results in this exploratory study.

467 Lastly, one major limitation of our study was the fact that almost two thirds of SAU and
468 SSLO IMI were detected in the first postpartum milk sample. This suggests that IMI may have
469 been acquired before calving. This finding is not surprising given the challenges to control
470 mastitis without antibiotics (NMC, 2019) and the increased prevalence of SAU on organic dairy
471 farms (Pol and Ruegg, 2007; Cicconi-Hogan et al., 2013). All the above limits our ability to
472 determine if colonization of NASM with antimicrobial activity preceded the acquisition of SAU

473 or SUB IMI. Therefore, it is conceivable that other factors not explored in this study, such as the
474 presence of a close-up pen with poor bedding management, high stocking density, high fly
475 presence, and presence of IMI carried across lactations for multiparous cows, may be influencing
476 the results and explaining why some of these animals calved with IMI (Green et al., 2008; De
477 Vliegher et al., 2012; Fernandes et al., 2022).

478 ***External validity***

479 This study was conducted in two organic dairy farms located in Minnesota and Colorado.
480 Considering the substantial differences in the prevalence NASM species across different dairy
481 farms in both this and previous studies (De Visscher et al., 2016; Mahmmod et al., 2018), it is
482 prudent to restrict the generalization of results to dairy farms managed under similar conditions.
483 In addition, because antimicrobial activity differed across different NASM species and even
484 within the species, results should be confirmed including a larger number of isolates
485 representative of different species that can be found on the mammary gland of dairy cows.
486 Finally, to address the wide heterogeneity of NASM antimicrobial activity, strain-level
487 identification is required to identify clades of bacteria and antimicrobial peptides connected to
488 the presence of *in vitro* antimicrobial activity.

489 **CONCLUSIONS**

490 The presence of NASM isolates classified as "top 10" with lowest MIC against SAU was
491 highest in cows without SAU or SUB IMI (controls) compared to cows that had an IMI by these
492 microorganisms (cases) in early lactation. However, no evidence of an association was found
493 between presence of isolates with "top 10" lowest MIC against SUB and IMI status. In this
494 study, no evidence of an association was found between NASM counts on teat apex and presence
495 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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518 **DATA AVAILABILITY**

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

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677

678 **APPENDIX**

679 ***Tables***

680 **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)

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

684

685 **Table 2.** Bacterial counts on teat-apex (log10 CFU/square inch of gauze) of controls (referent)
686 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

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

690 **Table 3.** Presence of non-*aureus Staphylococci* and *Mammaliicocci* (NASM) isolates on teat-
691 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

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

694 **Table 4.** Taxonomic identification of isolates harbored from teat apex samples and submitted for
695 taxonomic identification using Matrix-Assisted Laser Desorption/Ionization-Time of Flight mass
696 spectrometer (n=275 isolates) and confirmation for non-*aureus Staphylococci* and
697 *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)

698

699 **Table 5.** Relationship between species identification and minimum inhibitory concentration
700 (MIC) of non-*aureus Staphylococci* and *Mammaliicocci* (NASM) against *Staphylococcus aureus*
701 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)	

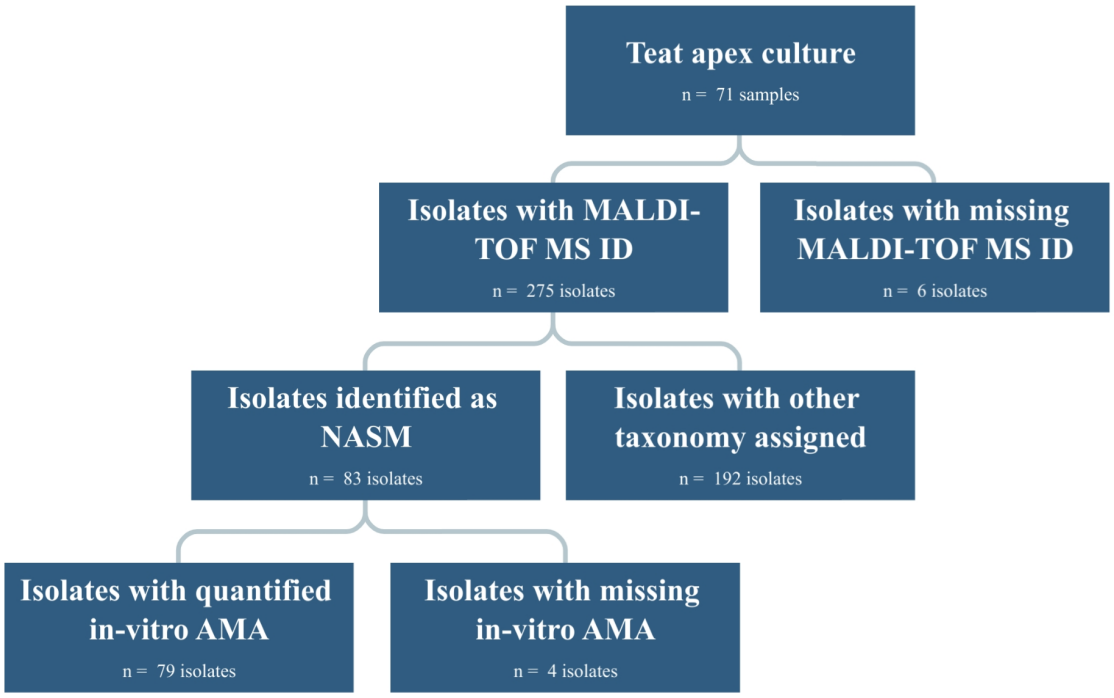
702 Other NASM: *Staph cohnii, Staph devreseii, Staph equorum.* Different super indexes within the
703 same column indicate statistical differences. Intraclass correlation coefficient cow-ID *Staph*
704 *aureus*: Not available, *Strep uberis*: Not available.Other: *Staph cohnii, Staph devreseii* and *Staph*
705 *equorum.* Only isolates in which MIC was determined were included for analysis; this included
706 a total of 75 isolates for *Staph aureus*: *Staph chromogenes:* 100% (19/19), *Staph haemolyticus*:
707 81.0% (17/21), *Staph sciuri*: 100% (6/6), *Staph succinus*: 100% (11/11), *Staph*
708 *xylosus/pseudoxylosus*: 100% (18/18), other NASM: 75.0% (3/4). For *Streptococcus uberis* MIC
709 was determined in 63 isolates: *Staph chromogenes:* 100% (19/19), *Staph haemolyticus*: 33.0%
710 (7/21), *Staph sciuri*: 100% (6/6), *Staph succinus*: 100.0% (11/11), *Staph xylosus/pseudoxylosus*:
711 100% (18/18), other NASM: 50.0% (2/4).

712 **Table 5.6.** Relationship between non-*aureus Staphylococci* and *Mammaliicocci* (NASM)
713 identified species and isolates classification as the top 10 with the lowest minimum inhibitory
714 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)	

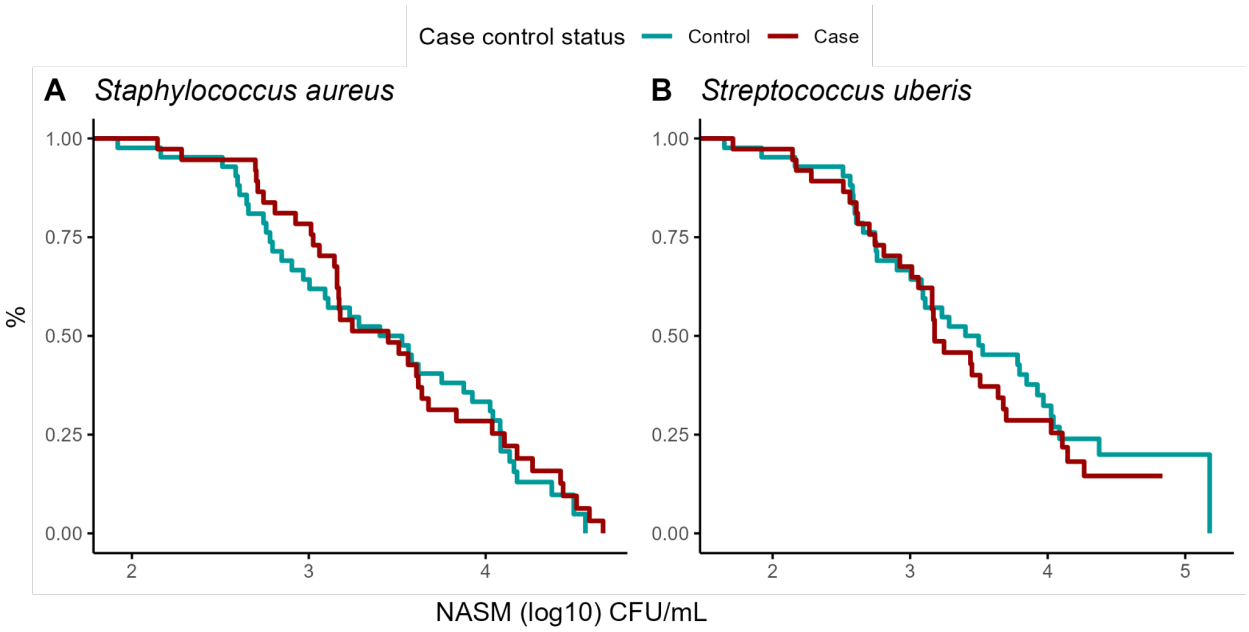
715 Other: *Staph cohnii, Staph devreseii* and *Staph equorum.*

716 ***Figures***



717

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



720

721 **Figure 2.** Kaplan meier plots showing presence of growth of *Staphylococcus aureus* and
722 *Streptococcus uberis* at different concentrations of non-*aureus Staphylococci* and
723 *Mammaliicocci* (NASM) on cases and controls (Referent group) (n=79 isolates). *Staphylococcus*
724 *aureus*: HR (95%CI): 0.92 (0.56, 1.49), *P* =0.72. *Streptococcus uberis*: HR (95%CI): 1.23 (0.74,
725 2.08), *P*=0.45.

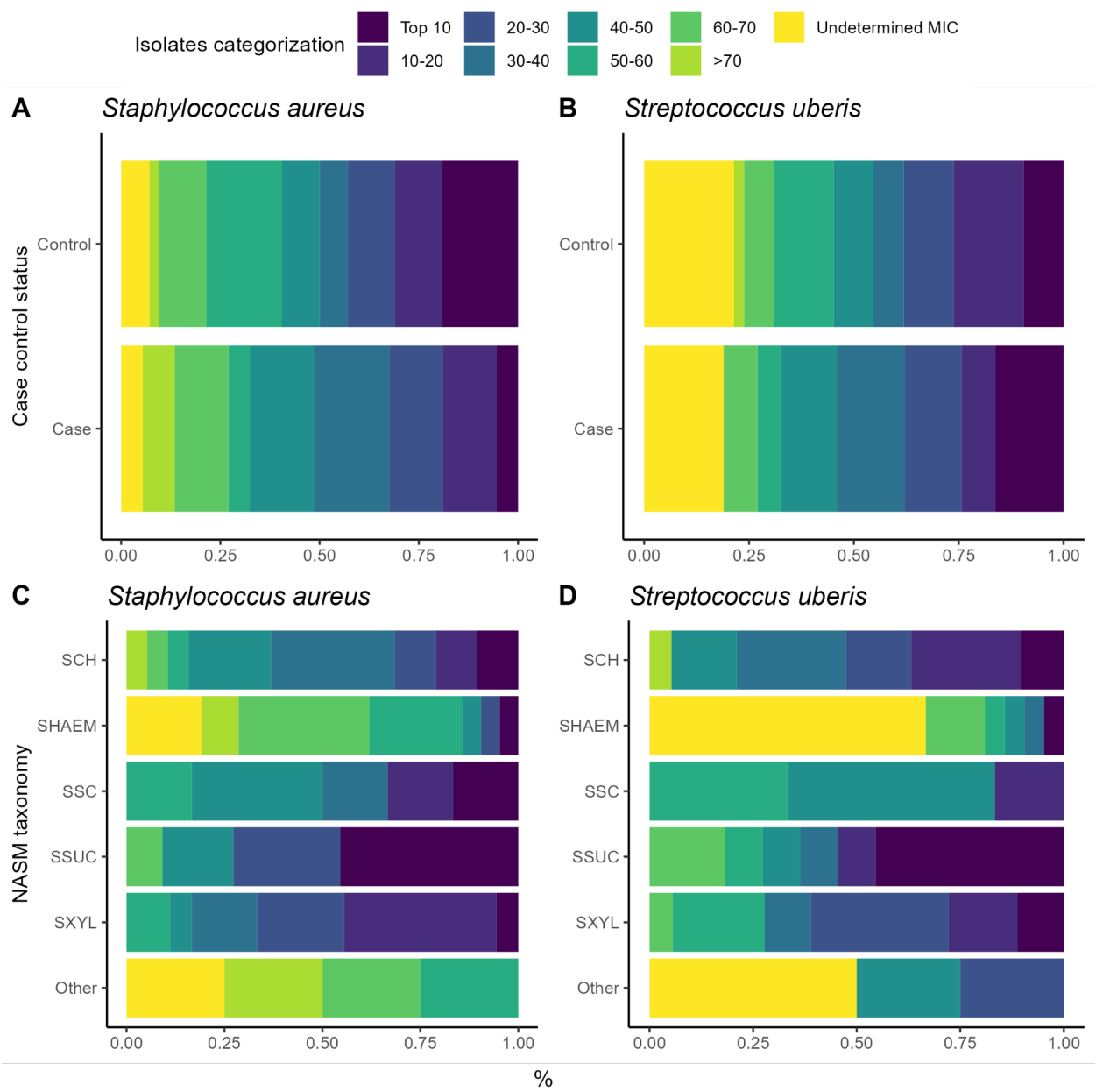


Figure 3. Ranking of 79 non-*aureus Staphylococci* and *Mammaliicocci* (NASM) isolates by minimum inhibitory concentration (MIC) against *Staphylococcus aureus* and *Streptococcus uberis* harbored from teat apex stratified by case control status (panel A/B) and assigned species (panel C/D) (n=79 isolates). 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.*

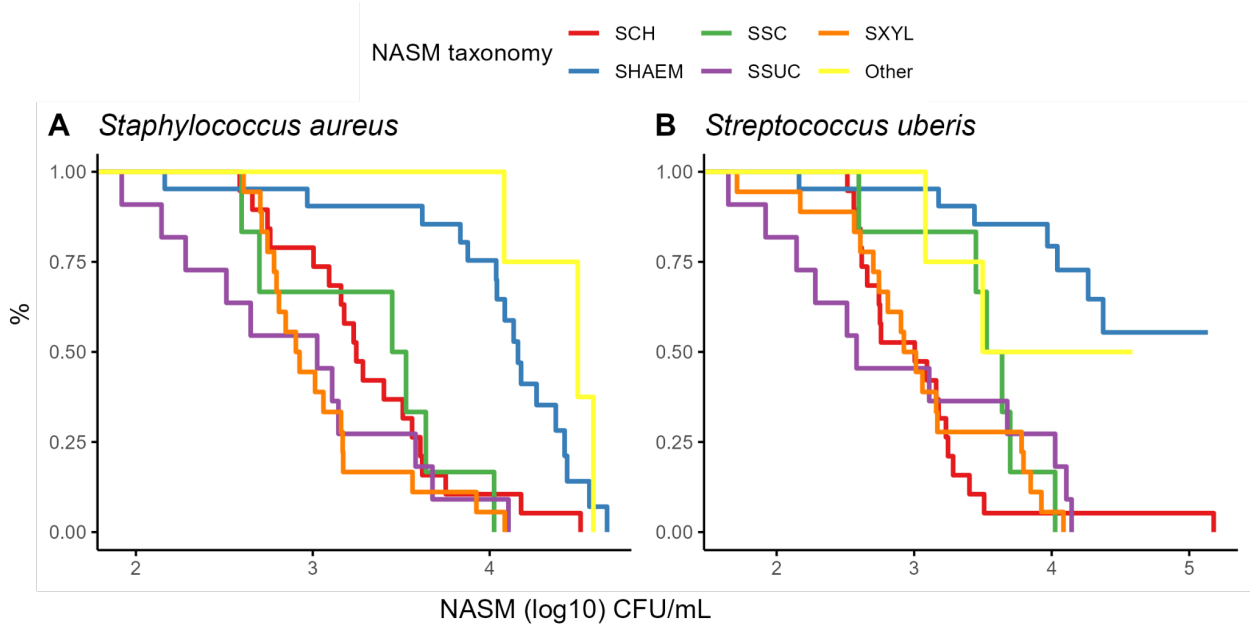
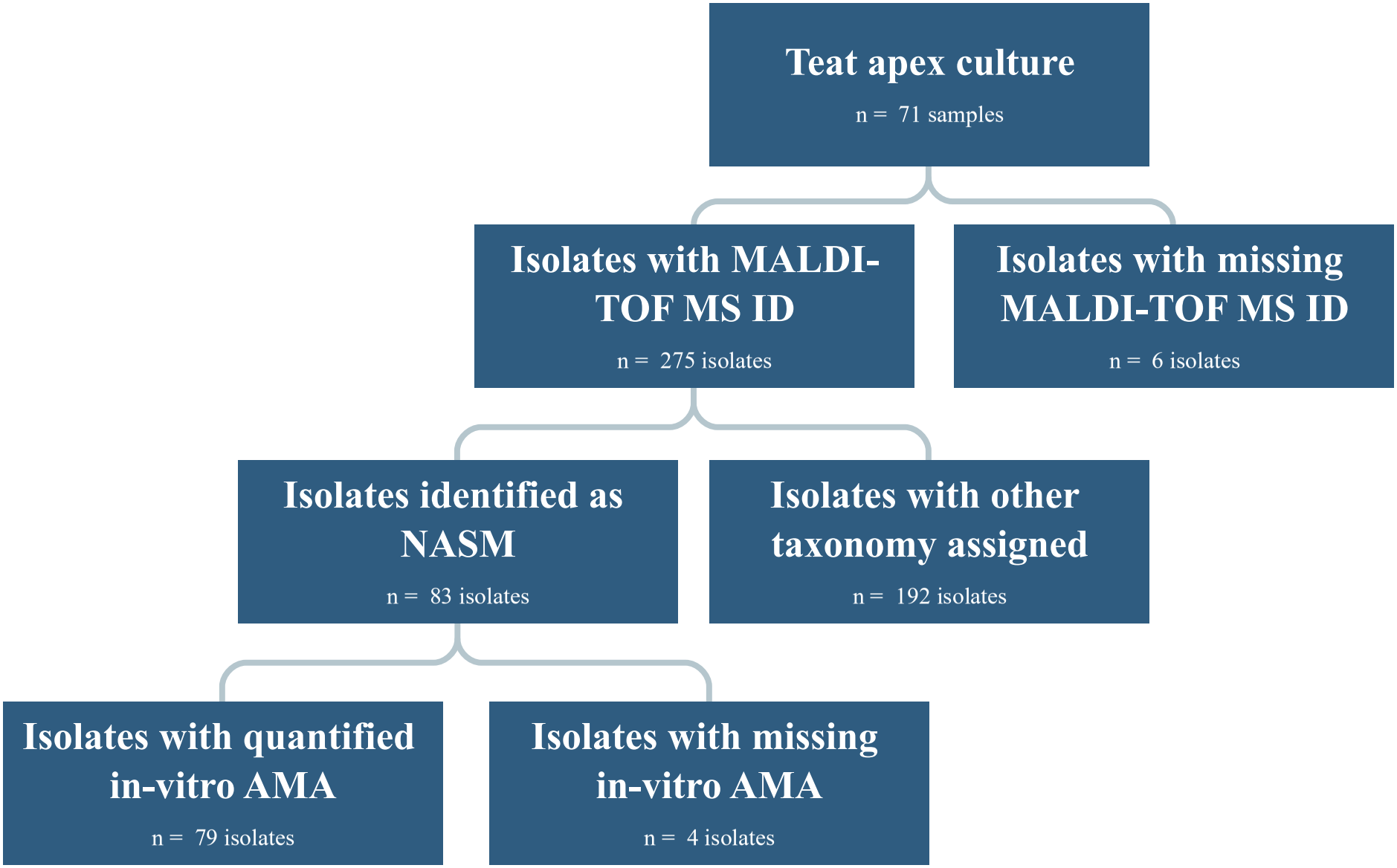
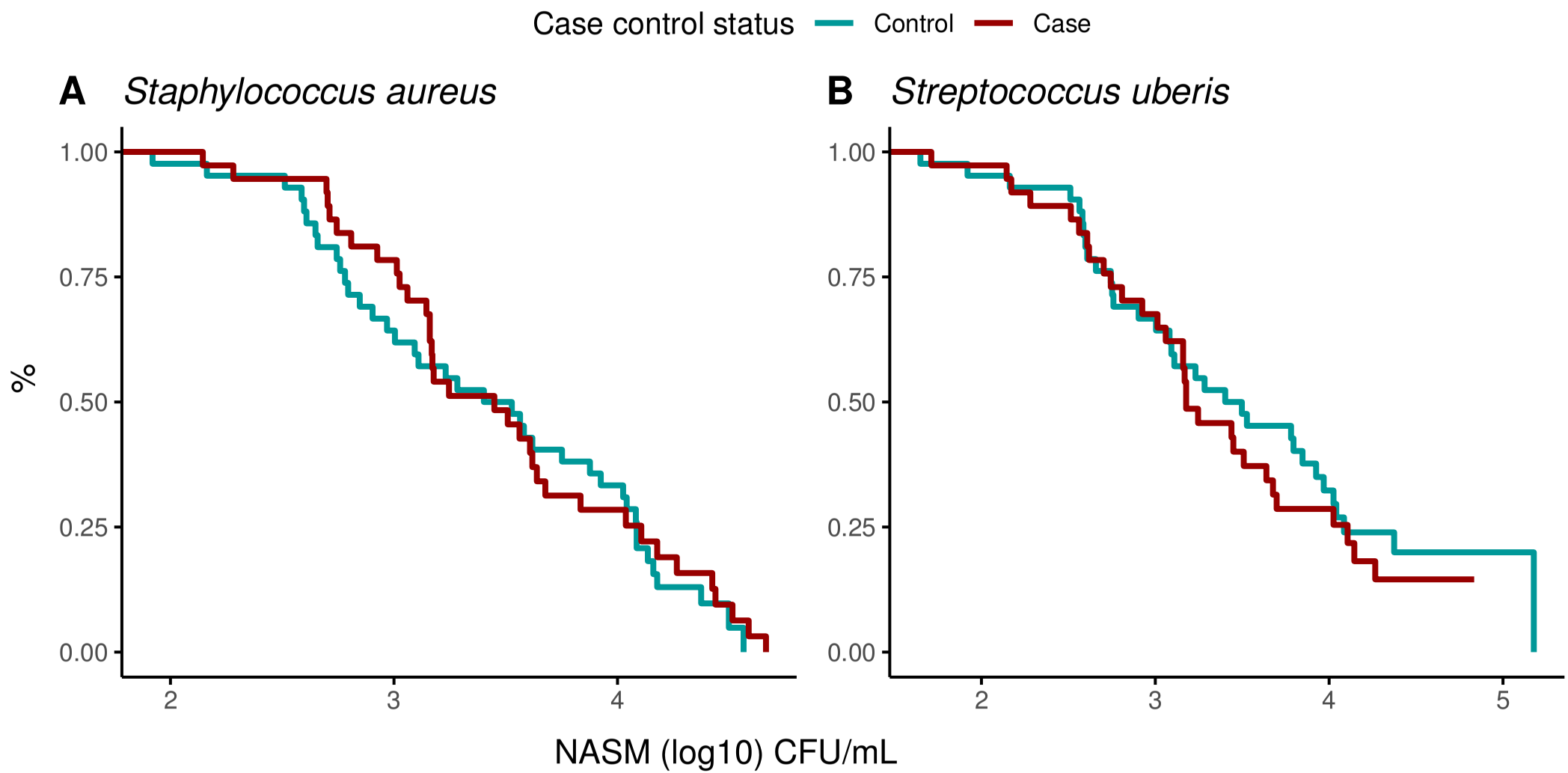
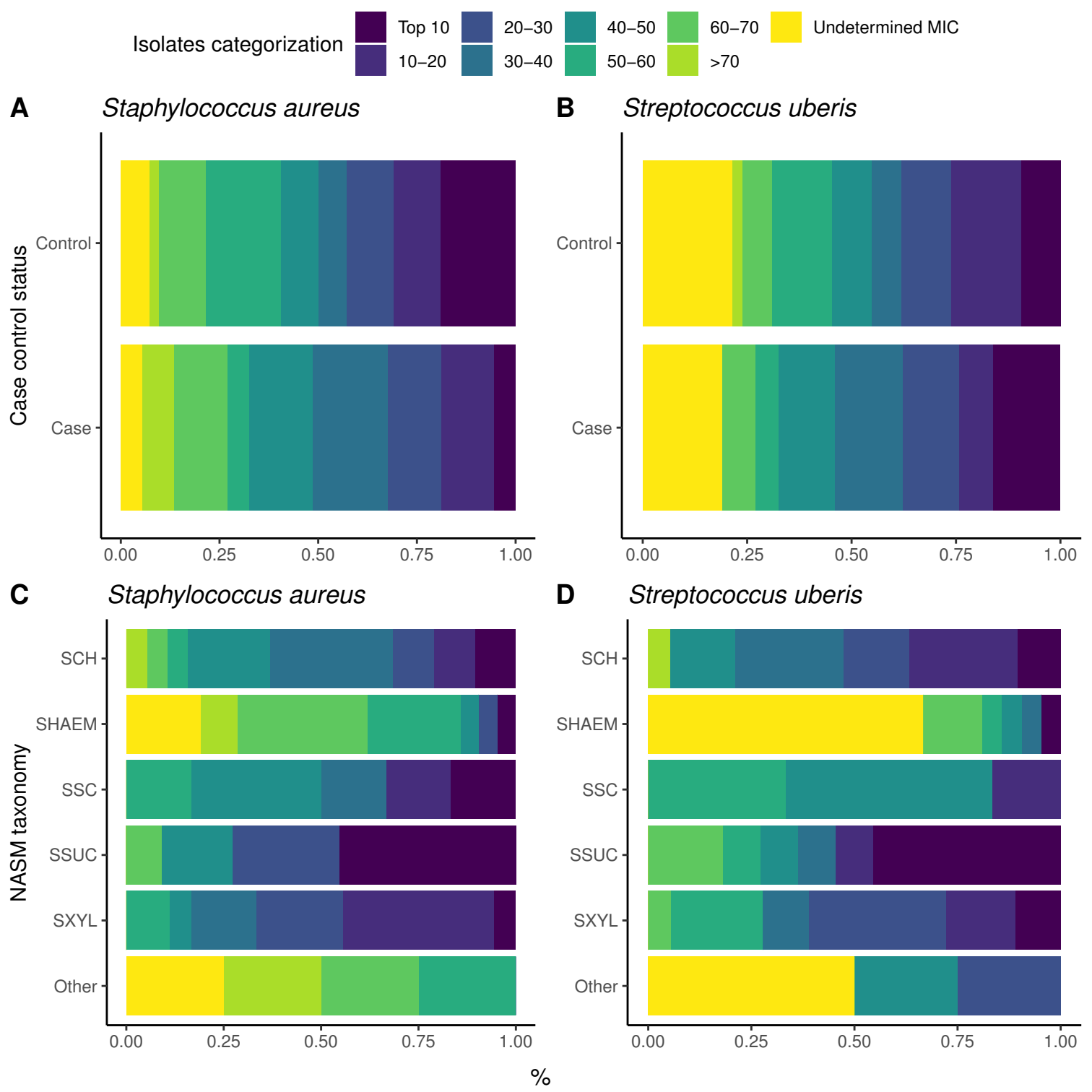
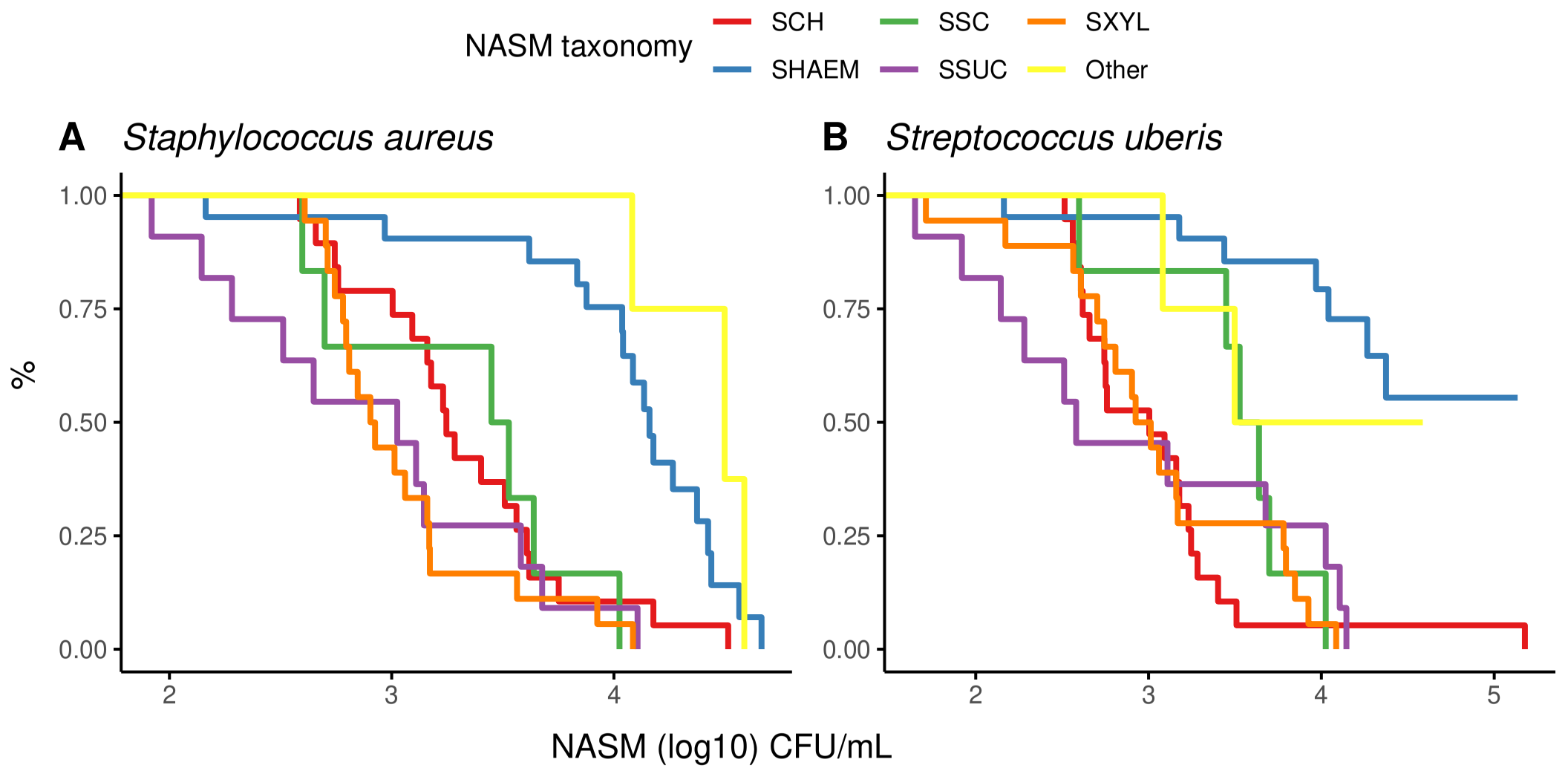


Figure 4. Kaplan meier plots showing presence of growth of *Staphylococcus aureus* and *Streptococcus uberis* at different concentrations of non-*aureus Staphylococci* and *Mammaliicocci* (NASM) for the different species isolated in this study (n=79 isolates). SCH: *Staphylococcus chromogenes*, SHAEM: *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.


Teat apex culture
n = 71 samples
Isolates with MALDI-TOF MS ID
n = 275 isolates
Isolates with missing MALDI-TOF MS ID
n = 6 isolates
Isolates identified as NASM
n = 83 isolates
Isolates with other taxonomy assigned
n = 192 isolates
Isolates with quantified in-vitro AMA
n = 79 isolates
Isolates with missing in-vitro AMA
n = 4 isolates


Case control status
Control
Case
A
Staphylococcus aureus
B
Streptococcus uberis
%
1.00
0.75
0.50
0.25
0.00
2
3
4
5
NASM (log10) CFU/mL


Isolates categorization
Top 10
10–20
20–30
30–40
40–50
50–60
60–70
>70
Undetermined MIC
A
Staphylococcus aureus
B
Streptococcus uberis
Case control status
Control
Case
0.00
0.25
0.50
0.75
1.00
C
Staphylococcus aureus
D
Streptococcus uberis
NASM taxonomy
SCH
SHAEM
SSC
SSUC
SXYL
Other
%


NASM taxonomy
SCH
SHAEM
SSC
SSUC
SXYL
Other
A Staphylococcus aureus
B Streptococcus uberis
%
1.00
0.75
0.50
0.25
0.00
2
3
4
5
NASM (log10) CFU/mL