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Longitudinal microbiological evaluation of subclinical non-aureus staphylococcal intramammary infections in a lentivirus-infected dairy goat herd☆

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ABSTRACT

The objectives of this study were to 1) correlate pre-partum teat skin colonization with non-aureus staphylococcal (NAS) intramammary infection (IMI) in early lactation, and 2) evaluate infection dynamics of subclinical NAS IMI in goats during lactation in a small ruminant lentivirus-infected herd. Pre-partum teat skin swabs (41 goats, 82 halves) and post-partum half-level milk samples (106 goats, 203 halves) were collected at various intervals starting at ≤10 days in milk (DIM) until ≥120 DIM. Teat skin colonization and IMI were defined by culture and strain-typing. The association between the pre-kidding udder-half teat skin sample status and early lactation IMI status for a given species was investigated using McNemar's exact test or logistic regression. Time to IMI elimination and time to new IMI were evaluated by discrete-time survival analysis. Halves with *S. caprae* isolated from teat skin prior to kidding had increased odds of *S. caprae* IMI ≤ 10 DIM. Time to IMI elimination varied as a function of NAS species. Intramammary infections detected > 10 DIM had a higher hazard of elimination (hazard ratio [HR] 5.6, 95% CI 2.8–11.2) than IMI detected ≤10 DIM. The presence of an IMI in the contralateral half was associated with a higher hazard of new IMI (HR 2.1, 95% CI 1.3–3.4) in an uninfected half. Further studies on interventional strategies targeting early IMI and IMI caused by persistent species are warranted.

1. Introduction

Staphylococci are the most common bacterial species causing intramammary infections (IMI) in dairy goats, and as many as 20 different staphylococcal species have been isolated from goat milk (Bergonier et al., 2003; Contreras et al., 2003). Among the staphylococci isolated from goat milk, *Staphylococcus aureus* is considered a major pathogen due to its association with clinical mastitis and increased somatic cell count (SCC; Contreras et al., 2003; Deinhofer and Pernthaner, 1995; Koop et al., 2012), while the non-aureus staphylococci (NAS), primarily coagulase-negative species, are considered less pathogenic. However, elevations in milk somatic cell score (SCS) can be affected by NAS species causing the IMI, duration of the IMI, and stage of infection (Moroni et al., 2005). The NAS species most commonly isolated from goats' milk including *Staphylococcus chromogenes*, *Staphylococcus xylosus*, *Staphylococcus epidermidis*, *Staphylococcus simulans*, and *Staphylococcus caprae* have been reported to cause persistent IMI (Contreras et al., 1997; Koop et al., 2012; Moroni et al., 2005). Because IMI with these species are common, elimination of IMI and reinfection of the udder-half by the same species, but different strain is possible. The use of strain-typing has not been reported in other studies describing NAS IMI persistence over time. Additionally, other than differences between staphylococcal species, previous studies did not report on factors possibly affecting the persistence of IMI, such as parity, stage of lactation,

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or small ruminant lentivirus (SRLV) infection. McDougall et al. (2014) reported a higher IMI cure rate (by any species) in early lactation. Small ruminant lentivirus seropositive status has been associated with bacterial IMI (Ryan et al., 1993; Smith and Cutlip, 1988), and it has been hypothesized that SRLV infection may be associated with a reduction in the mammary gland inflammatory response to bacterial infection (Sanchez et al., 2001), which could increase the risk of new IMI as well as facilitate the persistence of IMI.

Staphylococci are part of the normal mammalian skin flora. In cows, NAS species have been considered environmental, opportunistic, or udder-adapted based on frequency of isolation from environmental sources, body sites surfaces, or milk (De Visscher et al., 2014; Taponen et al., 2008). Additionally, the isolation of *S. chromogenes*, *S. simulans*, or *S. xylosus* from the teat skin of pre-partum heifers was associated with increased odds of IMI caused by the same species after calving (Adkins et al., 2018). There are very limited data on the staphylococcal species isolated from teat skin and other body sites of goats. One study reported that *S. xylosus, Staphylococcus haemolyticus*, and *Staphylococcus sciuri* were commonly isolated from the nasal mucosa or skin including the axillary fold, udder surface and teat, but rarely isolated from milk, whereas *S. epidermidis*, *S. chromogenes*, *S. caprae*, and *Staphylococcus warneri* were commonly isolated from body sites and milk (Valle et al., 1991). To the authors' knowledge, the association between isolation from teat skin and from milk of the same animal has not been reported.

The objectives of the present study were to correlate pre-partum teat skin colonization with NAS IMI in early lactation, and evaluate infection dynamics of NAS IMI in dairy goats during lactation using longitudinal data collection.

2. Materials and methods

2.1. Study design and population

This study was an observational, longitudinal study conducted on an approximately 1400-lactating goat dairy in year-round production in Missouri, USA, between March and December 2016. All procedures were approved by the University of Missouri Animal Care and Use Committee (Protocol #8290). The herd was predominantly composed of Saanen crossbred goats, with some lineage of Alpine, Toggenburg, and La Mancha breeds. The goats were machine-milked in a parlor twice daily. Teats were disinfected pre-milking and post-milking with iodine-based products. Prior to kidding, two groups of multiparous (n = 99) and primiparous (n = 72) goats, respectively, were enrolled in the study based on expected kidding dates. Once the goats kidded, they were moved into a fresh pen containing up to approximately 40 goats where they were housed for 2 d before being moved into the early lactation pen, which included up to approximately 250 goats.

2.2. Sample collection

Approximately 10 d prior to expected kidding date, teat skin swabs were collected from a subset of the enrolled goats (n = 60 goats; n = 22 primiparous and n = 38 multiparous) for bacterial culture. To facilitate sampling, the goats were moved into the milking parlor, and the udder was wiped with a clean, dry cloth (one per goat). All samples were collected by the same person, and gloves were worn at the time of sampling. Each individual teat was swabbed using a sterile cotton-tipped applicator (VetOne, Boise, ID, USA) by three passages over the teat barrel and apex. The swab was placed into a sterile glass tube, capped, transported on ice in a cooler to the laboratory, and stored at –20 °C until analyzed. Each goat ear tag ID and correlated tube ID were recorded; kidding dates and parity data were obtained from farm records.

At the time of kidding, weekly visits were made to the farm to collect milk samples for bacterial culture. Prior to routine milking, the udder was disinfected and dried according to the farm protocol, both teat ends were scrubbed with a gauze sponge soaked with 70% isopropyl alcohol, 2–3 streams of foremilk were discarded from each teat, and individual udder-half milk samples were aseptically collected into 5-ml sterile plastic tubes for bacterial culture. Samples were transported in a cooler on ice packs to the laboratory, and stored at −20 °C until analyzed.

Initial milk samples were collected within 10 d of kidding (≤10 days in milk [DIM]), then once weekly for two more weeks, and then approximately once monthly until at least 120 DIM. Colostrum was not excluded from microbiological culture. Where possible, goats that were still in the lactating herd were sampled toward the end of lactation to document late lactation infection status.

2.3. Bacterial culture

Only the teat skin swabs from goats that started their lactation were cultured. Aerobic bacterial culture of teat skin swabs was performed after thawing the cotton swab at room temperature. The thawed swab was used to inoculate the surface of a mannitol salt agar plate (MSA; Remel, Lenexa, KS, USA) and incubated at 37 °C for 24 h. After 24 h, up to ten colonies, including at least one of each morphologically distinct staphylococcal colony, were transferred onto Columbia blood agar (CBA; Remel, Lenexa, KS, USA) for isolation, and incubated at 37 °C for 24 h. The CBA plates were read at 24 h and the isolates were compared based on morphologic characteristics (colony size, color, and hemolysis). At least one isolate of each distinct morphology was stored in phosphate-buffered glycerol at −80 °C until further identification. When multiple isolates from a single swab had the same morphology on CBA, if ≤4 isolates had the same morphology, only one of them was selected and stored. However, to account for bacterial diversity, when > 4 isolates had the same morphology, 2 of these isolates were selected and stored.

Aerobic bacterial culture of milk was performed after thawing the milk at room temperature (˜22 °C). Approximately 10 µl of milk was plated with a cotton swab on CBA, then incubated at 37 °C for 24 h. The plates were read at 24 h, incubated at room temperature for 24 h, and a final read was performed at 48 h (Roberson et al., 1994). Preliminary identification of colonies was performed based on morphology, catalase test, and coagulase test according to published guidelines (Middleton et al., 2017). An IMI was defined as a culture yielding ≥1 colony forming units (CFU) per inoculum (˜100 cfu/ml). A mixed IMI was defined as a positive culture including two different colony types. Milk cultures yielding ≥3 different colony types were classified as contaminated. Isolates presumptively identified as staphylococci were subcultured on CBA and stored in phosphate-buffered glycerol at −80 °C for further characterization. Non-staphylococcal isolates were not further characterized.

2.4. Species identification

Identification of isolates from milk and from teat skin was initially performed using matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry accordingly to Bernier Gosselin et al. (2018) after growing the isolates in pure culture on CBA. Initially isolates were run using the direct method, and isolates that were not identified by the direct method were retested using the formic acid extraction method (Bernier Gosselin et al., 2018). For both methods, when an isolate was assigned a score of ≥2.0 using the proprietary software (Tomazi et al., 2014) it was considered adequately identified at the species level.

When isolates could not be identified with MALDI-TOF, PCR amplification and sequence analysis of either *rpoB* or *tuf* was performed to identify bacterial species. Lysates were prepared by mixing colonies with 100 µl of Tris-EDTA buffer in microcentrifuge tubes, which were stored at −20 °C until used for PCR amplification. Isolates were subjected to PCR amplification and sequence analysis of either *rpoB* or *tuf*

according to Drancourt and Raoult (2002) and Hwang et al. (2011), respectively. Polymerase chain reaction products were purified using a PureLink PCR purification kit (Invitrogen Life Technologies, Carlsbad, CA, USA), and DNA sequencing was performed at the University of Missouri DNA Core Facility. Gene sequences were compared to the GenBank database using the nucleotide-BLAST algorithm (https://blast.ncbi.nlm.nih.gov). Species identification was assigned when the sequence had ≥97% similarity with a database sequence for *rpoB* (Adkins et al., 2018), or ≥98% similarity and > 0.8% separation from the next species for *tuf* (Hwang et al., 2011). If PCR amplification and sequence analysis of both *rpoB* and *tuf* were unsuccessful, amplification and sequencing were repeated after DNA extraction was performed using a DNeasy kit (Qiagen, Valencia, CA, USA). If after this final step species identification was unsuccessful, the isolate was considered unidentified.

2.5. Strain-typing

When the same staphylococcal species was isolated from a prepartum teat skin swab and the first milk sample collected after kidding, i.e. ≤10 DIM, from the same udder-half, all isolates identified as the same species were characterized using pulsed-field gel electrophoresis (PFGE). Similarly, within udder-half, when an IMI caused by the same staphylococcal species was detected more than once, PFGE was performed on the isolates from the beginning and end of the sample sequence. Briefly, bacterial colonies were incorporated into agarose plugs. Bacterial lysis and DNA digestion with *Sma*I were performed according to Joo et al. (2001). A section of agarose plug containing the digested DNA was incorporated into a 1% agarose gel that was placed in the PFGE machine (CHEF-DR III System, Bio-Rad, Hercules, CA) and immersed in 0.5% Tris-borate-EDTA buffer at 15 °C. DNA fragments were separated by PFGE using a 120° ramp angle and 5–50 s pulse time for 20 h. Paired isolates were run side by side in the gel, and their banding patterns were visually compared. Isolates that had an identical number of bands of the same molecular weight were considered the same strain (Tenover et al., 1995). When the first and last isolates had different PFGE banding patterns, and the IMI lasted more than 2 sample periods, all intervening episodes that yielded the same staphylococcal species were also strain-typed using PFGE. Conversely, when the first and last isolates had the same PFGE banding pattern, it was assumed that any intervening samples also possessed the same strain as the first and last sample in the series.

2.6. SRLV status

Approximately 25–45 d prior to expected kidding date, whole blood samples were collected from the jugular vein of all goats enrolled in the study. Samples were allowed to clot, the serum was separated by centrifugation at 1700 × *g* for 10 min, transferred into a 5-ml plastic storage vial (Sarstedt, Nümbrecht, Germany) and stored at −20 °C until analysis. Each goat ear tag ID was recorded on the blood collection tube at the time of collection, then on the serum storage vial. Serum samples were thawed at room temperature and assayed in duplicate for presence of antibodies against SRLV using a commercial competitive ELISA kit according to the manufacturer's instructions (VMRD, Inc., Pullman, WA, USA). Sample optical density was read on a microplate absorbance spectrophotometer at 630 nm blanked on an empty well. Inhibition was reported as % relative to the optical density of negative controls. If duplicates yielded discrepant results, the assay was repeated. There was agreement between all duplicates when repeated. Sensitivity and specificity of this commercial test is reported as 100% and 96.4%, respectively (Herrmann et al., 2003).

2.7. Statistical analyses

Goats that had a clinical mastitis episode were excluded from the analyses. When an udder-half yielded a contaminated culture result or the species and strain-type could not be determined, these events were treated as missing data. Udder-halves with no milk sample or missing data at ≤10 DIM or at the terminal sampling before 120 DIM were likewise excluded. The following definitions were used to categorize IMI status:

- An uninfected udder-half was defined as a half free of staphylococcal IMI; halves with IMI caused by non-staphylococcal species were still considered at risk of a staphylococcal IMI.
- An IMI was defined as an udder-half with ≥1 sample with a culture positive for a staphylococcal species.
- A new IMI was defined as an IMI that was acquired by a previously uninfected half, i.e. after ≥1 sample negative for a staphylococcal species at the beginning of lactation.
- An IMI was considered eliminated when followed by at least two consecutive samples negative for the same strain, i.e. no growth or became infected with a different strain or different species and remained negative for the same species and/or strain as the original IMI for the remainder of the study period.
- An early IMI was defined as an IMI that was first diagnosed ≤10 DIM.

An udder-half was considered at risk of elimination (cure) at the sampling immediately following the sample when the IMI was diagnosed, and the time of elimination was the day of the first negative sample after the IMI was diagnosed. An uninfected half was considered at risk of a new IMI at the next sample after being considered uninfected, and the time of the new IMI was the day of the first positive sample. Only the first IMI event in each udder-half was considered in the analyses. If the first IMI event was a mixed staphylococcal IMI, the IMI and subsequent samples were censored from time to new IMI analyses and excluded from time to elimination analyses.

For all analyses, parity data were categorized as 1 or > 1. For descriptive analysis, IMI were further classified as eliminated < 30 d, persisting ≥30 d but eliminated before the end of the study, or lasting until the end of the study.

2.7.1. Relationship between isolation of staphylococci from the teat skin and IMI

For the teat swab data, the distribution of NAS species recovered was reported at the half level and goat level. For each species recovered from teat swabs and from milk samples collected at ≤10 DIM, the proportion of halves positive for a given species within each sample type were compared using the McNemar's exact test. When there were no cells in the contingency table with a 0 value, a logistic regression model with random effect at the goat level was performed. Additionally, data were computed at the goat level as the presence of a given species on the teat swab or in the milk sample of either of the teats. Similar to half-level analysis, for each NAS species, proportions of goats positive or negative on teat swabs or milk samples were compared using the McNemar's exact test.

2.7.2. Elimination of IMI

For analyses of IMI elimination, NAS species were categorized as *S. caprae*, *S. epidermidis*, *S. simulans*, *S. xylosus*, *S. lentus*, *Staphylococcus cohnii*, and species with less than 10 IMI per species were grouped together as "other NAS" (in this case, *S. chromogenes*, *S. equorum*, *Staphylococcus auricularis*, *Staphylococcus gallinarum*, *Staphylococcus succinus*, and *Staphylococcus arlettae*), with *S. simulans* as the reference category due to its high prevalence and tendency to cause persistent IMI. Within an IMI, for each observation (sample) where the IMI was at risk of elimination, days at risk of IMI elimination (DRE) were calculated as the number of days from the first positive sample to the day of the observation. For example, for an IMI that was eliminated on a follow-up sample 7 d after the IMI was first detected, DRE on the day of IMI elimination was 7. For an IMI persisting until the end of the study

with follow-up samples collected at 30-d intervals after the IMI was first detected, DRE on the days of follow-up samples would be 30, 60, 90, and 120, respectively. Time to IMI elimination was calculated as the number of days from the first positive sample to the first negative sample. All IMI with at least two follow-up samples that were not contaminated were included in the dataset. Intramammary infections were considered censored when the study ended before the IMI was eliminated. To account for censored data, a discrete-time survival analysis was performed, which took into account the varying length of time between sample collections. A generalized linear model assuming a binary distribution and using a complementary log-log link was used, with a fourth order polynomial function for DRE, and with NAS species involved, early IMI status and SRLV status as main predictors, and adjusted for confounding by parity. When possible (i.e. if model convergence could be obtained), interactions between DRE and each independent variable were evaluated. Robust variance estimates were computed to account for clustering by goat. Analyses were conducted using the SAS 9.4 GENMOD procedure (SAS Institute Inc., Cary, NC, USA). The model was as follows:

$$P(ELIM = 1) = \alpha_d + \beta_1 NAS + \beta_2 early + \beta_3 parity + \beta_4 SRLV \quad (1)$$

Where ELIM is the status of the IMI at the end of the study coded as 1 if eliminated and 0 if still present, and the probability (P) of elimination is modeled as a function of a baseline risk, α_d (a fourth order polynomial function of DRE), β_1 (coefficients for NAS species), β_2 (coefficients for early IMI status), β_3 (coefficients for parity category), and β_4 (coefficients for SRLV status) through the complementary log-log function. In order to evaluate the impact of a hypothetical informative censoring, two scenarios were: 1) all censored IMI were eliminated immediately after censoring and 2) all censored IMI persisted until 300 DIM.

2.7.3. New IMI

For new IMI detected during lactation, survival analyses were performed for all NAS species grouped together, and then independently for each NAS species that were associated with > 10 new IMI (in this case, *S. caprae*, *S. epidermidis*, *S. lentus*, and *S. arlettae*). Generalized linear mixed models assuming a binary distribution and using a complementary log-log link were again used, with a fourth order polynomial function of DIM, with IMI status (presence or absence of a NAS IMI) of the contralateral half on the same sampling occasion and SRLV status as main predictors, and adjusting for confounding by parity, and with a random intercept to account for clustering by goat. Interactions between independent variables were assessed, and interactions with DIM were evaluated when possible (i.e. if model convergence could be obtained). Analyses for NAS species as a group were conducted using SAS 9.4 GLIMMIX procedure. Due to a problem with model convergence in the analyses with individual NAS species, robust variance was used to account for clustering of observations by goat, and analyses were conducted using SAS 9.4 GENMOD procedure (SAS Institute Inc.). The model was as follows:

$$P(NEW = 1) = \alpha_{DIM} + \beta_1 otherhalf + \beta_2 parity + \beta_3 SRLV \quad (2)$$

Where NEW is the status of the uninfected half at the end of the study coded as 1 if a new IMI occurred and 0 if still free of IMI, and the probability (P) of new IMI is modeled as a function of a baseline risk, α_{DIM} (a fourth order polynomial function of DIM), β_1 (coefficient for IMI status of the contralateral half), β_2 (coefficient for parity category), and β_3 (coefficient for SRLV status) through the complementary log-log function. As described before, the impact of a hypothetical informative censoring was investigated using two scenarios where: 1) all censored halves acquired a new IMI immediately after censoring and 2) all censored halves remained free of IMI for 300 days. All analyses were performed using SAS 9.4 (SAS Institute Inc.).

3. Results

Of the 171 goats enrolled in the study before kidding, 134 goats (n = 74 multiparous and 60 primiparous) started their lactation. Among these 134 goats, two developed clinical mastitis, and 26 did not meet the follow-up criteria, i.e. sample at ≤10 DIM, and until ≥120 DIM. Among the remaining 106 goats, one half had a web teat and 8 halves on 8 goats did not meet the follow-up criteria and were eliminated from study, therefore 9 goats contributed a single udder-half to the study. Hence, 203 halves on 106 goats were studied. Primiparous goats represented 40.6% of the goats, and the median (range) parity among the multiparous goats was 2 (2–5). The kidding period of the enrolled goats spanned 54 days. Median (range) DIM on the first sample was 4 (1–9) d. Median (range) DIM at the last sample collection was 249 (121–266) d. Regarding SRLV status, 55 (52.4%) goats were seronegative and 50 (47.6%) were seropositive, and one goat had a missing serum sample. All primiparous goats (n = 43) and 12 multiparous goats were seronegative, and 50 (80.7%) multiparous goats were seropositive.

3.1. Teat skin isolation

Of the 60 goats with teat swabs collected, 41 goats (n = 16 primiparous and n = 25 multiparous) started their lactation. The median (range) time span between collection of teat swabs and kidding was 6 (2–39) d, with 78% of the teat skin swabs being collected ≤10 d before kidding. All swabs were positive for at least one staphylococcal species. A total of 410 staphylococcal isolates were recovered from 82 teats on 41 goats. The median (range) number of isolates stored per teat swab was 5 (1–10), and the median (range) number of different staphylococcal species recovered per teat swab was 3 (1–5). Five staphylococcal isolates could not be identified to the species level. At the goat level, when considering both teats, the number of different staphylococcal species recovered was one for one primiparous goat, two or three species for 13 goats (n = 5 primiparous and n = 8 multiparous), and four to six species for 27 goats (n = 10 primiparous and n = 17 multiparous). Most goats (n = 31) had a similar number of different species on each udder half (difference of ≤1 species between halves). Nine goats had a difference of 2 species between the halves, and one goat had a difference of 3 species. The number of species in common between both halves of a goat was 1 species (n = 19 goats), 2 species (n = 12 goats), 3 species (n = 6 goats), 4 species (n = 1 goat), and 3 goats had no species in common between halves. After exclusion of replicates, the most frequent species detected on teat skin was *S. lentus* (Table 1). Accordingly, *S. lentus* was also the species most commonly recovered from the teat skin on both halves of the same goat (n = 31), whereas other species that were isolated from both halves of a goat included *S. equorum* (n = 11), 5 goats each for *S. arlettae* and *S. xylosus*, 4 goats each for *S. caprae* and *S. nepalensis*, 3 goats for *S. simulans*, and 2 goats

Table 1
Proportion of staphylococcal species isolated at least once from 82 teat skin swabs from 41 goats pre-partum.

Species	Number of halves (%)	Number of goats (%)
Staphylococcus lentus	71 (86.6)	40 (97.6)
Staphylococcus equorum	38 (46.3)	28 (68.3)
Staphylococcus xylosus	31 (37.8)	26 (63.4)
Staphylococcus caprae	23 (28)	19 (46.3)
Staphylococcus arlettae	23 (28)	18 (43.9)
Staphylococcus simulans	13 (15.9)	10 (24.4)
Staphylococcus nepalensis	13 (15.9)	9 (22)
Staphylococcus cohnii	11 (13.4)	9 (22)
Staphylococcus epidermidis	2 (2.4)	2 (4.9)
Staphylococcus sciuri	1 (1.2)	1 (2.4)
Staphylococcus warneri	1 (1.2)	1 (2.4)
Staphylococcus haemolyticus	1 (1.2)	1 (2.4)

Table 2
Number of halves and goats culture positive or negative for non-aureus staphylococcal (NAS) species on the teat skin swab (S) and in the 1st milk (M) sample collected ≤ 10 days in milk organized by NAS species isolated.

	Half level						Goat level					
Species	S−M−	S−M+	S+M−	S+M+	P^a	OR[b]	S−M−	S−M+	S+M−	S+M+	P^a	OR[b]
S. lentus	11	0	64	3	< 0.01	–	2	0	34	3	< 0.01	–
S. equorum	40	2	36	0	< 0.01	–	12	1	25	1	< 0.01	0.48
S. xylosus	46	2	30	0	< 0.01	–	13	1	24	1	< 0.01	0.54
S. caprae	55	1	18	4	< 0.01	12.2	21	0	13	5	< 0.01	–
S. simulans	55	10	11	2	1.0	1.0	22	7	8	2	1.0	0.79
S. epidermidis	75	1	2	0	1.0	–	36	1	2	0	1. 0	–

[a] *P*-value for the McNemar's exact test.
[b] Odds ratio for the McNemar's exact test, e.g. an udder-half that was teat skin culture-positive for *S. caprae* was 12.2 times more likely to have a *S. caprae* IMI within 10 DIM. "– "indicates OR not calculated due to at least one 0 value in the contingency table.

for *S. cohnii*.

For comparison of teat skin isolation with IMI data, all goats with teat swabs that had at least one milk sample collected ≤10 DIM were included. Of the 82 teats with skin swab samples, 26 halves (31.7%) on 20 goats had a NAS IMI within 10 DIM (6 goats with bilateral IMI and 14 with unilateral IMI). Six of these goats were primiparous and 14 were multiparous. For 9 halves on 9 goats, the species causing the IMI was the same as one of the isolates recovered from teat skin, which included *S. caprae* (n = 4), *S. lentus* (n = 3), and *S. simulans* (n = 2). Contingency tables for each species are presented in Table 2. In the logistic regression models at the half level, the odds of having a *S. caprae* IMI after kidding was higher (OR 12.2, $P = 0.04$) for the halves whose pre-partum teat skin was culture-positive for that organism, whereas the association was not significant for *S. simulans* (OR 1.5, $P = 0.76$). Among the pairs of same-species isolates from teat skin and milk, only 2 of 4 pairs of *S. caprae* isolates and 1 of 2 pairs of *S. simulans* isolates were of the same strain-type, whereas all *S. lentus* pairs were different strains.

Historical data collected in the same herd from 17 of the goats also enrolled in the present study provided evidence for persistence of 5 *S. simulans* and one *S. chromogenes* IMI from the previous lactation. Data from the previous lactation was only available for seven goats that had teat skin swabs collected. Twelve udder-halves were culture-negative on the last available milk sample of the previous lactation. One udder-half had a *S. chromogenes* IMI that was also detected at ≤10 DIM but not recovered from teat swab, and one udder-half had a *S. simulans* strain that was recovered from milk in the previous lactation, from the pre-partum teat skin swab, and from milk at ≤10 DIM.

3.2. IMI prevalence and persistence

Fifty-nine halves (43 goats) had an early IMI (≤10 DIM), 54 halves (44 goats) were initially negative and acquired an IMI during the follow-up period, and 66 halves (50 goats) did not have a NAS IMI detected during the study. Additionally, 19 halves (19 goats) had an IMI with insufficient follow-up to determine elimination status, and 5 halves (5 goats) had a mixed NAS IMI on the first positive samples, and were therefore excluded. Staphylococcal IMI detected at ≤10 DIM were caused by *S. simulans* (25/59; 42.4%), *S. xylosus* (11/59; 18.6%), *S. caprae* (7/59; 11.9%), *S. lentus* (5/59; 8.5%), *S. chromogenes* (2/59; 3.4%), *S. cohnii* (2/59; 3.4%), *S. epidermidis* (2/59; 3.4%), *S. equorum* (2/59; 3.4%), *S. aureus* (1/59; 1.7%), *S. auricularis* (1/59; 1.7%), and *S. succinus* (1/59; 1.7%). Median (range) DIM for new IMI acquired during the follow-up period was 63 (11–121) d. New IMI were caused by *S. caprae* (13/54; 24.1%), *S. epidermidis* (9/54; 16.7%), *S. equorum* (8/54; 14.8%), *S. lentus* (8/54; 14.8%), *S. arlettae* (6/54; 11.1%), *S. simulans* (4/54; 7.4%), *S. cohnii* (2/54; 3.7%), *S. xylosus* (2/54; 3.7%), *S. gallinarum* (1/54; 1.9%), and *S. succinus* (1/54; 1.9%).

Based on species-level identification, 67 IMI were persistent for ≥1 sample and staphylococcal isolates from these IMI were subjected to strain-typing. Based on species and strain-typing, 69 (61.6%) IMI were eliminated at some point during the follow-up period, i.e. became negative for the same species and strain-type on at least two consecutive milk samples, whereas 43 (38.4%) persisted until the end of the study. A single *S. aureus* IMI that was detected at more than one sampling was eliminated from the analyses because the isolates did not yield a readable PFGE banding pattern. Numbers and proportions of the different NAS species IMI that were eliminated or not eliminated during the study period are presented in Table 3. The Kaplan-Meier failure curves for different NAS species are shown in Fig. 2. Among IMI detected at ≤10 DIM, 44.1% (26/59) were eliminated before the end of the study, whereas 81.5% (44/54) of IMI detected > 10 DIM were eliminated before the end of the study.

Among 66 same-species udder-half IMI that were successfully assigned a strain-type, 19.7% (13/66) had different strains isolated from the first and last positive samples, including 20% (3/15) of *S. caprae* IMI, 50% (1/2) of *S. chromogenes* IMI, 50% (4/8) of *S. epidermidis* IMI, 33.3% (1/3) of *S. lentus* IMI, 8.3% (2/24) of *S. simulans* IMI, and 18.2% (2/11) of *S. xylosus* IMI. Nine of these 13 IMI lasted more than two sample periods and thus had additional isolates available for strain-typing. Six IMI were classified as eliminated after the first positive sample (or within 30 d), and three were classified as eliminated after two samples ≥30 d apart with the same strain, including two IMI that showed banding patterns changing back and forth over the course of the IMI (Fig. 1). Among the 13 IMI with different strains, the isolates of four IMI had banding patterns that differed by ≤3 bands, and the isolates of the remaining 9 IMI had banding patterns that differed by

Table 3
Number (proportion within species) of intramammary infections (IMI) eliminated after the first positive sample or < 30 d between the first and last positive samples, IMI eliminated after ≥2 positive samples ≥30 d apart, and IMI that lasted until the end of the follow-up period organized by species.

Species	Total	Eliminated[a] after 1 sample or < 30 d	Eliminated[a] after ≥2 positive samples ≥30 d apart	Lasted until end of study
S. simulans	29	8 (27.6)	2 (6.9)	19 (65.5)
S. caprae	20	8 (40)	3 (15)	9 (45)
S. xylosus	13	4 (30.8)	2 (15.4)	7 (53.8)
S. lentus	13	12 (92.3)	0	1 (7.7)
S. epidermidis	11	6 (54.5)	1 (9.1)	4 (36.4)
S. equorum	10	10 (100)	0	0
S. arlettae	6	6 (100)	0	0
S. cohnii	4	3 (75)	0	1 (25)
S. chromogenes	2	1 (50)	0	1 (50)
S. succinus	2	1 (50)	1 (50)	0
S. gallinarum	1	0	0	1 (100)
S. auricularis	1	1 (100)	0	0
Total	112	60 (53.6)	9 (8.0)	43 (38.4)

[a] IMI elimination was defined as two samples negative for the same strain.

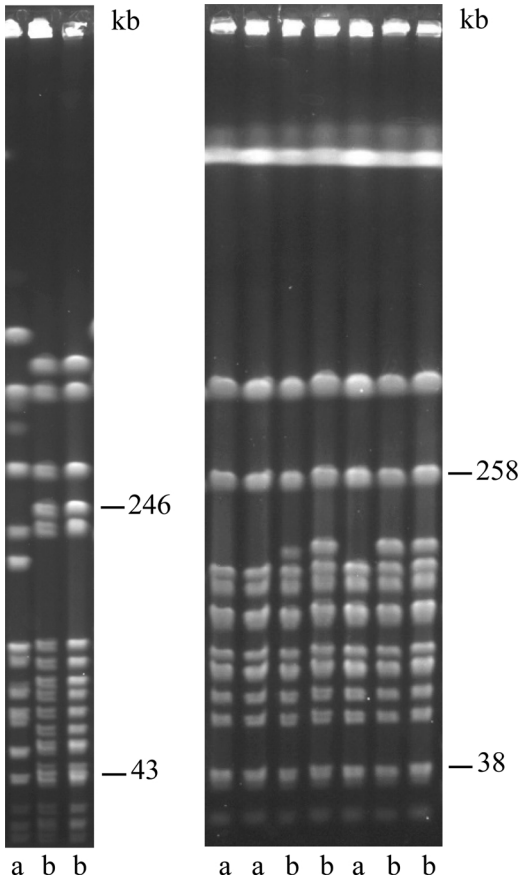

Fig. 1. DNA banding patterns obtained by pulsed-field gel electrophoresis of bacterial isolates collected from intramammary infections caused by *S. epidermidis* (left) and *S. simulans* (right). Each lane represents isolates from different sample periods of the same udder-half. Within each electrophoretogram, different letters indicate different strains. The low number of bands that differ (right) suggests that the isolates are closely related.

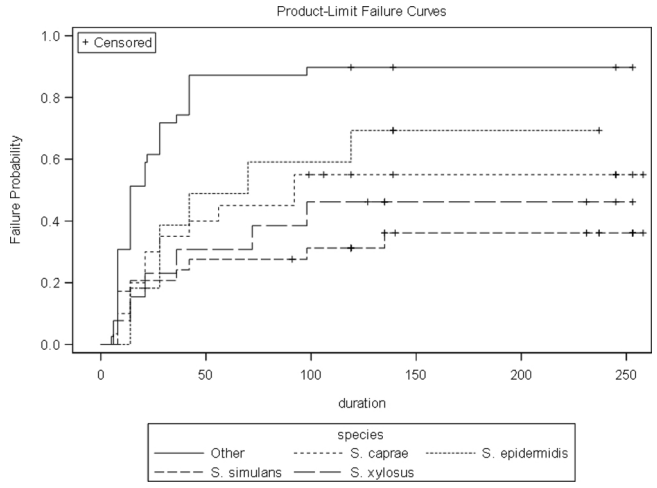

Fig. 2. Kaplan-Meier failure curve (in this case, failure = elimination of intramammary infection [IMI]) showing time to IMI elimination ("duration", in days) by non-aureus staphylococcal species categories, based on data from 112 IMI. "Other" species included *S. auricularis* (n = 1), *S. arlettae* (n = 6), *S. cohnii* (n = 4), *S. chromogenes* (n = 2), *S. equorum* (n = 10), *S. gallinarum* (n = 1), *S. lentus* (n = 13), and *S. succinus* (n = 2).

≥9 bands. Among other species causing IMI lasting more than one sample period, 100% of isolates from IMI caused by *S. cohnii* (n = 1), *S. gallinarum* (n = 1), and *S. succinus* (n = 1) were the same strain at the beginning and end of the IMI.

Some goats (n = 11) had bilateral IMI with the same species of staphylococci for which strain-typing was performed on the staphylococci from both halves as a consequence of the halves having a persistent IMI or a teat skin swab of the same species. For such occurrences, the banding patterns obtained from the strains isolated from both udder-halves were also compared. Among these goats, 75% (3/4) with bilateral *S. caprae* IMI, 17% (1/6) with bilateral *S. simulans* IMI, and one goat with bilateral *S. cohnii* IMI had the same strain in both halves.

3.3. Hazard of IMI elimination

From the 59 halves with an IMI ≤10 DIM and 54 halves that acquired an IMI during the follow-up period (> 10 DIM), after exclusion of one half whose IMI could not be fully characterized due to unsuccessful strain-typing, 112 udder-half IMI were included in the elimination dataset. Time to elimination was not associated with SRLV status ($P = 0.10$), therefore SRLV status was not included. There were no significant interactions between independent variables or between these variables and DRE. In the final model, the hazard of IMI elimination was higher (hazard ratio [HR] 5.6, 95% CI 2.8–11.2, $P < 0.01$) in halves with IMI detected > 10 DIM than in halves with IMI detected ≤ 10 DIM (Table 4). Staphylococcal species was associated with time to elimination ($P < 0.01$). After adjustment for multiple comparisons, "other NAS" IMI had a higher hazard of elimination than *S. caprae* (HR 4.6, $P < 0.01$) and *S. simulans* (HR 7.0, $P < 0.01$). Additionally, hazard of elimination of *S. cohnii* IMI was higher than that of *S. simulans* IMI (HR 6.0, $P < 0.01$). When evaluating the impact of a hypothetical informative censoring, both scenarios evaluated resulted in HRs similar to that of the final model. However, with the scenario where all censored IMI were eliminated on the day of censoring, other NAS IMI still had a higher hazard of elimination than *S. simulans* (HR 4.0, $P = 0.02$), but not different than *S. caprae* (HR 3.1, $P = 0.11$).

3.4. Hazard of new IMI

In the analyses for all NAS species as a group, hazard of new IMI was

Table 4
Generalized linear model on the probability of intramammary infection (IMI) elimination as a function of non-aureus staphylococcal species category and IMI being detected ≤10 or > 10 days in milk, with a fourth order polynomial function of days at risk of IMI elimination (DRE) and adjusting for parity, based on observations from 112 udder half-level IMI.

Effect	Estimate	Standard Error	*P*-value	HR
Intercept	−1.69	0.77	0.03	
DRE	−0.06	0.09	0.53	
DRE ^{2}	0.0004	0.003	0.88	
DRE ^{3}	0.0000	0.0000	0.85	
DRE ^{4}	−0.0000	0.0000	0.63	
NAS species			< 0.01	
Other NAS[a]	1.95	0.43	< 0.01	7.0
S. cohnii	1.80	0.55	< 0.01	6.0
S. lentus	1.67	0.64	< 0.01	5.3
S. epidermidis	1.00	0.55	0.07	2.7
S. xylosus	0.54	0.52	0.31	1.7
S. caprae	0.43	0.43	0.33	1.5
S. simulans	Reference	—	—	Reference
IMI detected > 10 DIM	1.72	0.36	< 0.01	5.6
Parity > 1[b]	−0.32	0.31	0.31	0.7

[a] Other non-aureus staphylococcal species included *S. arlettae* (n = 6), *S. auricularis* (n = 1), *S. chromogenes* (n = 2), *S. equorum* (n = 10), *S. gallinarum* (n = 1), and *S. succinus* (n = 2).
[b] Variable included as confounding variable.

associated ($P < 0.01$) with the presence of a NAS IMI in the contralateral half, but not with parity ($P = 0.18$) or SRLV status ($P = 0.10$). The effect of SRLV status was therefore not included in the final model. There were no significant interactions between independent variables or between these variables and DIM. In the final model, the hazard of new IMI was higher (HR 2.1, 95% CI 1.3–3.4, $P < 0.01$) in halves for which the contralateral half had a NAS IMI. When evaluating the impact of a hypothetical informative censoring, the model with the scenario where all censored halves were censored at 300 DIM did not differ from the final model. However, in the model with the scenario where all censored halves had a new IMI on the day of censoring, there was a significant interaction between parity and time (polynomial function of DIM). The HR for the presence of a NAS IMI in the contralateral half remained almost unchanged (1.7; 95% CI 1.0–2.6, $P = 0.04$). In the analyses for the four individual species evaluated, only the hazard of new IMI caused by *S. caprae* was higher (HR 5.2, $P < 0.01$) in halves for which the contralateral half had a NAS IMI. Although the specific NAS species of the contralateral IMI was not included in the model, among eight new *S. caprae* that were associated with contralateral IMI, the species isolated in the contralateral half was *S. caprae* (n = 5), *S. simulans* (n = 2), and *S. arlettae* (n = 1). Strains of *S. caprae* from each half were compared in three goats, and were the same in both halves of two goats.

4. Discussion

The most common staphylococcal species recovered from teat skin in descending order were *S. lentus*, *S. equorum*, *S. xylosus*, *S. arlettae*, *S. caprae*, and *S. simulans*. A previous study reported a different species distribution with *S. lentus* and *S. caprae* being isolated in low prevalence (Valle et al., 1991). This could reflect the different methodologies used such as culture media, although most staphylococcal species isolated from goats have been reported to grow on MSA (De Visscher et al., 2013), and methods of speciating the bacterial isolates. This could also be attributed to different environmental factors or udder hygiene practices on the farms where the goats were studied, especially since the teat skin of goats in the Valle et al. (1991) study were evaluated during lactation in contrast to the goats in the present study which were sampled during the non-lactating period. In cows, staphylococcal species frequently isolated from the environment or extramammary sites, but rarely from IMI have been classified as environmental species, while those frequently isolated from IMI, but infrequently from other sources have been classified as udder-adapted, and species isolated from both sources with similar frequencies have been classified as opportunistic pathogens (De Visscher et al., 2014; Taponen et al., 2008). Based on this classification, and with the limitation that teat skin was the only extramammary site evaluated in the present study, *S. epidermidis* was rarely isolated from the teat skin, but was among the commonly isolated species from milk, and would therefore be classified as udder-adapted. In contrast, *S. caprae*, *S. simulans*, and *S. xylosus* were commonly recovered from milk, but infrequently isolated from teat skin and would therefore be classified as either opportunistic or udder-adapted pathogens. This classification is in agreement with that of *S. caprae*, but in disagreement for *S. xylosus* and *S. epidermidis* that would have been considered environmental and opportunistic, respectively, based on a previous study (Valle et al., 1991). Finally, *S. arlettae*, *S. equorum*, and *S. lentus* were commonly isolated from teat skin and less commonly in milk, and would be considered either opportunistic or environmental. These classifications are further supported by the very low frequency of persistent IMI among these three species. Frequency of isolation of various staphylococcal species from cattle teat skin has been reported to vary among herds (De Visscher et al., 2014), but was not evaluated in the present study due to inclusion of a single herd. Due to the low number of udder-halves and goats with an IMI detected at ≤10 DIM that were teat skin swab positive for staphylococci prior to kidding (26 halves on 20 goats), statistical power was limited. Moreover, the clustering of halves by goat could not be taken into account by the McNemar's exact test used for comparison, but was accounted for in the logistic regression models for *S. caprae* and *S. simulans*. Nevertheless, udder-halves with *S. caprae* isolated from teat skin had higher odds of *S. caprae* IMI in early lactation, similar to what has been reported for *S. chromogenes*, *S. simulans*, and *S. xylosus* in heifers (Adkins et al., 2018). Teat skin colonization by *S. caprae* may play a role in the development of IMI, but the recovery of the same strain in only 50% of cases suggests that investigation of a larger number of isolates from teat skin, other animal and environmental sources, and from goats with or without an IMI in the previous lactation is warranted.

Staphylococcus simulans, *S. caprae*, *S. epidermidis*, and *S. xylosus* were the most prevalent cause of NAS IMI in this study and tended to persist in the mammary gland, similar to previous reports (Contreras et al., 1997; Koop et al., 2012; Moroni et al., 2005). *Staphylococcus simulans* and *S. caprae* IMI were more likely to persist than other NAS that had a lower prevalence, based on elimination hazard ratios, although this finding may have been impacted by informative censoring. Koop et al. (2012) reported a majority of *S. caprae* (53%) and *S. xylosus* (60%) IMI detected in early lactation to still be present in late lactation, whereas only 35% of *S. epidermidis* and 35% of *S. simulans* IMI persisted during the same period. The definition of IMI elimination in the present study, with ≥2 negative samples, also affected the reported proportion of eliminated IMI. For instance, some IMI persisted then had a single negative sample before the end of the study and were considered as lasting until the end of the study, but may have been considered eliminated if further follow-up had been available or if elimination had been defined as ≥1 negative sample. The definition of cure based on ≥2 negative samples has been previously used in goats (McDougall et al., 2010) and advocated in cows (Dohoo et al., 2011), and was expected to decrease the risk of misclassification bias due to false-negative culture results. Similarly, an IMI was defined using a threshold of ˜100 cfu/ml to maximize the sensitivity of the test. Thresholds used in the literature range from ≥100 cfu/ml to ≥500 cfu/ml (Koop et al., 2012; Moroni et al., 2005), but their accuracy in goats has not been validated due to the lack of a reference test.

In the current study, besides NAS species causing the IMI, factors evaluated in association with the probability of IMI elimination included stage of lactation at the time IMI was first detected, parity, and SRLV status. A few underlying factors can be hypothesized to explain the higher hazard of elimination of IMI detected > 10 DIM as compared to early IMI. Carryover of an IMI from the previous lactation or acquisition of an IMI during the non-lactating period may impact persistence in the following lactation as the IMI may be well-established in the gland and hence IMI first detected at ≤10 DIM may represent an already persistent IMI. Alternatively, supposing a given udder-half was previously uninfected and acquired an IMI between kidding and the first sampling, factors related to early lactation such as goat's immunity may favor the persistence of an IMI that would otherwise be eliminated if acquired later in lactation. Because seroconversion to SRLV may take several months to occur (de Andres et al., 2005; Barquero et al., 2013), the use of serology as a proxy for SRLV infection may have resulted in false-negatives, notably among primiparous goats. The lack of association between SRLV status and hazard of new IMI or IMI elimination might be attributable to a large majority of goats actually being infected with SRLV, and inclusion of truly negative goats, e.g. from an SRLV-free herd, could have yielded different results.

A significant finding of the present study was that 19.7% of apparently persistent IMI diagnosed based on species-level bacterial identification were actually caused by different strains when using 100% similarity of PFGE patterns. Misclassifications based on species-level identification alone have similarly been reported in cows (Fry et al., 2014), and is likely due to elimination of an IMI, followed by re-infection with a different strain of the same species. When the strains are closely related based on a low number (2–3) of different bands, it is also possible that genetic mutations may have occurred over time

within the bacterial population causing the infection (Tenover et al., 1995). Additionally, there may have been co-infection by two strains, but because only one colony was selected from the plate for subculture, only one of these strains was selected for characterization from a given milk sample, which resulted in the changes seen in genotype among samplings (Fig. 1).

A higher hazard of new IMI was associated with the presence of a NAS IMI in the contralateral half in the models for all NAS species and for *S. caprae*, which corroborates previous findings by McDougall et al. (2014) for undifferentiated CNS species. Regardless of the species causing the IMI in the contralateral half, this finding may reflect increased IMI susceptibility at the goat level, or contagiousness. Contagion between halves was supported by the observation of a few cases of new IMI by the same strain of *S. caprae* isolated from the contralateral half. However, further studies with a larger number of paired halves with IMI caused by the same staphylococcal species are needed.

In conclusion, the findings of this study on both the dynamics of NAS IMI and the distribution of NAS species in milk and on teat skin are suggestive of host adaptation of *S. caprae*, *S. simulans*, and *S. xylosus* in goats. This study provides evidence to justify the use of strain-typing to characterize persistent IMI, in a research setting, but strain-typing may not be necessary in the day-to-day on-farm management of staphylococcal IMI. Further investigation is warranted regarding interventional strategies targeting IMI detected in early lactation, including carryover from a previous lactation and IMI caused by *S. caprae*, *S. simulans*, and *S. xylosus*.

Conflict of interest statement

Declarations of interest: none.

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