ABSTRACT

Previous studies have shown that organically-raised dairy cows have an increased prevalence of *Staphylococcus aureus* compared with conventionally-raised dairy cows. However, little information exists about the dynamics of IMI in primiparous cows during early lactation on organic dairy farms. The objective of this study was to describe the IMI dynamics of primiparous cows on certified organic farms during early lactation. This longitudinal study enrolled 503 primiparous cows from 5 organic dairy farms from February 2019 to January 2020. Quarter-level milk samples were collected aseptically on a weekly basis during the first 5 weeks of lactation. Samples were pooled by cow and time point into composite samples inside a sterilized laminar hood and submitted for microbiological culture. For each of the different microorganisms identified, we estimated the prevalence in each postpartum sample, period prevalence (PP), cumulative incidence (CI) and persistence of IMI. Logistic regression models were used to investigate whether the prevalence of IMI differed by farm or sampling time points and whether IMI persistence differed between detected microorganisms. Our findings revealed a high prevalence of *Staphylococcus aureus* (PP = 18.9%), non- *aureus Staphylococcus* and closely related *Mammaliicoccal* species (PP = 52.1%), and *Streptococcus* spp. and *Streptococcus*-like organisms (PP = 32.1%) within the study population. The prevalence of these microorganisms varied significantly between farms. *Staphylococcus aureus* and *Staphylococcus chromogenes* exhibited significantly higher IMI persistence compared with other detected bacterial taxa, confirming the divergent epidemiological behavior in terms of IMI chronicity across different microorganisms. This study improves our understanding of the epidemiology of mastitis-causing pathogens in organically-raised primiparous cows, which can be used to tailor mastitis control plans for this unique yet growing subpopulation of dairy cows.

Key words: Organic dairy farms, Primiparous cows, Intramammary infections

INTRODUCTION

Recently, there has been increased demand for organic dairy products from consumers (Greene and McBride, 2015; Gundala and Singh, 2021). Although organic dairy production represents a small proportion of the overall milk sales in the US, demand continues to grow annually (USDA, 2021). US farms that produce organic-certified milk operate under different regulations than conventional dairy farms, including restrictions on the use of antimicrobials. These additional restrictions can create challenges in maintaining udder health in organically-raised cows compared with conventionally-managed cows (Ruegg, 2008; NMC, 2019).

Mastitis is the main reason for administration of antimicrobials to dairy cows in conventional farms in the US (USDA, 2022). Indeed, the use of intramammary antibiotics is a key component of many mastitis control plans on conventional farms. This is especially true at dry-off (Halasa et al., 2009a), and for the treatment of cases caused by microorganisms with a contagious epidemiology and high adaptation to the mammary gland (Barlow, 2011). The fact that antibiotics are a key component of mastitis control and treatment on conventional farms could indicate that organically raised cows may be at increased risk of mastitis because of restrictions on antibiotic use on organic farms (Ruegg, 2008; NMC, 2019).

While the epidemiology of mastitis on organic farms has not been extensively studied, available reports suggest they have an elevated prevalence of *Staphylococ-
*Staphylococcus aureus* compared with conventional dairy farms (Pol and Ruegg, 2007; Cicconi-Hogan et al., 2013). Bulk somatic cell count (SCC) has also been reported to be higher on organic compared with conventional farms (Zwald et al., 2004; Levison et al., 2016), though conflicting reports do exist (Vaarst et al., 2001; Valle et al., 2007; Stiglbauer et al., 2013). In contrast, the incidence of clinical mastitis on organic dairy farms has been reported to be lower than conventional farms (Hamilton et al., 2006; Valle et al., 2007; Richert et al., 2013). Additionally, no differences have been found in the incidence of subclinical mastitis (Hardeng and Edge, 2001) or individual SCC (Mullen et al., 2013) on organic versus conventional farms. Such reports suggest that there may be differences in mastitis epidemiology between conventional and organic dairy farms.

First-lactation cows represent a particular concern in terms of mastitis epidemiology, as they constitute a significant investment during the rearing phase and serve as a replacement for older cows that leave the herd (Boulton et al., 2017). These costs are even higher for organic dairy farms, especially those that are transitioning from organic to conventional dairy farming, because the available pool of organically raised calves is much more constrained and raising an organic-certified cows has additional costs. Studies in conventional herds consistently report that primiparous cows have a greater incidence of clinical mastitis in early lactation compared with multiparous cows (De Vliegher et al., 2012). In addition, primiparous cows with high SCC in early lactation have an increased SCC during the entire first lactation (De Vliegher et al., 2004); in organically managed primiparous cows, this high SCC may also carry over into subsequent lactations (Fernandes et al., 2021).

It should also be noted that the management practices and herd characteristics of organic dairies vary dramatically across the US and compared with conventionally managed farms (Stiglbauer et al., 2013). The US has experienced the growth of relatively large organic dairy farms, associated with increased profitability as herd size increases (Walsh et al., 2020). These operations typically employ different management strategies than the smaller organic dairy farms that characterized the US organic dairy industry until recently (Stiglbauer et al., 2013). Observations regarding mastitis in organic dairy herds have typically originated from smaller herds (Cicconi-Hogan et al., 2013; Levison et al., 2016) and may not extrapolate well to larger herds.

Given the increasing number of organic farms in the US, there is a growing need to better understand the dynamics of mastitis in these farms, particularly those with large herd size. Specifically, very little is known about the prevalence and characteristics of IMI in early lactation primiparous dairy cows raised under organic conditions. Therefore, the overall goal of this observational study was to describe the IMI dynamics of primiparous cows on certified organic farms during early lactation. Specific objectives were to estimate the prevalence and incidence of IMI with microorganisms of interest for udder health; and to describe their persistence in the mammary gland during the first 5 weeks of lactation.

**MATERIALS AND METHODS**

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

**Herd inclusion criteria**

Only USDA organic certified dairy farms were enrolled in this study. Farms were selected from different US regions and herd sizes. Enrollment was based on willingness to participate in the study, availability of electronic farm records and proximity to the Universities involved in the study. For this longitudinal observational study, we enrolled 503 primiparous cows from 5 organic dairy farms (Farm A = 162, Farm B = 122, Farm C = 130, Farm D = 23, Farm E = 66). The 5 dairy farms included in this study were selected based on their willingness to participate in the study. A total of 20 organic dairy farms were contacted by the beginning of the study. All cows that calved for the first time between February 2019 and January 2020 were eligible for enrollment. This study is part of a larger research initiative to investigate potential associations between the udder microbiome and udder health (Dean et al., 2021). For this purpose, cows were enrolled 8 weeks before calving and followed up during the first 5 weeks of lactation. In this study, we focus on the milk samples collected at calving and during lactation.

**Milk sampling**

Milk samples were collected by the research team. All research technicians collecting milk samples at different farms were trained to collected aseptic milk samples before the beginning of the study. Aseptic quarter milk samples were collected on a weekly basis during early lactation.
lactation following procedures described by the National Mastitis Council (NMC, 2017). Briefly, 3 to 4 streams of milk were discarded after pre-dipping. Teat-ends were then thoroughly scrubbed with gauze squares soaked in 70% ethanol. Wearing clean gloves, quarter-level milk samples were collected in separate tubes. All samples were collected before morning milking. Samples were stored on ice immediately upon collection and frozen at −20°C within 4 h of collection.

**Milk pooling**

Before submission of samples for milk culture, all available quarter samples from each cow were pooled into a single sample inside a sterilized laminar hood. Briefly, quarter samples were thawed overnight at 4°C. After homogenization, 2 mL of milk was extracted from each quarter samples and dispensed into a single sterile plastic vial. The resultant composite milk samples were then submitted to the Laboratory for Udder Health at the University of Minnesota for milk culture.

**Milk culture**

Using a cotton swab, milk (approximately 100 μL) was plated onto Columbia CNA agar with 5% sheep blood (CNA) and MacConkey agar. Agar plates were incubated in aerobic conditions at 37°C for 42 to 48 h. Subsequently, plates were visually examined by a trained technician, and the identity of representative colonies was further investigated. Milk samples were defined as contaminated when more than 3 distinct isolates were identified across the 2 utilized plates (Dean et al., 2022). Taxonomic assignment of isolates was accomplished in non-contaminated milk samples using a Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer (MS) (MALDI Microflex LT Biotyper, Bruker Daltonics Inc.) as previously described (Jahan et al., 2021). Briefly the peak profiles of each of the isolates were compared with a reference spectra Biotyper reference library (Microflex version 7854; last updated on 02/19/2019). Following manufacturer’s recommendations, confidence scores were used in the following way: > 2.0: species-level diagnosis; 1.8 to 2: genus-level diagnosis and < 1.8: MALDI-TOF diagnosis not recorded and traditional identification methods used. An IMI was defined as a composite sample containing one or more colony forming units (i.e., 10 cfu/mL) of any cultured isolate. Microorganisms were grouped into different taxonomic groups: *Staphylococcus aureus*; non-*aureus Staphylococcus* spp. and the closely related *Mammalian cocci* species (NASM); *Streptococcus*-like organisms (SLO, comprising *Enterococcus* spp., *Lactococcus* spp. and *Micrococcus* spp.); *Streptococcus* spp. and *Streptococcus*-like organisms (SSLO, which include members from the *Streptococcus* genus as well as the SLO); gram-negative bacteria (i.e., *Escherichia* spp., *Klebsiella* spp., *Enterobacter* spp., *Pseudomonas* spp., *Enterobacter* spp., *Pantoea* spp.) and “others” (i.e., microorganisms that did not belong to any of the previous groups). Milk samples with no calving date information, contaminated milk samples, milk samples from cows with no sample in the first 14 DIM, and milk samples corresponding to a 6th postpartum sample and/or collected after 35 DIM were excluded from the analysis (Figure 1).

**Definition of postpartum sample**

Given complexities of farm management and sampling logistics, the interval between sample collections was not always perfectly consistent for every enrolled animal, i.e., the sample-to-sample interval was not always exactly 7 d. Consequently, for some cows, 2 samples may have been collected within 1 week of each other (i.e., 6 d apart). Additionally, some animals could not be sampled during the first postpartum week, and/or the milk samples in that period were contaminated. Given these considerations and our desire to understand IMI persistence, we decided to use the order in which the postpartum samples were collected as a surrogate for postpartum week (i.e., the first sample collected was considered “postpartum sample 1,” the second sample “postpartum sample 2,” etc…). To understand the distribution of DIM relative to sample collections, see Supplementary Figure 1. A total of 424 cows were included in the analysis, from which 20 out of 424 (4.7%) had the results available from 1 sample only, 31 out of 424 (7.3%) from 2 samples and 373 out of 424 had the results available from 3 or more samples (88.0%).

**Statistical analysis**

Statistical analysis was performed using R (https://www.r-project.org/; version 4.1.2). The statistical code used for this study, including all relevant outputs, can be found online (https://fepenamosca.github.io/IMI-organic-dairies.github.io/). Before the start of analysis, electronic and paper data were manually compared with correct any errors in sample labeling or collected records (e.g., incorrect animal tags, farm names, date of sample collection and calving dates). When inconsistencies were present in the electronic records, the errors were corrected based on the cow-ID, sampling date recorded on milk vials, and sampling paper records (i.e., gold standard records).
**Estimation of prevalence, cumulative incidence, and persistence of intramammary infections.** Period prevalence (PP) of IMI was calculated as the proportion of animals that had an IMI during the postpartum period. Additionally, prevalence of IMI at calving was defined as animals that had an IMI in the first postpartum sample. The proportion of IMI by each microorganism that was already present in the first postpartum sample was reported. Cumulative incidence (CI) was defined as the proportion of animals at risk that acquired a new IMI by a particular microbe at any given sampling following the first-sampling (e.g., sample 2, 3, 4 or 5), but without an IMI by that microorganism in the first postpartum sample. Postpartum sample prevalence was calculated as the proportion of animals that had an IMI at a specific postpartum sample. Persistence of IMI was calculated by summing the number of postpartum samples in which cows harbored the same microorganism. Persistent-IMI was defined as harboring the same microorganism for 2 or more samples after calving, regardless of whether the IMI occurred in consecutive samples or not. Only cows with at least 2 samples collected during the follow-up period (n = 404) were included in the analysis of persistence, CI, and the proportion of IMI identified in the first postpartum sample.

**Associations between farm, postpartum sample and intramammary infections.** To investigate differences in IMI prevalence across postpartum samples and farms, we utilized multivariable modeling. Association between postpartum sample (explanatory variable) and prevalence of IMI for each bacterial group.
(dependent variable) was assessed using mixed logistic regression as implemented in ‘lme4’ (Bates et al., 2015). Farm-ID and cow-ID were included as fixed and random effects in the models, respectively, to account for the non-independence of observations within each sampling unit. Association between farm (explanatory variable) and PP of IMI (dependent variable) was investigated using logistic regression.

Association between bacterial group and persistence of intramammary infection. The association between bacterial group in the mammary gland was assessed using mixed logistic regression as implemented in ‘lme4’ (Bates et al., 2015). Potential confounding due to different number of samples available for each cow was accounted for by including this variable as a covariate in the model. Since each animal could have multiple IMI during the postpartum period, cow ID was added as a random effect in the model to account for non-independence of observations within each cow.

For logistic regression models, odds ratios were calculated by exponentiating the coefficients from the model, and Wald 95% confidence intervals were determined using the ‘Confint’ function from the ‘car’ package (Fox et al., 2022). Multiple comparisons were accounted for using Tukey adjustment as implemented in the ‘emmeans’ package (Lenth et al., 2022). The amount of variability explained by the random effects was assessed by estimating the intraclass correlation coefficient as implemented in the ‘performance’ package (Lüdecke et al., 2021). In some instances, the estimated variance of random effects was zero, making it impossible to compute the intraclass correlation coefficient, which was denoted as not available.

**RESULTS**

**Herd characteristics**

Herd characteristics for enrolled farms are shown in Table 1. Enrolled farms were in Colorado (n = 1), Texas (n = 1), Minnesota (n = 2) and New Mexico (n = 1). Herd size ranged from 100 and 3,000 milking cows. All the farms allowed access to pasture and cows consumed at least 30% of their dry-matter intake (DMI) from pasture when possible. The housing systems differed between farms, with cows in Texas and New Mexico housed in dry lot pens; cows in Colorado and one Minnesota farm in a free stall barn; and cows in the other Minnesota farm having access to a compost barn and out-wintering lot during the winter.

**Prevalence of any microorganisms**

Among the enrolled cows, 84.7% (359/424) had an IMI during the postpartum period. The prevalence of IMI did not vary significantly across the postpartum period (P = 0.25) and was 59.2% (251/424) in sample 1, 56.4% (228/404) in sample 2, 60.9% (227/373) in sample 3, 53.8% (176/325) in sample 4, and 55.9% (85/152) in sample 5 (Table 2). The intraclass correlation coefficient suggested that 38.4% of the variability on the IMI prevalence was explained by cow-ID.

**Staphylococcus aureus**

During the postpartum period, 80 out of the 424 cows (18.9%) had a *Staphylococcus aureus*-IMI, with significant differences between farms (P < 0.05, Figure 3). For instance, in farm D none of the cows had a *Staphylococcus aureus*-IMI, whereas in farm E almost half of the cows (46.2%, 24/52) had a *Staphylococcus aureus*-IMI (Figure 3). The prevalence of *Staphylococcus aureus* remained relatively stable throughout the postpartum period in all enrolled farms, 14.6% (62/424), 13.6% (55/404), 13.9% (52/373), 13.4% (43/320) and 12.5% (19/152) in postpartum sample 1 to 5, respectively (P = 0.86, Table 2). *Staphylococcus aureus* was detected in the first sample after calving in 14.6% (62/424) of the animals, while the CI was considerably lower (5.3%, 18/342, Figure 2). In fact, 76.6% (59/77) of *Staphylococcus aureus*-IMI during the postpartum period were already present in the first postpartum sample.

**NASM**

The PP of NASM-IMI was 52.1% (221/424) (Figure 3). Among NASM, *Staphylococcus chromogenes* was...
the most frequently isolated in all farms (Figure 3). The PP of *Staphylococcus chromogenes*-IMI was 38.0% (161/424), with no statistically significant difference between farms (PP range: 31.5 to 48.1%, *P* = 0.16) or across the postpartum period (PP = 0.12) (Table 2). At first sampling, 25.5% (108/424) of the animals had *Staphylococcus chromogenes*-IMI, while 17.9% (53/296) of the cows at risk acquired an IMI by this microorganism in subsequent samples. The percentage of *Staphylococcus chromogenes*-IMI harbored on the first sample was 66.2% (104/157). Other NASM (i.e., NASM non-*chromogenes*) showed a numerically lower PP (PP = 22.6% [96/424]) and divergent distribution across the enrolled farms (P < 0.001) (Figure 3). These micro-

### Table 2. Culture results by postpartum sample (PS) (n = 1,673)

<table>
<thead>
<tr>
<th>Culture Result</th>
<th>PS 1 (n = 424)</th>
<th>PS 2 (n = 404)</th>
<th>PS 3 (n = 373)</th>
<th>PS 4 (n = 320)</th>
<th>PS 5 (n = 152)</th>
<th>P-value ^b</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staph. aureus</em></td>
<td>62 (14.6%)</td>
<td>55 (13.6%)</td>
<td>52 (13.9%)</td>
<td>43 (13.4%)</td>
<td>19 (12.5%)</td>
<td>0.86</td>
</tr>
<tr>
<td>NASM</td>
<td>132 (31.1%)</td>
<td>127 (31.4%)</td>
<td>116 (31.1%)</td>
<td>79 (24.7%)</td>
<td>44 (28.9%)</td>
<td>0.16</td>
</tr>
<tr>
<td><em>Staph. chromogenes</em></td>
<td>108 (25.5%)</td>
<td>91 (22.5%)</td>
<td>92 (24.7%)</td>
<td>63 (19.7%)</td>
<td>33 (21.7%)</td>
<td>0.63</td>
</tr>
<tr>
<td>NASM non-<em>chromogenes</em></td>
<td>30 (7.1%)</td>
<td>42 (10.4%)</td>
<td>31 (8.3%)</td>
<td>21 (6.6%)</td>
<td>13 (8.6%)</td>
<td>0.50</td>
</tr>
</tbody>
</table>

^b Type-III P-value assessing the association between the postpartum sample and presence of IMI by a specific microorganism, accounting for farm-ID and cow-ID as a random effect. Different superindices within the same row represent significant differences across different postpartum samples. All models accounted for farm-ID as a fixed effect and cow-ID as a random effect. Intraclass correlation coefficient Cov-ID: Any-IMI = 0.38, *Staphylococcus aureus*-IMI = 0.98, *Staphylococcus chromogenes*-IMI = 0.85, NASM non-*chromogenes*-IMI = 0.48 *Streptococcus* spp.-IMI = 0.93, *Streptococcus*-like organisms-IMI = 0.30, Gram-negative bacteria-IMI = Not available, Others-IMI = 0.14. 

Journal of Dairy Science Vol. TBC No. TBC, TBC
organisms included: *Staphylococcus epidermidis* (PP = 0.2% [1/424]), *Staphylococcus haemolyticus* (PP = 2.1% [9/424]), *Staphylococcus hominis* (PP = 0.7% [3/424]), *Staphylococcus sciuri* (PP = 0.9% [4/424]), *Staphylococcus xylosus*/saprophyticus (PP = 2.4% [10/424]) and unspeciated *Staphylococcus* spp. (PP = 17.9% [76/424]). The prevalence of NASM non-*chromogenes* in the first postpartum sample was 7.1% (30/424), and the CI in the subsequent postpartum period was 17.6% (66/374). (Figure 2). Only 27.5% (25/91) of the IMI caused by these microorganisms were found in the first postpartum sample.

**SSLO**

The PP of SSLO was 32.1% (136/424). Among SSLO, *Streptococcus* spp. had a PP of 16.7% (71/424) which differed significantly between farms (P < 0.001, Figure 3) and postpartum samples (P = 0.008) (Table 2). *Streptococcus* spp. were found at calving in 11.6% (39/424) of the enrolled animals and 6.2% (22/355) of the cows without *Streptococcus* spp.-IMI on the first sample acquired a new *Streptococcus* spp.-IMI during the following postpartum period (Figure 2). An important proportion of *Streptococcus* spp.-IMI during the postpartum period (67.6% [37/61]) were already present in the first postpartum sample. *Streptococcus dysgalactiae* was the predominant species within the *Streptococcus* genus (PP: 11.1% [47/424]), while *Streptococcus uberis* (PP: 2.1% [9/424]) and unspeciated *Streptococcus* spp. (PP: 7.5% [32/424]) showed a numerically lower PP. The distribution of different species within the SSLO group varied across farms as shown in Figure 3. In our study, 17.0% (72/424) of the cows harbored SLO-IMI during early lactation. The prevalence of SLO varied significantly across farms (P < 0.001, Figure 3), but not between samples collected at different postpartum time points (P = 0.63). The most prevalent species among this group included *Aerococcus* spp. (5.4% [23/424], and *Enterococcus* spp. (10.1% [43/424]), while *Lactococcus* spp. (1.4% [6/424]) and *Micrococcus* spp. (0.7% [3/424]) showed a low PP. Among the samples taken at calving, SLO species were found in 5.9% (25/424), whereas SLO-IMI CI was 12.4% (47/379). SLO-IMI in the first sample represented 33.8% (24/71) of all SLO-positive samples.

**Gram-negative microorganisms**

Prevalence of gram-negative bacteria was significantly different between farms (P < 0.001, Figure 3). These microorganisms had a low PP in our study.
with the exception of farm C that showed a statistically (vs. farm A and B) or numerically (vs. farm C and D) higher prevalence compared with other farms (PP: 20.7% [23/111]) (Figure 3). The predominant bacterial genus within gram-negative organisms was Escherichia spp. (PP = 3.3% [14/424]). Klebsiella oxytoca-IMI was identified only in Farm C and in very low frequency (PP = 0.7% [3/424]). Other gram-negative organisms isolated from milk samples in this study showed a low PP and included Pseudomonas spp. (0.7% [3/424]), Enterobacter spp. (0.5% [2/424]), Pantoea spp. (0.7% [3/424]), Citrobacter freundii (0.2% [1/424]), Serratia spp. (0.7% [3/424]), and other unidentified gram-negative organisms (1.2% [5/424]).

Gram-negative bacteria had a low prevalence throughout the follow-up period with no significant difference across postpartum samples ($P = 0.58$). Additionally, gram-negative microorganisms were harbored in 2.8% (12/424) of milk samples in the first postpartum sample, representing 32.4% (12/37) of gram-negative IMI, while 6.4% (25/392) of the cows at risk acquired a new gram-negative IMI during the subsequent postpartum period (Figure 2).

‘Others’ comprise a diverse group of microorganisms that do not belong to any of the previously mentioned taxa. In this longitudinal study, 32.5% (138/424) of the animals acquired an IMI associated with this group during the follow-up period. The PP of IMI caused by microorganisms belonging to this group varied significantly across farms ($P < 0.001$, Figure 3) and ranged from 9.5% (Farm D, 2/21) to 72.1% (Farm C, 80/111). The most frequently isolated microorganisms in this group were Bacillus spp. (PP = 20.5% [87/424]) and Corynebacterium spp. (PP: 13.9% [59/424]). Other microorganisms from this group showed a very low prevalence during the study period (PP <1%).

### Persistence of intramammary infections

Association between bacterial group and persistence of IMI were assessed to understand the epidemiology of the microorganisms associated with IMI in this observational study (Figure 4). Staphylococcus aureus exhibited high persistence, with 27.3% (21/77) and 59.7% (46/77) of the IMI caused by this microorganism being found in 2 and 3 or more samples, respectively. Similarly, 108 of the 157 (68.8%) cases of Staphylococcus chromogenes persisted for 2 or more samples after calving. In con-
Contrast, the majority of NASM non-\textit{chromogenes} caused transient-IMI and were only observed in 1 of the postpartum samples from each cow, i.e., 24.5\% (24/98). For \textit{Streptococcus} spp., 24 out of 45 (53.3\%) \textit{Streptococcus dysgalactiae}-IMI (the most frequently isolated SSLO in our study) were persistent. \textit{Streptococcus uberis} and unspecified \textit{Streptococcus} spp. had 50.0\% (4/8) and 31.3\% (10/32) of cases classified as persistent, respectively. Persistence for SLO-IMI was low, as these microorganisms showed predominantly short-lived IMI. Only 9.5\% (8/84) of the SLO-IMI persisted for 2 or more samples. All IMI caused by gram-negative organisms were transient, as none of them were identified in more than 1 postpartum sample from the same cow. Other microorganisms not belonging to any of these groups also showed low persistence during the follow-up period (18.5\%, 34 out of 184 IMI), including \textit{Bacillus} spp. and \textit{Corynebacterium} spp.

Mixed logistic regression modeling indicated that bacterial group ($P < 0.001$) and number of postpartum samples (OR [95\%CI]: 1.80 [1.36–2.38], $P < 0.001$) were associated with the odds of IMI persistence (Table 3). Farm was not statistically significantly associated with IMI persistence ($P = 0.31$). After adjusting for multiple comparisons, \textit{Staphylococcus aureus} (Adj. risk [95\%CI]: 0.89 [0.79–0.94]) showed a higher persistence than \textit{Staphylococcus chromogenes} (Adj. risk [95\%CI]: 0.72 [0.64–0.79]) ($P = 0.06$), while both microorganisms showed significantly higher odds of persistence compared with other bacterial groups: NASM non-\textit{chromogenes} (Adj. risk [95\%CI]: 0.26 [0.18–0.36], $P < 0.001$); \textit{Streptococcus} spp. (Adj. risk [95\%CI]: 0.44 [0.32–0.56], $P < 0.001$); SLO (Adj. risk [95\%CI]: 0.10 [0.05–0.20], $P < 0.001$); other microorganisms (Adj. risk [95\%CI]: 0.20 [0.13–0.28], $P < 0.001$). Additionally, \textit{Streptococcus} spp. showed a higher persistence compared with SLO (OR [95\%CI]: 6.67 [1.82–24.49], $P < 0.001$), while no statistically significant difference was found between these groups and NASM non-\textit{chromogenes} ($P > 0.05$).

**DISCUSSION**

This multi-site longitudinal study described the temporal prevalence dynamics and distribution of microorganisms associated with IMI in organically managed primiparous cows during early lactation. In our enrolled herds, 84.7\% of the animals had an IMI during the postpartum period. The prevalence of IMI during the postpartum period remained relatively stable and ranged between 39.1\% and 46.3\% in the different postpartum samples (1st to 5th sample). This is comparable to the 48.7\% prevalence in early lactation primiparous cows in conventional dairy farms in Europe (Piepers et al., 2010), and higher than that reported for quarter-level IMI in other epidemiological studies conducted in conventionally managed primiparous cows, which ranged from 14.2\% to 32.1\% (Supré et al., 2011; Nitz et al., 2020; Valkenier et al., 2020).

**Dynamics and distribution microorganisms leading to intramammary infections**

In our study, the prevalence of \textit{Staphylococcus aureus} was higher than that reported in other observational studies in conventionally managed first-lactation dairy cows, which ranges from 0.3\% to 7.0\% (Piepers et al., 2010; Nitz et al., 2020; Valkenier et al., 2020). Indeed, previous studies have shown an increased prevalence of \textit{Staphylococcus aureus} in organic compared with conventional dairies (Pol and Ruegg, 2007; Cicconi-Hogan et al., 2013) and may be associated with the restrictions placed on organic farms regarding the use of antimicrobials (NMC, 2019). Importantly, the majority of \textit{Staphylococcus aureus}-IMI (76.6\%) were already present in the first sample after calving, in agreement with previous studies reporting a high prevalence of \textit{Staphylococcus aureus}-IMI in primiparous cows before calving (0.4\% to 14.9\%) (De Vliegher et al., 2012). This suggests that prepartum management may play a role in the acquisition of \textit{Staphylococcus aureus}-IMI (Phillips et al., 2021). The mechanisms that facilitate the transmission of \textit{Staphylococcus aureus} to primiparous cows before calving are not completely elucidated. It has been suggested that potential pathways of transmission may involve the ability of \textit{Staphylococcus aureus} to colonize other body sites (De Vliegher et al., 2012), or be transmitted through vectors like flies (Gillespie et al., 1999). Although certain strains affecting primiparous cows could be of environmental origin (Zadoks et al., 2011), identical strains have been reported to affect multiparous and primiparous cows, suggesting that within herd transmission across these groups is plausible (Tenhagen et al., 2007).

Non-\textit{aureus} \textit{Staphylococci} and closely related \textit{Mammi liococcal} species are the most frequently isolated microorganisms associated with IMI in conventionally managed first-lactation cows (De Buck et al., 2021). In our study, NASM were also the most prevalent microorganisms detected in milk samples in early lactation. Surprisingly, we did not identify a statistically significant association between postpartum sample and NASM-IMI, which contradicts previous studies that showed an increased prevalence of NASM-IMI in the first days after calving in comparison to the subsequent postpartum period (Valkenier et al., 2020). Among identified NASM species, \textit{Staphylococcus chromogenes} was the most frequently isolated in all enrolled farms, consistent with reports from conventional dairy farms.
Figure 4. Persistence in the mammary gland by bacterial group (n = 685). Data from 404 cows was included in this figure. Number of samples: number of samples that the same microorganism from a bacterial group was harbored during the postpartum period. NASM non-chromogenes = Non-aureus Staphylococci and closely related Mammaliicoccal species that were not Staphylococcus chromogenes. Other = Other microorganisms not included in the previous groups.
Table 3. Mixed logistic regression model assessing the association between Bacterial group (Explanatory variable) and odds of persistent infection (Harbor same microorganism in milk in 2 or more milk samples during follow-up period). Each observation in this model represents one IMI (n = 685)

<table>
<thead>
<tr>
<th>Bacterial group</th>
<th>n</th>
<th>Adjusted Risk (95% CI)</th>
<th>Estimate (SE)</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>77</td>
<td>0.89 (0.79-0.94)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Referent</td>
<td>Referent</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Staphylococcus chromogenes</td>
<td>157</td>
<td>0.72 (0.64-0.79)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-1.09 (0.39)</td>
<td>0.34 (0.16-0.72)</td>
<td></td>
</tr>
<tr>
<td>NASM non-chromogenes</td>
<td>98</td>
<td>0.26 (0.18-0.36)&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>-3.10 (0.43)</td>
<td>0.05 (0.02-0.10)</td>
<td></td>
</tr>
<tr>
<td>Streptococcus spp.</td>
<td>85</td>
<td>0.44 (0.32-0.56)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-2.29 (0.42)</td>
<td>0.10 (0.04-0.23)</td>
<td></td>
</tr>
<tr>
<td>Streptococcus-like organisms</td>
<td>84</td>
<td>0.10 (0.05-0.20)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-4.19 (0.53)</td>
<td>0.02 (0.01-0.04)</td>
<td></td>
</tr>
<tr>
<td>Other organisms</td>
<td>184</td>
<td>0.20 (0.13-0.28)&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>-3.46 (0.42)</td>
<td>0.03 (0.01-0.07)</td>
<td></td>
</tr>
<tr>
<td>Num. samples per cow</td>
<td>—</td>
<td>—</td>
<td>0.59 (0.14)</td>
<td>1.80 (1.36-2.38)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>


Cow-ID was added as a random effect (Variance [SD]: 1.88 e-14 [1.37 e −7], Intraclass correlation coefficient = Not available). Gram-negative organisms-IMI (n = 36) were not included in this model, because they had zero cases of persistent-IMI. ‡ Different superindices within the same column represent significant differences across bacterial groups.

(De Buck et al., 2021). In previous epidemiological studies, the prevalence of *Staphylococcus chromogenes* in first-lactation cows ranged between 5.5% and 13% (Supré et al., 2011; De Visscher et al., 2016; Valckenier et al., 2020) and was higher in first-lactation compared with multiparous cows (De Visscher et al., 2016; Nitz et al., 2018). Other NASM species (i.e., NASM non-chromogenes) showed a lower prevalence overall, with differences in prevalence across the enrolled farms. It has been reported that most of these NASM species have a poor adaptation to the mammary gland and a predominantly environmental epidemiology (De Buck et al., 2021). For example, previous studies reported that these microorganisms showed a higher prevalence in environmental niches compared with milk samples (De Visscher et al., 2014; Wuytack et al., 2020), which may potentially explain the lower prevalence and differential distribution of these microorganisms across farms (De Buck et al., 2021). In general, differences in microorganism prevalence between the farms in our study was not surprising given the numerous differences in management, including bedding materials, housing systems and/or geographical regions of farms that were enrolled in our study (Table 1).

The SSLO are an important cause of clinical mastitis and a concern to dairy farmers around the world (Kabelitz et al., 2021). In the present observational study, the prevalence of SSLO was higher than that reported in conventionally managed first-lactation animals (0.6% - 5.0%) (Piepers et al., 2010; Nitz et al., 2020; Valckenier et al., 2020). *Streptococcus spp.* were the most prevalent genus within SSLO. The PP of *Streptococcus* spp. showed important differences across farms, suggesting that differential management and housing systems could have an impact on its prevalence. Following a similar pattern to *Staphylococcus aureus*, Farm B and E showed a higher prevalence of *Streptococcus* spp. compared with the other farms. This suggests a predominance of pathogens adapted to the mammary gland. Indeed, organic dairy farms face challenges to deal with host-adapted microorganisms due to the restrictions placed on the use of antimicrobials and the potential difficulties finding replacements for culled animals (NMC, 2019). Among *Streptococcus* spp., *Streptococcus dysgalactiae* was the most prevalent. *Streptococcus dysgalactiae* is recognized as an important mastitis pathogen, usually classified as an intermediate pathogen (Kabelitz et al., 2021), in which environmental and host-associated strains are frequently present within a dairy farm (Wente and Krömker, 2020). The SLO represents a diverse group of microorganisms that are commonly misdiagnosed as *Streptococcus* spp. While these microorganisms are thought to be environmental (Hogan and Smith, 2012), little is known about the epidemiology of different species within this group. The SLO-IMI have been associated with an increase in the risk of subclinical mastitis, indicating their importance for udder health (Rowe et al., 2021).

Gram-negative microorganisms are an important cause of clinical mastitis in conventionally managed dairy cows (Lago et al., 2011; Royster et al., 2014). However, the prevalence of gram-negative bacteria is usually reported to be low (0% - 8.5%) (Piepers et al., 2010; Nitz et al., 2020; Valckenier et al., 2020), due to the poor adaptation of gram-negative microorganisms to the mammary gland (Todhunter et al., 1991; Klaas...
and Zadoks, 2018). Only 8.7% of the enrolled cows had gram-negative-IMI, showing a similar low prevalence in all farms except for farm C. Consistently, farm C also showed a higher prevalence of SLO. While the specific factors that led to these differences are unknown, it is possible that factors related to the housing system had an impact on the prevalence of these microorganisms in farm C. Dry-lots are thought to increase cow exposure to environmental hazards such as rain, wind or sun, since cows are less sheltered than in indoor confinement systems. This higher exposure can impact animal health and welfare (Chen et al., 2017), which could partially explain the higher prevalence of environmental microorganisms in farm C. However, if dry-lot housing was the primary driver we would also expect similar dynamics in farm B, but that was not the case, suggesting the presence of other unknown factors that may have influenced the prevalence of gram-negative bacteria in the different farms of this study.

**Persistence of intramammary infections**

The prevalence of IMI at any given time point represents both acquisition of new IMI and IMI persistence. Different strategies can be used in both organic and conventional dairy farms to reduce the risk of new IMI (Ruegg, 2017; NMC, 2019). Nonetheless, antibiotics are the most frequently used strategy in conventional dairy farms for managing IMI after they are acquired (Halasa et al., 2009a; Ruegg, 2021); this strategy is not available to certified organic dairies unless the treated cows are removed from the herd (NMC, 2019). Our study highlights how IMI persistence varies among different bacterial species. These differences may have implications for the epidemiology of mastitis-causing microorganisms, prognosis of infected quarters and decision-making in dairy farms; and these results are especially crucial for organic-certified dairy farms in which antibiotics are not used for control and herd-level management of mastitis. Not surprisingly, a large proportion of *Staphylococcus aureus*-IMI in our study were persistent (Adj. risk: 89%), conforming to the idea that this bacterium has high adaptation to the mammary gland and an ability to persist within it for long periods of time (Barkema et al., 2006). *Staphylococcus chromogenes* also showed a high persistence in the mammary gland (Adj. risk: 72%) which agrees with previous reports (Piessens et al., 2011; Supré et al., 2011; Vanderhaeghen et al., 2015). *Streptococcus* spp. showed a moderate likelihood of persistence in the mammary gland, with almost half of the IMI being classified as persistent (Adj. risk: 44%). This could be attributed to the fact that *Streptococcus dysgalactiae*, the most prevalent *Streptococcus* species in our study, is known for its ability to survive in the mammary gland and persist for long periods (Calvino and Oliver, 1998; Kabelitz et al., 2021), although some strains can be environmental (Wente and Krömker, 2020). In contrast to *Streptococcus* spp. a low proportion of SLO-IMI were persistent, suggesting a poor adaptation to the mammary gland, which agrees with their reported environmental epidemiology (Hogan and Smith, 2012). All of the gram-negative-IMI were only present in the mammary gland for 1 postpartum sample (i.e., transient-IMI), which can be explained by the poor host adaptation and predominantly environmental epidemiology of gram-negative microorganisms (Todhunter et al., 1991; Klaas and Zadoks, 2018). The exception to this is *Klebsiella* spp., which had low prevalence in the present study (detected in 3 out of 424 animals).

**Study implications and internal validity**

The prevalence and distribution of microorganisms associated with early lactation IMI in organic dairy farms described in our study does not agree with previous reports from conventional dairy farms. Our study was not designed to identify the drivers of these differences, and many factors could be responsible for these findings. Some of these factors may be related to organic production practices (such as antimicrobial use, as discuss), but others could be due to differential cow demographics and management between organic and conventional farms. For example, it has been reported that organic farms have a higher average parity (Hardeng and Edge, 2001; Stiglbauer et al., 2013) which is associated with an increased prevalence of contagious mastitis pathogens and particularly *Staphylococcus aureus* (De Vliegher et al., 2012; Ziesch and Krömker, 2016), that could potentially be transmitted to first-lactation cows before and after calving (De Vliegher et al., 2012; Dufour et al., 2012). Furthermore, organic dairies are less likely to routinely measure SCC, which may lead to subpar identification of chronically infected cows that can play a key role in the dissemination of IMI organisms (Stiglbauer et al., 2013). The management and environment in which cows are raised also has an impact on udder health. The possible exposure to mastitis bacteria and the environment can be assessed using the udder hygiene score (Schreiner and Ruegg, 2003), which may be used to be lower in organic farms (Ellis et al., 2007). Antimicrobials and teat sealants can still be used to safeguard udder health on conventionally maintained cows during the dry period (Berry and Hillerton, 2007; Halasa et al., 2009b), but their use is not permitted under organic management (NMC, 2019).
One important strength of this study is the longitudinal approach of sampling the same animals multiple times after calving, which allows for the investigation of not only IMI prevalence, but also the likelihood of IMI acquisition (incidence) and IMI persistence during the first 5 weeks of lactation. In addition, the use of MALDI-TOF MS provides a higher taxonomic resolution than traditionally utilized biochemical methods (Royster et al., 2014), allowing species-level identification for many of the isolates (Jahan et al., 2021). Limitations of this study include the potential misclassification of true IMI as contaminated samples due to mixed infections across quarters (Dean et al., 2022), that could result in an understimation of the true IMI prevalence. Likewise, the use of composite milk samples could lead to an underestimation of the prevalence of IMI due to a dilution effect (Reyher and Dohoo, 2011). In an effort to overcome these constraints, this study utilized an IMI definition of 10 cfu/mL of any culturable isolate in contrast to the commonly used 100 cfu/mL, hence increasing the likelihood of detecting an IMI in the processed milk samples (Dohoo et al., 2011). In addition, contamination was defined as more than 3 different isolates in a given sample to reduce the misclassification of mixed inter-quarter infections as contaminated milk samples (Dean et al., 2022). The absence of strain-typing techniques in this study represents another limitation for the investigation of IMI-persistence (Simar et al., 2021). This could lead to an IMI being categorized as persistent when, in fact, different strains or species within the same taxonomic group could be causing an infection across multiple sampling points. Therefore, our results could be potentially overestimating the persistence of IMI in such cases. Lastly, in our study, MALDI-TOF MS failed to identify a considerable number of isolates at the species level. For instance, 78.4% (422/538) of NASM isolates were identified up to the species level, which is consistent with previous studies (Rowe et al., 2019, 2020). The taxonomic resolution for the identification of these microorganisms may have been enhanced by using lower confidence score thresholds, such as 1.7 for taxonomic assignment at the species level, as previously examined (Cameron et al., 2017). As an example, we still had access to data from a small proportion of the isolates processed using MALDI-TOF MS that were not speciated (10.3% [12/116]). Among these isolates, 3 (25%) could have been identified up to the species level if the cut-off value for MALDI-TOF MS identification had been lowered to 1.7. These isolates would have been identified as *Staphylococcus xylosus*, *Staphylococcus haemolyticus* and *Staphylococcus warneri*. However, to be consistent with the manufacturer recommendation and enable comparisons between this and other studies performed in North America we decided to utilize a 2.0 confidence score for species level identification (Jahan et al., 2021). Nonetheless, to overcome the limited ability to identify the NASM and other mastitis microorganisms species using MALDI-TOF MS, the use of alternate cut-off points (Cameron et al., 2017) and the use of enlarged databases (Cameron et al., 2018) should be considered to promote reproducibility across and within research institutions.

**External validity**

This study presents results for 5 organic dairy farms in the Midwest and Southwest regions, including farms with large and small herd sizes. Therefore, we consider our study findings relevant to a diversity of organic dairy farms in the United States. However, it is worth noting that only a few of the farms contacted by the research team agreed to participate in this study. Since the underlying reasons that led to participation are unknown, this could potentially lead to a type of selection bias known as non-response bias (Groves and Peytcheva, 2008). In addition, more studies are needed to investigate IMI dynamics in both primiparous and multiparous dairy cows in a larger number of organic dairy farms, to identify potential farm-level risk factors that could be driving the observed differences in the prevalence and distribution of mastitis pathogens between the enrolled herds.

**CONCLUSIONS**

This study provides a detailed description of the microorganisms leading to IMI in early lactation first-lactation dairy cows on organic dairy farms. Our results showed a high prevalence of *Staphylococcus aureus*, NASM and SSLO in the enrolled animals during the start of their first lactation. The prevalence of these microorganisms differed by herd. Additionally, certain bacterial microorganisms, especially *Staphylococcus aureus* and *Staphylococcus chromogenes*, showed a high prevalence at calving and high persistence in the mammary gland, which suggests that prepartum management should be a focus of IMI prevention and control in organically reared primiparous cows. Lastly, some IMI species showed a predominance of persistent-IMI (e.g., *Staphylococcus aureus*, *Staphylococcus chromogenes* and *Streptococcus spp.*), while others caused primarily transient-IMI (e.g., NASM non-*chromogenes*, SLO, gram-negative microorganisms). This study allows us to better understand the epidemiology of mastitis-causing pathogens in organically managed first-lactation cows, which is a necessary step toward developing tailored...
mastitis controls for this unique and growing subpopulation of US dairy cows.

ACKNOWLEDGMENTS

The authors would like to thank the Laboratory for Udder Health at the University of Minnesota for performing milk cultures; the numerous students, faculty and staff for their assistance with sample collection; and the owners and managers from the five participating herds who made this study possible. Felipe Peña-Mosca sampled in Minnesota, performed laboratory work, cleaned and wrangled the data, performed statistical analysis and prepared the initial and final draft of the manuscript. Chris Dean sampled in Minnesota, performed laboratory work, collaborated in data processing and edited the manuscript. Vinicius Machado conceptualized the study, coordinated sampling in Texas and New Mexico and edited the manuscript. Leticia Fernandes sampled in Texas and New Mexico and edited the manuscript. Pablo Pinedo conceptualized the study, coordinated sampling in Colorado and edited the manuscript. Enrique Doster sampled in Colorado and edited the manuscript. Bradley Heins conceptualized the study, coordinated sampling in Minnesota and edited the manuscript. Kirsten Sharpe sampled in Minnesota and edited the manuscript. Carol Baumann sampled in Minnesota, performed laboratory work and edited the manuscript. Tui Ray was performed laboratory work and edited the manuscript. Acir Antunes sampled in Minnesota and Colorado and edited the manuscript. Thomas Wehri sampled in Minnesota, performed laboratory work and edited the manuscript. Noelle Noyes conceptualized the study, funding acquisition, coordinated sampling and laboratory work and edited the manuscript. Luciano Caixeta was involved in study conceptualization, funding acquisition, coordinated sampling and laboratory work and edited the manuscript. The authors have not stated any conflicts of interest. This study was funded by the Organic Agriculture Research and Extension Initiative (OREI) from the National Institute of Food and Agriculture (Grant Number: 2018-51300-28563). The first author, Felipe Peña-Mosca, was partially funded by Fulbright and Agencia Nacional de Investigación e Innovación from Uruguay (Scholarship Number: POS_FUL_2019_1_1008441).

REFERENCES


Peña-Mosca et al.: Investigation of intramammary...


