***Materials and methods***

STROBE-VET (Strengthening the Reporting of Observational Studies in Epidemiology–Veterinary Extension) statement guidelines were followed in the reporting of this study (O'Connor et al., 2016). Animal use for this project was approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC; protocol #19-001).

*Sample origination*

Samples included in the current study were collected during a longitudinal, cross-sectional observational study of 10 certified organic dairy farms in Vermont (US) carried out in Winter 2019-2020. Enrolled farms were a non-probability subsample of certified organic dairies in Vermont which had participated in previous studies, and inclusion criteria included: 1) milking between 35-120 cows and 2) using either a tiestall barn bedded with shavings/sawdust or a bedded pack system to house lactating dairy cows. The inclusive term “bedded pack” is used here to encompass both aerobically composting bedded packs and deep bedded packs, and was defined as an enclosed loose housing facility deeply bedded with organic material (Jeffrey et al., 2024). In these systems, bedding and waste accumulate throughout the 6–8-month period of time when cows are housed on it and is only removed once a year. As facility type was a primary predictor of interest for NASM IMI outcomes, an equal number of herds (5) using TS and BP were enrolled. Around the time of the first farm visit, herd records were captured from the record processing center working with 9 of the participating herds (Lancaster DHIA, Manheim, PA; Dairy One Co-Op. Inc., Ithaca, NY) to obtain freshening date and parity for the current lactation. Freshening date and parity for 1 herd was obtained from personal communication with the producer who kept written records. The goal was to enroll 35 cows of varying parity in early- to mid-lactation from each herd for the duration of the study. In 1 herd with approximately 35 lactating cows, all cows were sampled. In 8 herds with ≥ 35 cows and with available DHIA data, a stratified random approach was used with cows stratified by SCC, lactation number, and DIM and then randomly selected across these variables. In 1 herd with ≥ 35 cows and no DHIA data, the producer generated a list of 35 cows in early lactation so that they would continue to be milking for the duration of the study. Cows that were unable to be sampled at a follow-up visit (dried off, left the herd) were replaced with another lactating cow dictated by convenience. At each farm visit, duplicate quarter-milk samples were aseptically collected from each lactating quarter immediately before milking for all enrolled cows according to NMC guidelines (NMC, 2017). Briefly, after routine pre-milking teat disinfection was completed, researchers (wearing clean disposable gloves) scrubbed teat ends and the distal third of teats with 70% isopropyl alcohol-moistened gauze swabs until teat ends were visibly clean, stripped the quarters (discarding 3-5 squirts of foremilk), and sequentially collected approximately 5-6 mL of milk into each of two sterile 11-mL flip-top vials. Samples were kept on ice in a cooler during transport until stored temporarily overnight at 4°C in the laboratory, where an aliquot was frozen for SCC measurement and the remaining milk sample was processed for bacteriological culture.

*Questionnaire administration*

A questionnaire was administered for all enrolled farms to collect information about housing and bedding management, as well as other practices on the farm that could impact mastitis risk. Eight of the 10 enrolled herds had completed the questionnaire as part of the previous study carried out in Spring 2019 (Jeffrey et al., 2024), and were asked if any changes had occurred in management practices at the start of the current study (Winter 2019). The 2 newly-enrolled herds completed the questionnaire shortly after completion of the current study, as soon as was allowable with university COVID-19 restrictions (Summer 2020). The study questionnaire was largely adapted from a previously published survey (Stiglbauer et al., 2013), with additional questions specific to the current study. Questions about mastitis risk explored producer concerns about bedding/mastitis risk; mastitis control, identification and record keeping; milking facilities, procedures, and hygiene practices; information about diet, vitamin and mineral supplementation, and water source; typical calving and periparturient practices; and fly control. Questions about housing and bedding management included describing type of housing system used for both lactating and dry cows; classification and description of any bedding material used; and bedding management practices for each housing type used. The questionnaire also collected some basic herd information (production numbers; number of lactating, dry, and youngstock; breed; record-keeping systems). The questionnaire and interview protocols were registered with the University of Vermont Institutional Review Board (IRB certification 19-0057), and created and administered on a tablet using KoboCollect software (KoboCollect, 2019).

*Aerobic culture of milk samples and determination of bacteriological status*

Standard aerobic bacteriological culture of quarter-milk was performed in duplicate within 24 hours of collection to identify bacterial species present in the sample. After being homogenized by gentle inversion, tryptic soy agar plates with 5% sheep blood (Northeast Laboratory, Waterville, ME) were inoculated with 10 μL of milk using disposable plastic inoculating loops. Plates were then incubated in aerobic conditions at 37°C before being read at approximately 48 hrs.

Aerobic culture results of both samples were then used together to determine the overall bacteriological status of each quarter-milk sample into the following categories: 1) “no significant growth,” when there was no growth on both plates, or ≤ 200 CFU/mL on one plate and no growth on the other plate, or ≤ 200 CFU/mL on both plates and morphology of isolates on each plate was different; 2) “pure culture,” when there was ≥ 100 CFU/mL of a particular isolate identified with the same morphology on both plates; 3) “mixed culture,” when there was ≥ 100 CFU/mL of two phenotypically-distinct isolates identified, each growing on both plates; 4) “contaminated,” when ≥ 1 of the 2 samples had more than 2 morphologically distinct isolates growing on a plate; 5) and “indeterminate,” when the set of quarter-milk samples did not meet the criteria for any of the previous categories (e.g., missing duplicate). Quarter-day observations were included in this study when the bacteriological status of a quarter on a given day could be determined.

*Speciation of bacterial isolates*

Isolates from both pure and mixed culture quarter-milk samples were selected and grown in pure culture on blood agar. Standard benchtop tests were done to presumptively identify bacteria following NMC procedure guidelines, including differential growth on selective media, colony morphology, hemolytic pattern, catalase reaction, Gram stain, and coagulase testing (NMC, 2017). Isolates were preserved in tryptic soy broth with a final concentration of 15% glycerol in cryovials and stored at -80°C. Frozen isolates were sent overnight on ice to the University of Missouri for speciation using MALDI-TOF mass spectrometry (Microflex, Bruker Daltonics) with Flex Control software (Bruker Daltonics). The protocol for identifying bacterial isolates with MALDI-TOF mass spectrometry has been described previously (Haw et al., 2024). Briefly, the plate extraction method was used in duplicate, and generated spectra were assigned a score based on similarity with spectra in the manufacturer’s database (MBT 8468 MSP Library), as well as the University of Missouri laboratory custom database (Adkins et al., 2018). The confidence levels used for NASM species identification were applied as previously described (Cameron et al., 2017), in which ≥ 1.7 was used for species-level identification and < 1.7 was classified as inconclusive. Suspect staphylococci and mammaliicocci isolates unable to be identified to the species level and those identified as *Staphylococcus agnetis* or *Staphylococcus hyicus* by MALDI-TOF were speciated using *tuf*gene sequences with a cut-off of 98% identity as previously described (Hwang et al., 2011). For all non-NASM isolates, species-level identification was considered reliable when a MALDI-TOF analysis score on at least 1 of the duplicates was ≥ 2.0, and scores between 1.7 and 1.99 resulted in only genus-level identification. If an isolate analysis score was <1.7, the isolate was re-cultured and re-analyzed in duplicate. For isolates with scores that remained below this threshold, other identification methods were used. These included both traditional identification methods (differential growth on selective media, colony morphology, catalase reaction, Gram stain), as well as PCR-based amplicon sequencing (16S rRNA gene (Weisburg et al., 1991); *rpob* gene (Drancourt et al., 2004); cut-off of 97% identity for genus-level identification, 98% identity for species-level identification).

*Determination of IMI status and selection of data set*

Using the bacteriological status and speciation information, a quarter-day IMI status was assigned to each quarter observation: 1) “healthy,” when there was no significant growth; 2) “single pathogen infection,” when ≥ 100 CFU/mL of a particular pathogen was identified in pure culture on both plates (interpretation in series; Dohoo et al., 2011); 3) “mixed infection,” when ≥ 100 CFU/mL of 2 different pathogens were identified in mixed culture on both plates; and 4) “unknown” if the sample status had been identified as contaminated or indeterminate as previously described.

A quarter-day observation was included in the final data set if: 1) the IMI status was classified as healthy *or* infected with a single SaM species for any of the most frequently observed SaM species (≥ 5 observed IMI); 2) it was collected from a cow ≤ 305 DIM at time of observation; and 3) it had an associated quarter-level SCC measurement. Figure 1 depicts the selection of the final data set of quarter-day observations using these criteria.

*Statistical analysis*

The quarter-day IMI status, cow parity and DIM data were organized into a spreadsheet (what did we actually use for this project?) (Microsoft Excel, Redmond, WA) and imported into the R Statistical Programming Environment (R Development Core Team, 2023) for analysis. The quarter-level prevalence for each farm visit was calculated by dividing the number of quarters infected with a particular pathogen (or grouping of similar pathogens) by the total number of sampled quarters at risk where IMI status could be determined for that farm visit. Median and range of quarter-level prevalence for each herd was then calculated using all consecutive visits to a particular farm. Median and range of quarter-level prevalence for tiestalls and bedded packs were calculated over all 15 visits to each facility type, respectively. Overall median and range of quarter-level prevalence were calculated using all 30 visits to the 10 farms.

Adkins, P. R. F., S. Dufour, J. N. Spain, M. J. Calcutt, T. J. Reilly, G. C. Stewart, and J. R. Middleton. 2018. Molecular characterization of non-aureus Staphylococcus spp. from heifer intramammary infections and body sites. J. Dairy Sci. 101(6):5388-5403.

Cameron, M., H. W. Barkema, J. De Buck, S. De Vliegher, M. Chaffer, J. Lewis, and G. P. Keefe. 2017. Identification of bovine-associated coagulase-negative staphylococci by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using a direct transfer protocol. J. Dairy Sci. 100(3):2137-2147.

Dohoo, I., S. Andersen, R. Dingwell, K. Hand, D. Kelton, K. Leslie, Y. Schukken, and S. Godden. 2011. Diagnosing intramammary infections: Comparison of multiple versus single quarter milk samples for the identification of intramammary infections in lactating dairy cows. J. Dairy Sci. 94(11):5515-5522.

Drancourt, M., V. Roux, P.-E. Fournier, and D. Raoult. 2004. rpoB Gene Sequence-Based Identification of Aerobic Gram-Positive Cocci of the Genera Streptococcus, Enterococcus, Gemella, Abiotrophia, and Granulicatella. Journal of Clinical Microbiology 42(2):497-504.

Haw, S. R., P. R. F. Adkins, V. Bernier Gosselin, S. E. Poock, and J. R. Middleton. 2024. Intramammary infections in lactating Jersey cows: Prevalence of microbial organisms and association with milk somatic cell count and persistence of infection. J. Dairy Sci. 107(5):3157-3167.

Hwang, S. M., M. S. Kim, K. U. Park, J. Song, and E. C. Kim. 2011. Tuf gene sequence analysis has greater discriminatory power than 16S rRNA sequence analysis in identification of clinical isolates of coagulase-negative staphylococci. J Clin Microbiol 49(12):4142-4149.

Jeffrey, C. E., T. Andrews, S. M. Godden, D. A. Neher, and J. W. Barlow. 2024. Relationship Between Facility Type and Bulk Tank Milk Bacteriology, Udder Health, Udder Hygiene, and Milk Production on Vermont Organic Dairy Farms. J. Dairy Sci.

KoboCollect: Simple, Robust and Powerful Tools for Data Collection. 2019 <http://www.kobotoolbox.org>.

National Mastitis Council. 2017. Laboratory Handbook on Bovine Mastitis. Third ed. National Mastitis Council, Inc., New Prague, MI.

O'Connor, A. M., J. M. Sargeant, I. R. Dohoo, H. N. Erb, M. Cevallos, M. Egger, A. K. Ersbøll, S. W. Martin, L. R. Nielsen, D. L. Pearl, D. U. Pfeiffer, J. Sanchez, M. E. Torrence, H. Vigre, C. Waldner, and M. P. Ward. 2016. Explanation and Elaboration Document for the STROBE-Vet Statement: Strengthening the Reporting of Observational Studies in Epidemiology-Veterinary Extension. J Vet Intern Med 30(6):1896-1928.

R Development Core Team. 2023. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Stiglbauer, K. E., K. M. Cicconi-Hogan, R. Richert, Y. H. Schukken, P. L. Ruegg, and M. Gamroth. 2013. Assessment of herd management on organic and conventional dairy farms in the United States. J. Dairy Sci. 96(2):1290-1300.

Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173(2):697-703.